

US EPA ARCHIVE DOCUMENT



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY  
WASHINGTON, D.C. 20460

OFFICE OF  
PREVENTION, PESTICIDES AND  
TOXIC SUBSTANCES

Date:

3/24/99

**MEMORANDUM**

**SUBJECT: Atrazine and Simazine-** Reviews of five studies examining: 1) Short-term effects on the rat estrus cycle; 2) Antiestrogenicity in rats; 3) *In vitro* antagonism of estrogen action and *in vitro* binding with the estrogen receptor; 4) Estrogenic responses in MCF-7 human breast cancer cells; 5) Estrogenic responses *in vivo* (rat) and *in vitro*.

**DP Barcode:** D253661  
**Case:** 838869  
**Submission:** S557393  
**Chemical:** Atrazine and Simazine  
**Chemical No.** 063 740  
**PC No.:** 080803 080807

**FROM:** Anna P. Bearden, Ph. D.  
Registration Action Branch III  
Health Effects Division (7509C)

**Thru:** Stephen Dapson, Ph. D.  
Branch Senior Scientist  
Registration Action Branch III, HED (7509C)

**To:** Catherine Eiden  
RCAB, Health Effects Division (7509C)  
and  
Jackie McQueen  
SRRD (7508W)

**Registrant:** Novartis Crop Protection  
P.O. Box 18300  
Greensboro, NC 27419-8300

**Action Requested:** Review 5 studies: 1) Short-term effects chlorotriazines on the rat estrus cycle; 2) Possible antiestrogenic properties of chloro-s-triazines; 3) Chloro-s-triazine antagonism of estrogen action: limited interaction with estrogen receptor binding; 4) Failure of atrazine and simazine to induce estrogenic responses in MCF-7 human breast cancer cells; 5) Failure of chloro-S-triazine-derived compounds to induce estrogen receptor-mediated responses *in vivo* and *in vitro*. All five studies are special studies and are non-guideline.

**Response:** Short-term effects on the rat estrus cycle (MRID no. 43598614) was reviewed and found to be acceptable. Antiestrogenicity in rats (MRID no. 43598617) was reviewed and found to be acceptable. *In vitro* antagonism of estrogen action and *in vitro* binding with the estrogen receptor (MRID no. 43598618) was reviewed and found to be acceptable. Estrogenic responses in MCF-7 human breast cancer cells (MRID no. 43598619) was reviewed and found to be acceptable. Estrogenic responses *in vivo* (rat) and *in vitro* (MRID no. 4394403) was reviewed and found to be acceptable.

**Reviewers:**

**EPA:** Primary reviewer for all studies - Anna Bearden  
Secondary reviewer for all studies - Roger Hawks

Data evaluation records (DERs) are attached and the executive summaries are as follows:

*Short-term effects chlorotriazines on estrus in female Sprague-Dawley and Fischer 344 rats. (MRID 43598614.) Published in Journal of Toxicology and Environmental Health. 43: 155-167.*

Executive Summary:

In a special study (MRID 43598614) on *in vivo* endocrine effects, atrazine and simazine (>96 % a.i.) were administered to 11 female rats/dose/strain (both Sprague-Dawley and Fischer 344 rats were used) by oral gavage at dose levels of 0, 100, and 300 mg/kg/day for 14 to 23 days depending on time to achieve proestrus. Some animals did not achieve proestrus and were sacrificed at day 23. Body weights and vaginal smears were taken daily. Blood was collected at termination for analysis of the following blood plasma hormones: estradiol, progesterone, corticosterone, prolactin. Ovaries, uterus, and adrenals were weighed at necropsy. Vaginal cytology, estrus cycle length, and percent days in cycle were observed.

One Sprague-Dawley and one Fischer (F) 344 rat in the 300 mg/kg/day atrazine group died during the study. One Sprague-Dawley (S.D.) in the 100 mg/kg/day simazine group died during the study. Body weight decreases of rats exposed to atrazine are statistically significant in both the low (-7.7% S.D.; -4.4% F) and high dose groups (-22.0% S.D.; -14.2% F). Body weight decreases of rats exposed to simazine are statistically significant in the high dose group. Plasma levels of prolactin and corticosterone were not effected by either triazine in both strains of rat.

Sprague-Dawley and Fischer 344 female rats differ in their endocrine response to atrazine and simazine. Significant reductions in plasma levels of estradiol (-61% and -90% for low and high doses) were measured in atrazine treated Sprague-Dawley rats. These decreases were paralleled by significant reductions in uterine weight (-28% and -44% for low and high doses). Sprague-Dawley rats exposed to atrazine exhibited a significant increase in percentage of time spent in the estrous cycle (+14% in high dose) with a parallel decrease in the percentage of time spent in diestrus (-13% in high dose) relative to the untreated control. The relative increase in percentage of the estrous cycle spent in estrous exhibited by Sprague-Dawley rats is exemplified by dose dependent and significant increases in the cell density index of both cornified (+22% and +38% for the low and high dose) and nucleated (+42% for the high dose) epithelial vaginal cells relative to untreated control.

There were no statistically significant alterations in plasma hormone levels observed in the Fischer 344 rats. Although the mean values of plasma estradiol, progesterone, and prolactin decreased by 28% to 63% with atrazine exposure and increased 9% to 53% with simazine exposure, the relatively large standard deviations (from  $\pm$  30% to 184% of the mean) preclude significant differences. Contrary to Sprague-Dawley rats, following exposure to atrazine, the cell density index of cornified epithelial vaginal cells in Fischer 344 rats significantly decreased (-24% and -41% for the low and high dose) relative to the negative control with no effects on nucleation. In Fischer 344 rats, the decrease in cornified epithelial cells parallels the significant decrease in percentage of time spent in the estrous cycle (-24% for high dose) with parallel increase (although not statistically significant) in the percentage of time spent in diestrus (+11% for the high dose).

There were conflicting results between the plasma level of progesterone in Sprague-Dawley and Fischer 344 rats and between atrazine and simazine. These contrary observations result from the variability in the measurement of progesterone in plasma and are not toxicologically relevant.

**The LOAEL for systemic toxicity is 100 mg/kg/day for both atrazine and simazine, based on body weight effects and organ weight effects for atrazine. The NOAEL for toxicity cannot be determined.**

**The LOAEL for endocrine effects of atrazine is 100 mg/kg/day based on organ weight effects, plasma hormone changes (estradiol), estrus cycle lengthening, and vaginal cytology. The NOAEL for endocrine effects of atrazine cannot be determined.**

**The LOAEL for endocrine effects of simazine is 300 mg/kg/day based on organ weight effects and vaginal cytology. The NOAEL for endocrine effects of simazine is 100 mg/kg/day.**

This special study on the endocrine effects of atrazine and simazine in the rat is **Acceptable-nonguideline.**

Executive Summary:

This study (MRID no. 43598617) combines three *in vivo* assays to investigate the estrogenic effects of atrazine, simazine, and diaminochlorotriazine [DACT, (a.i. > 96%)] on body weight, body weight gain, uterine weight, progesterone receptor binding capacity, and thymidine incorporation on female Sprague-Dawley rats.

For body weight and uterine weight measurements, ovariectomized rats were dosed by oral gavage daily for 3 days at 20, 100, and 300 mg/kg/day in single exposure experiment and in co-exposure experiments with 2  $\mu$ g estradiol on day 2 and 3. Body weight gains were decreased for rats exposed to the vehicle control, 0.5% carboxymethylcellulose, (-3.5%) and to the positive control, 2  $\mu$ g estradiol, (-6.9%). Larger decreases in weight gain were observed in rats exposed to triazines. At 20 mg/kg/day weight losses ranged from 6-9%. At 100 mg/kg/day, weight losses ranged from 8-13.5%. At 300 mg/kg/day, weight losses ranged from 11-17%.

Chemicals with estrogenic activity cause proliferation and thickening of the uterine wall. The uterine weights of rats exposed to triazines were similar to vehicle control uterine weights. In co-exposure experiments with estradiol, exposure to atrazine and DACT at 100 and 300 mg/kg/day resulted in a dose-dependant and statistically significant decrease in uterine weight relative to the positive control. In co-exposure experiments with estradiol, uteri of rats exposed to simazine at 100 and 300 were significantly decreased relative to the positive control.

Thymidine incorporation was measured to assess the effect triazines on DNA synthesis in the uterus of exposed rats. Dose groups included: exposure to 300 mg/kg/day of triazine alone administered by oral gavage for 2 days; and exposure to 1, 10, 20, 50, 100, and 300 mg/kg/day of triazine administered by oral gavage for 2 days plus an injection of 0.15  $\mu$ g estradiol on day 2. Exposure to triazines alone at 300 mg/kg/day resulted in significant decrease relative to the vehicle control. Exposure to the positive control, 0.15  $\mu$ g estradiol, resulted in approximately 2.5 fold increase ( $p < 0.05$ ) in thymidine incorporation relative to the vehicle control. Exposure to 50, 100, and 300 mg/kg/day in co-exposure with 0.15  $\mu$ g estradiol resulted in a significant reduction in thymidine incorporation relative to the positive control.

Levels of inducible progesterone receptor were indirectly measured by the PR binding capacity to a radioactive ligand. The PR was isolated from uteri of rats exposed by oral gavage to atrazine, DACT, and simazine alone at 300 mg/kg/day for 2 days and to atrazine, DACT, and simazine at 50 and 300 mg/kg/day in co-exposure with 1  $\mu$ g/day estradiol for 2 days. The PR binding capacity in rat uteri exposed to 300 mg/kg/day triazine alone was statistically decreased relative to vehicle control. PR binding capacity decreased significantly relative to the positive control with exposure to atrazine, DACT, and simazine at 300 mg/kg/day in co-exposure experiments with 1  $\mu$ g estradiol.

**According to the effects on uterine weight, progesterone binding capacity, and thymidine incorporation at the concentrations tested in this study, atrazine, DACT, and simazine do not exhibit estrogenic activity.**

This study is classified as **acceptable-nonguideline** as a special study on *in vivo* and *in vitro* estrogenic effects.

*Chloro-s-triazine antagonism of estrogen action: limited interaction with estrogen receptor binding. (MRID 43598618). Published in Journal of Toxicology and Environmental Health. 43: 197-211.*

Executive Summary:

In a special study (MRID 43598618) on *in vitro* and *in vivo* competitive binding of atrazine, diaminochlorotriazine (DACT, a mammalian metabolite of both atrazine and simazine) and simazine (>96 % a.i. for all three compounds) to the estrogen receptor (ER), several different experiments were performed utilizing extracted cytosolic ER from adult female Sprague-Dawley rats. Experiments observed the displacement of radiolabeled estradiol by atrazine, simazine, and DACT under both equilibrium (simultaneous exposure to triazine and radiolabeled estradiol at 4°C) and at disequilibrium conditions (pre-exposure to triazine at 25°C for 30 min. prior to exposure to radiolabeled estradiol). Results of equilibrium experiments indicated that atrazine, simazine, and DACT did not displace any radiolabeled estradiol whereas the positive control exhibited a dose-dependant displacement of the radiolabeled estradiol. Under disequilibrium conditions in a time-course experiment, uterine cytosolic extracts were first incubated with triazine at 100 µM for 30 min. prior to exposure to radiolabeled estradiol for 5-150 minutes. With time radiolabeled estradiol displaced triazine that had bound during the initial exposure. In a dose-response experiment under disequilibrium conditions, uterine cytosolic extracts were first incubated with triazine at concentrations 10<sup>-9</sup> to 10<sup>-3</sup> M or unlabeled estradiol at 10<sup>-11</sup> to 10<sup>-7</sup> M prior to exposure to radiolabeled estradiol. The IC<sub>50</sub> for unlabeled estradiol was approximately 10<sup>-9</sup> M whereas the IC<sub>50</sub>'s for the triazine were 20 µM for atrazine and 100 µM for simazine and DACT.

An additional experiment under disequilibrium conditions was performed to mimic a Scatchard type analysis using constant molar excess of triazine (100X estradiol, 10,000X atrazine, or 10,000X simazine) relative to the radiolabeled estradiol (0.2 nM, 0.5 nM, 1.5 nM, and 5.0 nM). Results indicated the triazines competed with the radioligand better at lower concentrations of tracer. For example, there was 60% displacement of 0.5 nM radiolabeled estradiol when co-exposed with 5 µM atrazine whereas there was 11% displacement of 5 nM radiolabeled estradiol when co-exposed with 50 µM atrazine. In the Scatchard-type plot, dissociation constants were produced; 0.5 nM in the presence of 100-fold molar excess of estradiol and 1 nM in the presence of 10,000 fold molar excess of atrazine and simazine. The x-intercepts of the plot were approximately equal (210 fmol/mg) in the presence of estradiol and simazine indicating competitive binding. The x-intercept for the atrazine plot was slightly less (158 fmol/mg) indicating the potential for competitive binding but also some noncompetitive binding under the disequilibrium conditions of this study.

Experiments using uteri extracted with KCl to extract the total ER and without KCl to extract the ER not bound or only loosely bound to chromatin were performed using cytosol fractions incubated with 50  $\mu$ M triazine or 500 nM estradiol under disequilibrium conditions followed by separation of the sample by sucrose density centrifugation. One hundred twenty five mL fractions were removed and counted for radioactivity. The authors generated plots representing the fraction number versus the radioactivity in the given fraction in order to observe which fraction exhibited the binding. In the extractions without KCl, the receptor binding peaks were detected in the 7-8S fraction for both estradiol and atrazine. Atrazine displaced an average of 10.7% of the radiolabeled estradiol whereas estradiol displaced all of the radiolabeled tracer. In the conditions favoring the extraction of total ER, activated and unactivated, binding was detected in the 4-5S fraction. Atrazine displaced an average of 29.6% of the radiolabeled estradiol whereas estradiol displaced all of the radiolabeled tracer.

Making the assumption that living rats contain the activated form of the ER, in order to test whether triazines competed more effectively against the transformed ER, ovariectomized rats were dosed with triazines prior to uterine dissection and competitive binding experiments. The binding of radiolabeled estradiol was reduced by an average of 33%, 39%, and 24% for atrazine, simazine, and DACT, respectively ( $p < 0.05$  vs. vehicle controls) in the 300 mg/kg/day group. In the 50 mg/kg/day group, binding of radiolabeled estradiol was reduced (not statistically significant) by 18, 21, and 13% .

**Overall the results indicate that atrazine, triazine, and DACT do exhibit some competitive binding with estradiol but only under conditions which favor triazine binding.**

This special study on *in vitro* and *in vivo* competitive binding of atrazine, DACT and simazine binding to the estrogen receptor in the rat is **Acceptable-nonguideline**.

*Failure of atrazine and simazine to induce estrogenic responses in MCF-7 human breast cancer cells. (MRID 43598619)*

Executive Summary:

This study (MRID no. 43598619) combines the following *in vitro* assays to investigate the estrogenic and antiestrogenic activity of atrazine and simazine: binding to hepatocyte Ah receptor; proliferation of MCF-7 (human breast cancer cell line) cells; gel electrophoresis mobility shift assay to measure levels of progesterone receptor; and MCF-7 transfection using a luciferase reporter gene.

Competitive binding to the aryl hydrocarbon (Ah) receptor was measured using TCDD as the competitor. This assay is performed because some dioxins which are strong Ah receptor agonists have also been shown to exhibit antiestrogenic activity. Cytosol from male rat hepatocytes containing Ah receptor was isolated and incubated with the positive control, radiolabeled 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), plus either atrazine or simazine. With addition of hydroxylapatite to this mixture, high molecular weight compounds, such as test compound bound to the Ah receptor will precipitate out of solution. Subsequent centrifugation of this solution will result in the Ah receptor, and anything bound to it, being spun out into a pellet. By counting the radioactivity in the pellet, it is possible to measure the amount of radiolabeled TCDD displaced indicating competitive inhibition with the triazine. Competitive binding was measured as the decrease in radioactivity versus the positive control. At concentrations up to 10,000 nM, atrazine and simazine did not displace TCDD from the Ah receptor.

A cell proliferation assay using the MCF-7 cell line was performed using the following doses: 10  $\mu$ M, 1.0  $\mu$ M, 0.1  $\mu$ M, and 0.01  $\mu$ M for atrazine and simazine and 1 nM for estradiol. Experiments using individual chemicals plus triazine were performed. Results indicated that the positive control increased the density of MCF-7 cells two fold relative to the negative control after 11 days. Exposure to atrazine and simazine alone neither increased nor decreased cell number at day 11 relative to the negative control. Results of co-exposure experiments indicate that cell number did not change relative to the positive control.

Estradiol has been shown to increase the level of progesterone receptor in mammalian cells. This assay measures the affect of estradiol and/or triazine on the binding capacity of a high affinity ligand (R5020) to the progesterone receptor which an indirect measure of progesterone level. The gel electrophoresis mobility shift assay utilized indirectly measures progesterone receptor (PR) levels in MCF-7 cells following exposure to atrazine, simazine, or estradiol for 3 days. Polyacrylamide gel electrophoresis can separate molecules, such as DNA, or complexes of molecules, such as protein-DNA complexes, based on size. The DNA used in this study is a radioactive oligonucleotide of a specific sequence which binds to the PR. This sequence is called a progesterone response element (PRE). PR isolated from MCF-7 cells is first incubated with R5020. PR-R5020 complex was then incubated with radiolabeled PRE. The R5020-PR-PRE complex was then run on a polyacrylamide gel. The unbound PRE was separated from the bound complex based on differences in mobility through the gel. Doses used were 1  $\mu$ M for atrazine and simazine and 10 nM for estradiol. Exposure to estradiol resulted in a 3.5-fold increase in reactivity of the R5020-PR-PRE complex relative to the negative control. The radioactivity in the R5020-PR-PRE band following exposure to atrazine and simazine was similar to negative control. The radioactivity in the band representing the R5020-PR-PRE complex was measured to get an indirect measure of PR levels in MCF-7 cells. Results indirectly indicate that the levels of PR did not increase or decrease with exposure to atrazine or simazine.



Transfection experiments were performed where the human estrogen receptor along with the luciferase expression system were inserted into MCF-7. The MCF-7 cell assay measured luciferase activity (*i.e.*, light production) and is quantitative in nature (*i.e.*, concentration of estrogenic chemical is directly related to light production). Doses used in the luciferase experiments were  $10^{-13}$  M to  $10^{-8}$  M estradiol and  $10^{-9}$  M to  $10^{-5}$  M atrazine and simazine. Atrazine and simazine did not induce luciferase activity above background at concentrations as high as  $10^{-5}$  M (concentrations above  $10^{-5}$  M are toxic to MCF-7 cells). Exposure to the positive control, estradiol, resulted in significant luciferase activity beginning at  $10^{-12}$  M. Light production increased exponentially up to  $10^{-8}$  M where light production plateaued.

**Neither atrazine nor simazine displayed estrogenic activity or interacted with the Ah receptor in the set of experiments described in this paper.**

This study is classified as **Acceptable-nonguideline** as a special study on *in vitro* estrogenic effects.

*Failure of chloro-S-triazine-derived compounds to induce estrogen receptor-mediated responses in vivo and in vitro. (MRID 4394403). Published in Fundamental and Applied Toxicology. 30:93-101.*

Executive Summary:

This study (MRID no. 4394403) combines three *in vivo* and four *in vitro* assays to investigate the estrogenic and antiestrogenic activity of atrazine and simazine. *In vivo* experiments, 4 or 5 female Sprague-Dawley rats/dose were dosed orally or by intraperitoneal injection. In order to test for estrogenic activity, rats were exposed to atrazine (> 97% a.i.) or simazine (> 97% a.i.) at 50, 150, and 300 mg/kg/day or estradiol (positive control) at 10  $\mu$ g/kg/day. In order to test for antiestrogenic activity, co-exposure experiments using atrazine or simazine plus estradiol were performed using the same concentrations. Following a three day exposure, the following *in vivo* endpoints were measured: uterine weight, progesterone receptor levels and uterine peroxidase.

Exposure to estradiol results statistically significant increased uterine weights, increased levels of the progesterone receptor, and increased activity of uterine peroxidase. Exposure to atrazine and simazine individually resulted in negative effects indicating antiestrogenic activity are shown in slightly reduced uterine weights, statistically significantly reduced levels of the progesterone receptor, and statistically significantly reduced activity of uterine peroxidase.

The following *in vitro* assays were performed: proliferation of MCF-7 (human breast cancer cell line) cells; gel electrophoresis mobility shift assay to measure levels of progesterone receptor; MCF-7 transfection using the luciferase reporter gene; and yeast transfection using a selective media expression system.

Doses for the cell proliferation assay when tested individually and in combination were 10  $\mu$ M, 1.0  $\mu$ M, 0.1  $\mu$ M, and 0.01  $\mu$ M for atrazine and simazine and 1 nM for estradiol. Results indicated that the positive control significantly increased the density of MCF-7 cells three fold relative to the negative control after 11 days. Exposure to atrazine and simazine alone neither increased nor decreased cell number at day 11 relative to the negative control. Results of co-exposure experiments indicate that cell number did not change relative to the positive control.

The gel electrophoresis mobility shift assay used in this study indirectly measures progesterone receptor (PR) levels in MCF-7 cells following exposure for 3 days. The oligonucleotide used contains progesterone response element (PRE) which binds to the PR. PR from MCF-7 cells was isolated and incubated with radiolabeled PRE. The PR-PRE complex was then run on a polyacrylamide gel to separate bound and unbound PRE based on differences in mobility through the gel. The radioactivity in the band representing the PR-DNA complex was measured as an indirect measure of PR levels. Doses used were 1  $\mu$ M for atrazine and simazine and 10 nM for estradiol. Results indicated that exposure to estradiol resulted in a 3.5-fold increase in reactivity of the DNA-PR complex relative to the negative control. The radioactivity in the DNA-PR band following exposure to atrazine and simazine was similar to negative control. These results indicate indirectly that the levels of PR did not increase or decrease with exposure to atrazine or simazine.

Two transfection experiments were performed where the human estrogen receptor was inserted into the genome of MCF-7 and yeast cells. The MCF-7 cell assay measured luciferase activity as a surrogate measure of estrogen receptor activity. Doses used were  $10^{-13}$ M to  $10^{-8}$ M estradiol and  $10^{-9}$ M to  $10^{-5}$ M atrazine and simazine. Atrazine and simazine did not induce luciferase activity above background at concentrations as high as  $10^{-5}$ M (concentrations above  $10^{-5}$ M are toxic to MCF-7 cells). Exposure to the positive control, estradiol, resulted in significant luciferase activity beginning at  $10^{-12}$ M and increased exponentially up to  $10^{-8}$ M where light production plateaued. Atrazine and simazine did not exhibit in estrogenic or antiestrogenic activity and did not interact directly with the estrogen receptor.

In the yeast transfection assay, the human estrogen receptor linked to a necessary amino acid was inserted into the yeast. Growth on selective media is a qualitative measure of activity of estrogen receptor. Yeast were dosed with 1 nM estradiol or 10  $\mu$ M atrazine or simazine. Yeast proliferated when exposed to estradiol but did not proliferate when exposed to atrazine or simazine. These results indicate that atrazine and simazine did not exhibit estrogenic activity and did not directly interact with the estrogen receptor.

**In conclusion, the results of these experiments indicate that *in vivo* atrazine and simazine exhibited some antiestrogenic activity but no estrogenic activity. Based on the *in vitro* results, this antiestrogenic activity is not the result of *direct* interaction with the estrogen receptor.**

This study is classified as **acceptable-nonguideline** as a special study on *in vivo* and *in vitro* estrogenic effects.

**SignOff Date:**  
**DP Barcode:**  
**HED DOC Number:**  
**Toxicology Branch:**

**3/24/99**  
**D253661**  
**013281**  
**RCAB**

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EPA Reviewer: Anna Bearden, PhD.  
EPA Secondary Reviewer: Roger Hawks, PhD.

\_\_\_\_\_, Date \_\_\_\_  
\_\_\_\_\_, Date \_\_\_\_

DATA EVALUATION RECORD

STUDY TYPE: *In vivo* endocrine effects in rats. Special study.

DP BARCODE: D253661  
P.C. CODE: 080803 (Atrazine); 080807 (Simazine)

SUBMISSION CODE: S557393  
TOX. CHEM. NO.: 063 Atrazine  
740 Simazine

TEST MATERIAL (PURITY): Atrazine and Simazine (>96%)

SYNONYMS: G-30027 (Atrazine); G-27692 (Simazine)

CITATION: Eldridge, J.C. *et al.* (1994) Short-term effects chlorotriazines on estrus in female Sprague-Dawley and Fischer 344 rats. Department of Physiology and Pharmacology, Wake Forest University and Department of Toxicology, Ciba-Geigy Corporation, Greensboro, NC . Laboratory report number: N/A. MRID 43598614. Published in *Journal of Toxicology and Environmental Health*. 43: 155-167.

SPONSOR: Ciba Crop Protection, Ciba-Geigy Corporation (presently Novartis), PO BOX 18300, Greensboro, NC 27419.

EXECUTIVE SUMMARY:

In a special study (MRID 43598614) on *in vivo* endocrine effects, atrazine and simazine (>96 % a.i.) were administered to 11 female rats/dose/strain (both Sprague-Dawley and Fischer 344 rats were used) by oral gavage at dose levels of 0, 100, and 300 mg/kg/day for 14 to 23 days depending on time to achieve proestrus. Some animals did not achieve proestrus and were sacrificed at day 23. Body weights and vaginal smears were taken daily. Blood was collected at termination for analysis of the following blood plasma hormones : estradiol, progesterone, corticosterone, prolactin. Ovaries, uterus, and adrenals were weighed at necropsy. Vaginal cytology, estrus cycle length, and percent days in cycle were observed.

One Sprague-Dawley and one Fischer (F) 344 rat in the 300 mg/kg/day atrazine group died during the study. One Sprague-Dawley (S.D.) in the 100 mg/kg/day simazine group died during the study. Body weight decreases of rats exposed to atrazine are statistically significant in both the low (-7.7% S.D.; -4.4% F) and high dose groups (-22.0% S.D.; -14.2% F). Body weight decreases of rats exposed to simazine are statistically significant in the high dose group. Plasma levels of prolactin and corticosterone were not effected by either triazine in both strains of rat.

Sprague-Dawley and Fischer 344 female rats differ in their endocrine response to atrazine and simazine. Significant reductions in plasma levels of estradiol (-61% and -90% for low and high doses) were measured in atrazine treated Sprague-Dawley rats. These decreases were paralleled

by significant reductions in uterine weight (-28% and -44% for low and high doses). Sprague-Dawley rats exposed to atrazine exhibited a significant increase in percentage of time spent in the estrous cycle (+14% in high dose) with a parallel decrease in the percentage of time spent in diestrus (-13% in high dose) relative to the untreated control. The relative increase in percentage of the estrous cycle spent in estrus exhibited by Sprague-Dawley rats is exemplified by dose dependent and significant increases in the cell density index of both cornified (+22% and +38% for the low and high dose) and nucleated (+42% for the high dose) epithelial vaginal cells relative to untreated control.

There were no statistically significant alterations in plasma hormone levels observed in the Fischer 344 rats. Although the mean values of plasma estradiol, progesterone, and prolactin decreased by 28% to 63% with atrazine exposure and increased 9% to 53% with simazine exposure, the relatively large standard deviations (from  $\pm 30\%$  to 184% of the mean) preclude significant differences. Contrary to Sprague-Dawley rats, following exposure to atrazine, the cell density index of cornified epithelial vaginal cells in Fischer 344 rats significantly decreased (-24% and -41% for the low and high dose) relative to the negative control with no effects on nucleation. In Fischer 344 rats, the decrease in cornified epithelial cells parallels the significant decrease in percentage of time spent in the estrous cycle (-24% for high dose) with parallel increase (although not statistically significant) in the percentage of time spent in diestrus (+11% for the high dose).

There were conflicting results between the plasma level of progesterone in Sprague-Dawley and Fischer 344 rats and between atrazine and simazine. These contrary observations result from the variability in the measurement of progesterone in plasma and are not toxicologically relevant.

**The LOAEL for systemic toxicity is 100 mg/kg/day for both atrazine and simazine, based on body weight effects and organ weight effects for atrazine. The NOAEL for toxicity cannot be determined.**

**The LOAEL for endocrine effects of atrazine is 100 mg/kg/day based on organ weight effects, plasma hormone changes (estradiol), estrus cycle lengthening, and vaginal cytology. The NOAEL for endocrine effects of atrazine cannot be determined.**

**The LOAEL for endocrine effects of simazine is 300 mg/kg/day based on organ weight effects and vaginal cytology. The NOAEL for endocrine effects of simazine is 100 mg/kg/day.**

This special study on the endocrine effects of atrazine and simazine in the rat is **Acceptable-nonguideline**.

COMPLIANCE: Signed and dated GLP and Data Confidentiality were provided. Quality Assurance and Flagging Statements were not provided. This study was published in the public literature and therefore was not subject to these requirements.

## I. MATERIALS AND METHODS

### A. MATERIALS:

1. Test Material: Atrazine and Simazine  
Description not given  
Lot/Batch #: Not given.  
Purity: > 96 % a.i.  
Stability of compound: Not given  
CAS #: 1912-24-9 Atrazine; 122-34- 9 Simazine
2. Vehicle control: Suspensions were made using 0.5% carboxymethylcellulose in tap water as the vehicle.
3. Test animals:  
Species: female rat  
Strain: Sprague-Dawley and Fischer 344  
Age and weight at study initiation: 10-13 weeks old; weight not given  
Source: Charles River Corporation, Raleigh, NC  
Housing: 2 per cage; All animals were kept in the same room.  
Diet: lab feed provided *ad libitum*  
Water: provided *ad libitum*  
Environmental conditions:   Temperature: Not given  
  Humidity: Not given  
  Air changes: Not given  
  Photoperiod: 14 hours light/ 10 hours dark  
Acclimation period: Not given

### B. STUDY DESIGN:

1. In life dates - Not given
2. Animal assignment

Animals were randomly assigned (method not given) such that mean weights and standard deviations for each dose group were the same. Vaginal cytology was performed daily for 2 weeks prior to initial treatment. Only animals exhibiting regular cycles were used in the study.

TABLE 1: STUDY DESIGN

Test Group	Conc. in Diet (mg/kg/day)	Number of animals
Control	0	11
Low	100	11
High	300	11

3. Dose selection rationale:  
Based on published studies reviewed by HED [Stevens, *et al.*, 1994 (MRID no. 43598611); Tennant *et al.*, 1994a, b (MRID no. 435986117 and 435986118); Wetzel, *et al.*, 1994 (MRID no. 43598615)] which have reported estrogenic and antiestrogenic responses resulting from exposure to atrazine and simazine at similar doses.
4. Diet preparation and analysis : Not given.  
Results : Homogeneity, Stability, and Concentration Analysis: Not evaluated
5. Statistics - Data were analyzed using Statistical Analysis System. Body and organ weight data were analyzed using a one-way ANOVA. One-way ANOVA of rank scores was performed on plasma hormone measurements to correct "nonnormal and/or heterogeneous variances." Cycle length data and vaginal cytology data were analyzed by 2-way ANOVA. If ANOVA was significant, Dunnett's test was used.

### C. METHODS:

1. Observations:  
Vaginal smears and body weight were obtained daily. Vaginal smears were observed daily for epithelial cell type. Cell density index of cornified and nucleated cells was calculated by adding the total scores for all observation days and normalizing for 10 days of observation. Epithelial cells which are "cornified" have changed from cuboidal to stratified squamous. Relative density was scored as 2 = heavy density; 1 = moderate density; 0 = light density.



Ovulation was defined by “the appearance of a heavy density of cornified cells followed the next day by a dense appearance of nucleated cells, secretory matrix, and leukocytes.” The length of the estrus cycle was defined as the number of days between ovulation. Estrus was characterized as “a day with very dense cornified cells...particularly if the following day’s pattern was not dense in cornified cells.” (MRID no. 42743902; Eldridge, J.C., *et al.* 1993). Proestrus occurred the day before estrus and was noted by “the first appearance of a mixed nucleated-cornified cell pattern that followed 1-2 days of few epithelial cells”(MRID no. 43598614 p. 158). Diestrus usually occurred the day after estrus and has been defined as “few epithelial cells of either type and, typically, an increased density of leukocytes; or dense nucleated epithelium, few cornified cells and often numerous leukocytes (usually the day after estrous).” (MRID no. 42743902; Eldridge, J.C., *et al.* 1993).

2. Administration of chemical:

Animals were administered 1 mL/100 g of body weight of either 10 or 30 mg/mL triazine suspension. Suspensions were made using 0.5% carboxymethylcellulose in tap water as the vehicle. Doses were administered daily by oral gavage.

3. Sacrifice, Blood Collection, and Hormone measurements:

Blood was collected at termination by inverting the carcass immediately following decapitation over a heparinized container.

The following hormones were analyzed by radioimmunoassay (Mahesh *et al.*, 1976) from the blood plasma: estradiol, progesterone, corticosterone, prolactin.

Doses were administered daily by oral gavage for a minimum of 14 to a maximum of 23 days depending on time to achieve proestrus. Animals were scheduled to be sacrificed at proestrus stage of the estrous cycle. Some animals did not achieve proestrus during the study and were sacrificed at day 23. Rats were killed by rapid decapitation.

5. Organ Weights:

The ovaries, uterus, and adrenals were collected and weighed at necropsy. Organ weights were compared to body weight to generate relative ratios.

## II. RESULTS

### A. Observations

#### 1. Toxicity -

No clinical signs were described in the study report.

#### 2. Mortality -

One Sprague-Dawley and one Fischer 344 in the 300 mg/kg/day atrazine group died during the study. One Sprague-Dawley in the 100 mg/kg/day simazine group died during the study. The study report did not describe the death of the animals.

### B. Body weight

As shown in Table 2, there is a dose dependant decrease in body weight and weight gain for both strains of rat exposed to atrazine and simazine. Body weight decreases of rats exposed to atrazine are statistically significant in both the low and high dose groups. Body weight decreases of rats exposed to simazine are statistically significant in the high dose group.

TABLE 2. BODY WEIGHT OF RATS EXPOSED TO ATRAZINE AND SIMAZINE.  
(MEANS AND STANDARD DEVIATIONS ARE GIVEN).

	Sprague-Dawley			Fischer344		
	0 mg/kg/day	100 mg/kg/day	300 mg/kg/day	0 mg/kg/day	100 mg/kg/day	300 mg/kg/day
<b>Atrazine</b>						
<b>Pretest (g)</b>	273.8 ± 18.2	270.7 ± 15.3	272.5 ± 19.0	159.8 ± 7.1	158.4 ± 4.5	160.8 ± 5.9
<b>Termination (g)</b>	288.7 ± 16.7	250.0 ± 14.4*	211.9 ± 20.5*	161.0 ± 6.5	151.5 ± 4.0*	138.4 ± 3.5*
<b>Weight gain (g)</b>	14.9 (+5.4%)	-20.7* (-7.7%)	-63.9* (-22.2%)	1.2 (+0.8%)	-6.9* (-4.4%)	-22.4* (-14.2%)
<b>Simazine</b>						
<b>Pretest (g)</b>	339.2 ± 20.0	336.6 ± 25.0	337.5 ± 22.3	167.5 ± 10.2	168.8 ± 6.4	166.9 ± 9.7
<b>Termination (g)</b>	348.3 ± 23.7	331.6 ± 25.0	319.3 ± 18.0*	170.0 ± 9.3	163.6 ± 7.0	160.3 ± 7.3*
<b>Weight gain (g)</b>	9.1 (+2.7%)	-5.0 (-1.5%)	-18.2* (-5.4%)	2.5 (+1.5%)	-5.2 (-3.3%)	-6.6* (-4.0%)

\*Significantly different from control ( $p < 0.05$ ). Data from MRID no. 43598614 p. 159-160.

#### E. Blood work

##### Plasma hormones-

As shown in Table 3, Sprague-Dawley rats exposed to atrazine and simazine showed a dose dependant decrease in estradiol. Plasma levels of progesterone increased with atrazine exposure and decreased with simazine exposure in Sprague-Dawley rats.

There were no statistically significant alterations in plasma hormone levels observed in the Fischer 344 rats. Although the mean values of plasma estradiol, progesterone, and prolactin decreased by 28% to 63% with atrazine exposure and increased 9% to 53% with simazine exposure, the relatively large standard deviations (from ± 30% to ± 184% of the mean) preclude significant differences. The raw data were not supplied in the study report for further evaluation of the toxicological significance of this data.

TABLE 3. PLASMA HORMONE LEVELS OF RATS EXPOSED TO ATRAZINE AND SIMAZINE. (MEANS AND PERCENT CHANGE RELATIVE TO CONTROL ARE GIVEN).

Plasma hormone	Sprague-Dawley			Fischer344		
	0 mg/kg/day (±SD)	100 mg/kg/day	300 mg/kg/day	0 mg/kg/day (±SD)	100 mg/kg/day	300 mg/kg/day
<b>Atrazine</b>						
Estradiol (pg/mL)	64.9± 87.5	25.2± 28.6* (-61%)	6.5± 6.7* (-90%)	29.4 ± 29.1	19.3± 17.6 (-34%)	11.1± 3.9 (-62%)
Progesterone (ng/mL)	5.9± 3.5	6.3± 2.2 (+7%)	12.6 ± 10.2* (+114%)	17.6± 10.9	12.7± 8.2 (-28%)	10.4± 9.9 (-41%)
Prolactin (ng/mL)	3.5± 5.5	3.0± 5.5 (-14%)	4.4± 7.0 (+26%)	17.1± 15.1	10.6± 13.0 (-38%)	6.2± 11.4 (-63%)
Corticosterone (ng/mL)	359.3 ± 241.3	345.2± 169.3 (-4%)	174.7± 89.5 (-52%)	635.4± 182.2	516.6± 520.7 (-19%)	665.3± 430.9 (+5%)
<b>Simazine</b>						
Estradiol (pg/mL)	27.7± 22.0	18.8± 19.4 (-32%)	16.3± 17.0 (-41%)	18.9± 18.5	20.5 ± 8.4 (+9%)	25.5 ± 21.9 (+35%)
Progesterone (ng/mL)	5.4 ± 1.4	4.3± 1.9 (-26%)	2.1± 0.8 (-61%)	6.5± 1.8	5.7 ± 1.0 (-12%)	5.8 ± 1.6 (-11%)
Prolactin (ng/mL)	2.7± 1.5	2.9± 1.7 (+7%)	2.6± 1.2 (-4%)	14.5± 7.7	16.5± 9.3 (+14%)	22.2± 8.4 (+53%)
Corticosterone (ng/mL)	180.6± 239.9	323.1± 149.3 (+79%)	82.3± 8.7 (-55%)	286.7± 241.4	206.0± 182.8 (-28%)	162.0± 147.2 (-43%)

\*Significantly different from control ( $p < 0.05$ ). Data from MRID no. 43598614 p. 162-163.

## G. Organ weights:

As shown in Table 4, rats in both strains exposed to atrazine exhibited statistically significant decreases organ weight and organ to body weight ratio in the ovaries and uteri and statistically significant increases for adrenal glands at both the low and high dose.

The organ weight and organ to body weight ratio of the adrenal glands of Fischer rats exposed to simazine showed an significant increase at the high dose. The ovaries and uteri of both strains were not effected by simazine exposure.

TABLE 4. ORGAN WEIGHTS AND ORGAN TO WEIGHT RATIOS OF RATS EXPOSED TO ATRAZINE AND SIMAZINE. MEANS AND STANDARD DEVIATIONS ARE GIVEN. VALUES IN PARENTHESES ARE PERCENT CHANGE RELATIVE TO CONTROL.

	0 mg/kg/day	Sprague- Dawley 100 mg/kg/day	300 mg/kg/day	0 mg/kg/day	Fischer344 100 mg/kg/day	300 mg/kg/day
<b>Atrazine</b>						
<b>Ovaries</b>						
Organ weight (mg)	120.8± 14.8	87.6* ± 10.5 (-27%)	69.7*± 4.4 (-42%)	84.4 ± 10.8	60.9* ± 16.6 (-28%)	54.7* ± 8.0 (-35%)
O/BW ratio	41.9 ± 5.4	35.1*± 4.4 (-16%)	33.1*± 3.4 (-21%)	52.5± 7.7	40.1*± 10.4 (-24%)	39.6*± 6.2 (-26%)
<b>Uterus</b>						
Organ weight (mg)	627.4± 101.3	449.1*± 81.5 (-28%)	352.4*± 61.4 (-44%)	415.1± 55.7	308.1*± 46.4 (-26%)	242.9* ± 67.9 (-41%)
O/BW ratio	218.8± 42.1	179.4* ± 29.1 (-19%)	167.9*± 34.8 (-23%)	257.7± 31.6	203.3*± 29.3 (-21%)	174.7*± 44.7 (-32%)
<b>Adrenal glands</b>						
Organ weight (mg)	60.2± 8.1	77.6*± 13.1 (+28%)	89.3*± 13.3 (+48%)	45.9± 4.9	64.7*± 11.9 (+41%)	71.8*± 4.4 (+56%)
O/BW ratio	20.9± 2.7	31.0* ± 5.1 (+48%)	42.2*± 5.0 (+102%)	28.5± 3.4	42.7*± 7.5 (+50%)	51.9*± 4.2 (+82%)
<b>Simazine</b>						
<b>Adrenal glands</b>						
Organ weight (mg)	73.4 ± 9.1	73.5± 13.2	80.9± 9.5 (+10%)	50.4± 11.0	49.8± 5.6	63.1*± 9.5 (+25%)
O/BW ratio	21.1± 2.7	21.1± 3.0	25.4 ± 3.3 (+20%)	29.8± 6.8	30.5 ± 3.9	39.4*± 5.4 (+32%)

\*Significantly different from control (p < 0.05). Data from MRID no. 43598614 p. 159-160.

3. Microscopic pathology -

## Vaginal cytology-

The values in Table 5 represent the average daily cell density index of one vaginal smear per animal normalized for 10 days of observation. Simazine did not effect the cell density index for vaginal cytology any dose level.

Effects on vaginal cytology of the epithelium with atrazine exposure are rat strain dependant. Sprague-Dawley rats exposed to atrazine showed a dose-dependant and statistically significant increase in cornification and nucleation of vaginal epithelial cells. Fischer 344 exposed to atrazine showed a dose-dependant and statistically significant decrease in cornification vaginal cells with no effects on nucleation.

TABLE 5. EFFECTS ON THE VAGINAL CYTOLOGY IN RATS EXPOSED TO ATRAZINE. MEANS AND STANDARD DEVIATION OF CELL DENSITY INDEX ADJUSTED FOR 10 DAYS OF OBSERVATION ARE GIVEN.

	0 mg/kg/day	Sprague- Dawley 100 mg/kg/day	300 mg/kg/day	0 mg/kg/day	Fischer 344 100 mg/kg/day	300 mg/kg/day
<b>Atrazine</b>						
<b>Cornified epithelial cells</b>	6.8± 0.8	8.3 ± 0.5*	9.4± 0.8*	5.8± 1.1	4.4± 0.8*	3.4 ± 1.1*
<b>Nucleated epithelial cells</b>	7.1± 1.1	8.1± 1.1	10.1± 1.4*	8.6 ± 0.9	9.4± 1.2	8.7± 0.9
<b>Simazine</b>						
<b>Cornified epithelial cells</b>	7.3± 2.0	6.4± 1.3	6.6 ± 1.3	6.6± 0.8	6.7± 1.6	7.0± 1.5
<b>Nucleated epithelial cells</b>	6.4± 1.9	6.9± 2.2	6.6± 1.1	7.1± 1.2	6.8± 1.2	6.4 ± 1.4

\*Significantly different from control (p < 0.05). Data from MRID no. 43598614 p. 164.

H. Estrous cycle duration.

As indicated by the statistical significance ( $p < 0.05$ ) of the estrous cycle length of Sprague-Dawley rats exposed to 100 and 300 mg/kg/day atrazine, the authors claim that there is a trend towards longer estrous cycles. The mean values of estrous cycle increase slightly with increasing triazine exposure but these increases are accompanied by relatively large standard deviations (about  $\pm 30\%$  of the mean) at all doses for both atrazine and simazine. Without the raw data to further evaluate this data, it is difficult to interpret the toxicological significance of this finding.

TABLE 6. LENGTH OF THE ESTROUS CYCLE IN RATS EXPOSED TO ATRAZINE. MEANS AND STANDARD DEVIATION GIVEN.

	Sprague-Dawley			Fischer344		
	0 mg/kg/day	100 mg/kg/day	300 mg/kg/day	0 mg/kg/day	100 mg/kg/day	300 mg/kg/day
<b>Atrazine</b>						
<b>Length of estrous cycle (days)</b>	4.2± 0.4	4.8± 1.1*	4.9± 1.5*	5.2± 0.7	5.7± 1.2	5.7± 1.7
<b>Simazine</b>						
<b>Length of estrous cycle (days)</b>	4.7± 1.0	5.1± 2.0	5.1± 1.7	5.0± 0.8	4.9± 1.1	5.4± 1.4

\*Significantly different from control ( $p < 0.05$ ). Data from MRID no. 43598614 p. 164.

I. Percent of the estrous cycle spent in different stages.

As shown in Table 7, the percent of cycle days in estrus, proestrus, or diestrus were not effected by simazine exposure relative to the negative control.

Effects of atrazine are rat strain dependant. There were no changes in length of proestrus with atrazine exposure. There is an statistically significant increase in the percent of cycle days in estrus which parallels a statistically significant decrease in the percent of cycle days in diestrus with exposure to atrazine at both the high and low dose for Sprague-Dawley rats.

For Fischer 344 rats, there is a dose-dependant and statistically significant decrease in the percent of cycle days in estrus and a parallel increase (not statistically significant) in the percent of cycle days in diestrus with exposure to atrazine at the high dose.

TABLE 7. EFFECTS ON THE PERCENT OF THE ESTROUS CYCLE. VALUES REPRESENT PERCENT OF TOTAL DAYS SPENT IN EACH STAGE OF THE ESTROUS CYCLE IN RATS EXPOSED TO ATRAZINE. MEANS AND STANDARD DEVIATIONS GIVEN.

Percent of total cycle	Sprague-Dawley			Fischer 344		
	0 mg/kg/day	100 mg/kg/day	300 mg/kg/day	0 mg/kg/day	100 mg/kg/day	300 mg/kg/day
<b>Atrazine</b>						
<b>Estrus</b>	21.3± 1.9	26.8 ± 4.5*	35.4± 5.2*	19.3± 3.7	17.1± 5.0	14.1± 4.7*
<b>Proestrus</b>	25.3± 1.0	25.2 ± 3.3	24.5± 4.3	20.5± 2.6	18.5± 2.4	18.6± 3.4
<b>Diestrus</b>	53.4± 1.8	48.1 ± 3.1*	40.1± 3.3*	60.2 ± 3.9	64.3± 5.7	67.1± 6.5
<b>Simazine</b>						
<b>Estrus</b>	25.2 ± 8.8	21.7± 5.6	20.5± 3.0	24.5± 3.7	24.9± 8.7	22.7± 4.6
<b>Proestrus</b>	23.8± 4.2	23.1± 5.5	24.1± 3.7	18.5± 2.1	23.1± 6.4	20.7± 3.9
<b>Diestrus</b>	51.1± 8.5	55.2± 7.6	55.3± 5.1	57.0 ± 4.7	52.1± 8.0	56.6± 4.9

\*Significantly different from control (p < 0.05). Data from MRID no. 43598614 p. 165.



### III. DISCUSSION

- A. Sprague-Dawley and Fischer 344 female rats differ in their endocrine response to atrazine and simazine. Significant reductions in plasma levels of estradiol (-61% and -90% for low and high doses) were measured in atrazine treated Sprague-Dawley rats. These decreases were paralleled by significant reduction in uterine weight (-28% and -44% for low and high doses). Sprague-Dawley rats exposed to atrazine exhibited a significant increase in percentage of time spent in the estrous cycle (+14% in high dose) with a parallel decrease in the percentage of time spent in diestrus (-13% in high dose) relative to the untreated control. Estrus has been characterized as "a day with very dense cornified cells...particularly if the following day's pattern was not dense in cornified cells." (MRID no. 42743902; Eldridge, J.C., *et al.* 1993). Based on this characterization, the relative increase in percentage of the estrous cycle spent in estrus exhibited by Sprague-Dawley rats is exemplified by dose dependent and significant increases in the cell density index of both cornified (+22% and +38% for the low and high dose) and nucleated (+ 42% for the high dose) epithelial vaginal cells relative to untreated control.

There were no statistically significant alterations in plasma hormone levels observed in the Fischer 344 rats. Although the mean values of plasma estradiol, progesterone, and prolactin decreased 28% to 63% with atrazine exposure and increased 9% to 53% with simazine exposure, the relatively large standard deviations (from  $\pm 30\%$  to 184% of the mean) undermine the toxicological significance of these alterations. The raw data was not supplied in the study report for further evaluation of the toxicological significance of this data. Contrary to Sprague-Dawley rats, following exposure to atrazine, the cell density index of cornified epithelial vaginal cells in Fischer 344 rats significantly decreased (-24% and -41% for the low and high dose) relative to the negative control with no effects on nucleated cells. Diestrus has been defined as "few epithelial cells of either type and, typically, an increased density of leukocytes; or dense nucleated epithelium, few cornified cells and often numerous leukocytes (usually the day after estrus)." (MRID no. 42743902; Eldridge, J.C., *et al.* 1993). In Fischer 344 rats, the decrease in cornified epithelial cells parallels the significant decrease in percentage of time spent in the estrous cycle (-24% for high dose) with parallel increase (although not statistically significant) in the percentage of time spent in diestrus (+11% for the high dose).

As indicated by the statistical significance ( $p < 0.05$ ) of the estrous cycle length of Sprague-Dawley rats exposed to 100 and 300 mg/kg/day atrazine, the authors claim that there is a trend towards longer estrous cycles. The mean values of estrous cycle increase slightly with increasing triazine exposure but these increases are accompanied by relatively large standard deviations (about  $\pm 30\%$  of the mean) at all doses for both atrazine and simazine. When considered with the vaginal cytology and plasma hormone results for Sprague-Dawley rats, the lengthening of the estrous cycle may be toxicologically related. Fischer 344 rats exhibited opposite vaginal cytology results than did the Sprague-Dawley rats but yet exhibited a similar trend in the total length of the estrous cycle. Without the raw data to further evaluate this data, it is difficult to interpret the toxicological significance of this finding.

There are conflicting results for plasma progesterone levels both between rat strains and triazine compound. According to Table 3, there is a significant increase in the plasma level in Sprague-Dawley rats of progesterone (+114% for the high dose) with atrazine exposure. With exposure to simazine, the level of plasma progesterone significantly decreased (-61%). Fischer rats exhibited a decrease in plasma progesterone with atrazine (-41% for high dose) and simazine (-11% for high dose) exposure. Additionally, the standard deviations for these values range from 30% to 184% of the mean indicating high variability within these samples. These observations result from the variability in the measurement of progesterone in plasma and are not toxicologically relevant.

Exposure to both atrazine and simazine resulted in toxic effects at both doses tested as shown by changes in body weight and body weight gain. Therefore, no NOAEL for atrazine or simazine toxicity could be established. A statistically significant increase in ovary and uterine weight and a statistically significant decrease in adrenal gland weight was observed with atrazine treatment at both dose levels. Therefore no NOAEL for endocrine effects of atrazine could be established.

#### B. Study deficiencies

Only 2 doses were used which limited assignment of NOAELs or LOAELs. Additionally both of the doses used significantly effected the body weights of the rats. It would have been useful to have a lower concentration to evaluate a more "real life" exposure concentration.

It would be useful to know the percent of animals which did not achieve proestrus.

The raw data was not provided making the evaluation of the estrous cycle length and plasma hormone level evaluation very difficult since standard deviations were large relative to the means. Pretest data of plasma hormone levels is also needed for adequate evaluation of this data. Raw data with corresponding animal number and housing assignment would also be helpful to evaluate housing effects. Raw data of actual number of days (instead of % of total days) spent in each part of the estrous cycle would be helpful also.

Explanation for the large variation in plasma hormone levels would be useful in the review of this data. Plasma hormone level vary greatly based on the globulin concentration. Measurements of globulin would also be helpful.

## IV. REFERENCES

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**Atrazine and Simazine**

***In vivo* endocrine effects. Special study.**

SignOff Date: 3/24/99  
DP Barcode: D253661  
HED DOC Number: 013281  
Toxicology Branch: RCAB

EPA Reviewer: Anna Bearden, Ph. D.  
EPA Secondary Reviewer: Roger Hawks, Ph. D.

\_\_\_\_\_, Date \_\_\_\_  
\_\_\_\_\_, Date \_\_\_\_

DATA EVALUATION RECORD

STUDY TYPE: *In vivo* estrogenicity. Special study.

DP BARCODE: 253661

P.C. CODE: 080803 (Atrazine)  
080807 (Simazine)

SUBMISSION CODE: S557393

TOX. CHEM. NO.: 063 Atrazine  
740 Simazine

TEST MATERIAL (PURITY): Atrazine, Diaminochlorotriazine (DACT), and Simazine (>96%)

SYNONYMS: G-30027 (Atrazine); G-28273 (DACT); G-27692 (Simazine)

CITATION: Tennant, M.K., *et al.* 1994. Possible antiestrogenic properties of chloro-s-triazines in rat uterus. Wake Forest University, Winston-Salem, NC and Department of Toxicology, Ciba Crop Protection Division, Ciba-Geigy Corporation, Greensboro, NC. MRID 43598617 Laboratory number: N/A. Published in *Journal of Toxicology and Environmental Health*. 43: 183-196.

SPONSOR: N/A

EXECUTIVE SUMMARY:

This study (MRID no. 43598617) combines three *in vivo* assays to investigate the estrogenic effects of atrazine, simazine, and diaminochlorotriazine [DACT, (a.i. > 96%)] on body weight, body weight gain, uterine weight, progesterone receptor binding capacity, and thymidine incorporation on female Sprague-Dawley rats.

For body weight and uterine weight measurements, ovariectomized rats were dosed by oral gavage daily for 3 days at 20, 100, and 300 mg/kg/day in single exposure experiment and in co-exposure experiments with 2 µg estradiol on day 2 and 3. Body weight gains were decreased for rats exposed to the vehicle control, 0.5% carboxymethylcellulose, (-3.5%) and to the positive control, 2 µg estradiol, (-6.9%). Larger decreases in weight gain were observed in rats exposed to triazines. At 20 mg/kg/day weight losses ranged from 6-9%. At 100 mg/kg/day, weight losses ranged from 8-13.5%. At 300 mg/kg/day, weight losses ranged from 11-17%.

Chemicals with estrogenic activity cause proliferation and thickening of the uterine wall. The uterine weights of rats exposed to triazines were similar to vehicle control uterine weights. In co-exposure experiments with estradiol, exposure to atrazine and DACT at 100 and 300 mg/kg/day resulted in a dose-dependant and statistically significant decrease in uterine weight relative to the positive control. In co-exposure experiments with estradiol, uteri of rats exposed to simazine at

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100 and 300 were significantly decreased relative to the positive control.

Thymidine incorporation was measured to assess the effect triazines on DNA synthesis in the uterus of exposed rats. Dose groups included: exposure to 300 mg/kg/day of triazine alone administered by oral gavage for 2 days; and exposure to 1, 10, 20, 50, 100, and 300 mg/kg/day of triazine administered by oral gavage for 2 days plus an injection of 0.15  $\mu$ g estradiol on day 2. Exposure to triazines alone at 300 mg/kg/day resulted in significant decrease relative to the vehicle control. Exposure to the positive control, 0.15  $\mu$ g estradiol, resulted in approximately 2.5 fold increase ( $p < 0.05$ ) in thymidine incorporation relative to the vehicle control. Exposure to 50, 100, and 300 mg/kg/day in co-exposure with 0.15  $\mu$ g estradiol resulted in a significant reduction in thymidine incorporation relative to the positive control.

Levels of inducible progesterone receptor were indirectly measured by the PR binding capacity to a radioactive ligand. The PR was isolated from uteri of rats exposed by oral gavage to atrazine, DACT, and simazine alone at 300 mg/kg/day for 2 days and to atrazine, DACT, and simazine at 50 and 300 mg/kg/day in co-exposure with 1  $\mu$ g/day estradiol for 2 days. The PR binding capacity in rat uteri exposed to 300 mg/kg/day triazine alone was statistically decreased relative to vehicle control. PR binding capacity decreased significantly relative to the positive control with exposure to atrazine, DACT, and simazine at 300 mg/kg/day in co-exposure experiments with 1  $\mu$ g estradiol.

**According to the effects on uterine weight, progesterone binding capacity, and thymidine incorporation at the concentrations tested in this study, atrazine, DACT, and simazine do not exhibit estrogenic activity.**

This study is classified as **acceptable-nonguideline** as a special study on *in vivo* and *in vitro* estrogenic effects.

**COMPLIANCE:** Signed and dated GLP, Quality Assurance, Data Confidentiality, and Flagging statements were not provided. This study was published in the public literature and therefore was not subject to these requirements.

## I. Organization of this DER

This study entails three different *in vivo* assays. Because of the diversity in experiment design, this DER is separated into specific sections describing different assays. Each section has a separate materials and methods, and results. There is one discussion at the end.

## II. Description of different assays.

### 1. Uterine weight assay

This assay measures the weight of uteri taken from ovariectomized young female rats which have been exposed to a test chemical for 3 days. As an *in vivo* assay, it incorporates important factors such as toxicokinetics and metabolism which are ignored in typical *in vitro* studies. Increases in uterine weight result from proliferation and thickening of the uterine wall induced by estrogen or chemicals with estrogenic activity.

### 2. Progesterone receptor assay

Progesterone receptor (PR) binding capacity is an indirect measure of PR levels in the cell were measured using the methods described by Lamb and Bullock (1983) and Dickerson and Safe (1992).

Estradiol has been shown to increase the binding capacity of the progesterone receptor. Binding capacity is an indirect measure of receptor level. This assay measures the affect of estradiol and/or triazine on the capacity of the progesterone receptor to bind to a high affinity ligand (R5020). In this study, rats were exposed to atrazine, simazine, and /or estradiol for 2 days. Following sacrifice, the dissected uteri are homogenized and then separated into cytosol and membrane fractions by ultracentrifugation. The cytosolic fraction contains the PR. A radiolabeled compound called R5020 is used to measure the concentration of PR in the sample. R5020 is a protein ligand for the PR which has high affinity for the PR. Radiolabeled R5020 will bind PR in the uterine tissue sample. After separating R5020-PR complex from unbound R5020, scintillation counts can be used to measure PR levels in the different dose groups. It is assumed that one molecule of R5020 binds to one molecule of PR.

### 3. Thymidine incorporation:

The method of determining the thymidine incorporation rate into uterine DNA utilized in this study was similar to Stormshak *et al.* (1976).

Thymidine is a nucleoside which contains an aromatic base called thymine and a sugar moiety. Thymidine can be phosphorylated into the nucleotide form which then can be incorporated into DNA. When cells divide they must replicate (*i.e.*, make a new copy) their DNA. During DNA replication, thymidine is integrated into the newly synthesized

strands. Therefore, incorporation of thymidine is in turn correlated with cell division. In this study, incorporation of radiolabeled thymidine into DNA is measured in the uteri of rats exposed to triazines and/or estradiol to measure synthesis of new DNA. Estradiol has been shown previously to increase DNA synthesis in uterine tissue of rats.

**III. Uterine Weight.**

**A. MATERIALS**

1. Test Material: Atrazine, Simazine, and DACT  
Description: Not given  
Lot/Batch #: Obtained by author from Ciba Plant Protection Division (Greensboro, NC).  
Purity: > 96% a.i.  
Stability of compounds: Not given  
CAS #: 1912-24-9 (Atrazine); 122-34-9 (Simazine); 3397-62-4 (DACT)
  
2. Vehicle and/or positive control:  
Two vehicle controls were used: tap water and 0.5% carboxymethyl cellulose.
  
3. Test animals: Species: female rat  
Strain: Sprague-Dawley  
Age and weight at study initiation: 21 days old, weight not given  
Source: Charles River Laboratories, Inc., Raleigh, NC  
Housing: 4 per cage  
Diet: not specified. Given ad libitum  
Water: not specified. Given ad libitum  
Environmental conditions: Temperature: Not given  
Humidity: Not given  
Air changes: Not given  
Photoperiod: Not given  
Acclimation period: Not given

**B. STUDY DESIGN:**

1. In life dates - Not given
  
2. Animal assignment  
  
Animals were assigned randomly. Method of animal assignment was not given.

TABLE 1: STUDY DESIGN FOR BODY AND UTERINE WEIGHT STUDIES

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Test Group	Dose to animal (mg/kg/day)	Number of Rats
Vehicle Control	--	6
Atrazine	20	5
Atrazine	100	6
Atrazine	300	6
DACT	20	5
DACT	100	5
DACT	300	6
Vehicle Control	--	8
Simazine	100	8
Simazine	300	8
2 $\mu$ g Estradiol + Vehicle	--	6
2 $\mu$ g Estradiol + Atrazine	20	6
2 $\mu$ g Estradiol + Atrazine	100	5
2 $\mu$ g Estradiol + Atrazine	300	5
2 $\mu$ g Estradiol + DACT	20	6
2 $\mu$ g Estradiol + DACT	100	6
2 $\mu$ g Estradiol + DACT	300	6
2 $\mu$ g Estradiol + Vehicle	--	8
2 $\mu$ g Estradiol + Simazine	100	8
2 $\mu$ g Estradiol + Simazine	300	8

3. Method:

Ovariectomized animals were dosed by oral gavage using 0.5% carboxymethylcellulose as the vehicle. Concentrations were prepared such that 1 mL suspension/100 g body weight resulted in the following doses: 20, 100, and 300 mg/kg/day. Body weights were taken at pretest and at the end of the study. Animals were dosed with triazine for 3 consecutive days. On days 2 and 3, triazine-treated animals were also dosed with 2  $\mu$ g estradiol orally in 0.1 mL of peanut oil.

Animals were euthanized by carbon dioxide asphyxiation 24 hours following the last treatment. The animals were dissected and the uteri removed, cleansed of connective tissue, and weighed.

5. Dose Selection:

Not given.

6. Statistical methods:

Data was analyzed using CRUNCH software and ANOVA. Significant comparisons were made with a post hoc Bonferroni test.

## C. RESULTS: BODY WEIGHTS AND BODY WEIGHT CHANGES

1. Body weight:

As shown in Table 2, although the pretest body weights are similar among the groups, there are significant body weight changes in all dose groups over the 3 days of exposure. Effects on body weight are observed for both the vehicle control and the vehicle + estradiol controls. For each triazine, there is a dose-dependant weight loss with increasing triazine exposure.

TABLE 2: BODY WEIGHT AND BODY WEIGHT CHANGE

Test Group	Dose to animal (mg/kg/day)	Pretest Body Weight (g)	Termination Body Weight (g)	Body Weight Change (g)	Percent Body Weight Change
Vehicle Control	--	312± 4	301± 7	-11	-3.5%
Atrazine	20	308± 6	289± 9	-19	-6.2%
Atrazine	100	317 ± 4	277± 7	-41	-12.6%
Atrazine	300	309± 6	266 ± 5*	-43	-14.0%
DACT	20	315± 3	286 ± 5	-29	-9.2%
DACT	100	321± 4	286 ± 5	-35	-10.9%
DACT	300	313± 6	260± 6*	-53	-16.9%
Vehicle Control	--	251 ± 5	253 ± 5	2	0
Simazine	100	254± 5	234± 5*	-20	-7.9%
Simazine	300	253 ± 5	225± 5*	-28	-11.1%
2 µg Estradiol + Vehicle	--	321± 6	299 ± 4	-22	-6.9%
2 µg Estradiol + Atrazine	20	320± 6	294± 4	-26	-8.1%
2 µg Estradiol + Atrazine	100	315± 5	277± 5*	-39	-12.1%
2 µg Estradiol + Atrazine	300	321± 7	279± 7*	-42	-13.1%
2 µg Estradiol + DACT	20	318± 5	295 ± 4	-23	-7.2%
2 µg Estradiol + DACT	100	312± 5	270 ± 3*	-42	-13.5%
2 µg Estradiol + DACT	300	314 ± 5	268 ± 4*	-46	-14.6%
2 µg Estradiol + Vehicle	--	238 ± 6	238 ± 6	0	0%
2 µg Estradiol + Simazine	100	235 ± 5	229 ± 5	-6	-2.6%
2 µg Estradiol + Simazine	300	235 ± 4	215 ± 5*	-20	-8.5%

\*Statistically significant at p &lt; 0.05.

2. Uterine weights:

Changes in uterine weight following a 3 day exposure is considered a sensitive *in vivo* endpoint for estrogenicity. No data is reported; only graphs are presented in the study report.

The uterine weights of rats exposed to atrazine, DACT, and simazine alone were similar to vehicle control uterine weights.

A separate experiment studied the effects of co-exposure with estradiol and atrazine, DACT, or simazine. Significant ( $p < 0.05$ ) and dose-dependant decreases in the uterine weight of rats exposed to both estradiol and atrazine or DACT were observed in the 100 and 300 mg/kg/day groups relative to the positive control. Although not dose-dependant, significant ( $p < 0.05$ ) decreases in uterine weight were observed in rats exposed to 100 and 300 mg/kg/day simazine relative to the positive control.

**IV. Progesterone Receptor**

A. MATERIALS

1. Test Material: See p. 4
2. Vehicle control: See p. 4
3. Test animals: See p. 4

## B. STUDY DESIGN: PROGESTERONE LEVEL STUDIES

TABLE 3: STUDY DESIGN FOR PROGESTERONE LEVEL STUDIES

Test Group	Dose to animal (mg/kg/day)	Number of Rats
Vehicle Control	--	18
Atrazine	300	18
DACT	300	18
Simazine	300	18
2 $\mu$ g Estradiol + Vehicle	--	Not given
2 $\mu$ g Estradiol + Atrazine	50	12
2 $\mu$ g Estradiol + Atrazine	300	12
2 $\mu$ g Estradiol + DACT	50	18
2 $\mu$ g Estradiol + DACT	300	20
2 $\mu$ g Estradiol + Simazine	50	12
2 $\mu$ g Estradiol + Simazine	300	12

1. In life dates - Not given

2. Animal assignment: Animals were assigned randomly. Method of animal assignment was not given.

3. Method:

Animals were dose by oral gavage using 0.5% carboxymethylcellulose as the vehicle. Concentrations were prepared such that 1 mL suspension/100 g body weight resulted in the following doses: 50 and 300 mg/kg/day. Animals were weighed at pretest and at the end of the treatment (weights not given in study report). Animals were treated with triazines for 2 consecutive days. Two hours following triazine treatment, the rats were injected subcutaneously with 1  $\mu$ g estradiol in 0.25 mL 10% ethanol/90% saline vehicle.

Animals were euthanized by carbon dioxide asphyxiation after the 2<sup>nd</sup> estradiol injection. The animals were dissected and the uteri removed, cleansed of connective tissue, and weighed.

Uteri were placed in 0.5 mL of TEG buffer (TEG buffer contains Tris, EDTA, and dithiothreitol, glycerol, and sodium molybdate. The function of this buffer is to reduce the strength of the outer membrane of the cells in the uterine tissue samples to improve the effectiveness of the homogenation procedure.) The uterine tissue was then homogenated using a tissue grinder. The cytosol was incubated with [<sup>3</sup>H]R5020 in the presence and absence of progesterone. Following 18 hour incubation at 4°C, samples were treated with dextran coated charcoal and centrifuged. The supernatant which contained radiolabeled R5020 bound to PR was counted using a scintillation counter.

5. Dose Selection:

Not given.

6. Statistical methods:

Data was analyzed using CRUNCH software and ANOVA. Significant comparisons were made with a post hoc Bonferroni test.

C. RESULTS:

Raw data and/or means and standard deviations are not reported in this report. Data was only presented as a figure. Statistical significance was calculated by authors.

The PR binding capacity (as measured by binding to radioactive R5020) in rats exposed to atrazine, DACT, and simazine at 300 mg/kg/day alone were statistically decreased ( $p < 0.05$ ) relative to vehicle control.

In co-exposure experiments with 1  $\mu$ g estradiol, the PR binding capacity in rats exposed to atrazine, DACT, and simazine at 50 mg/kg/day were not different from the positive control. PR binding capacity decreased significantly ( $p < 0.05$ ) with exposure to atrazine, DACT, and simazine at 300 mg/kg/day in co-exposure experiments with 1  $\mu$ g estradiol.

V. Thymidine incorporation

A. MATERIALS

1. Test Material: See p. 4
2. Vehicle and/or positive control: See p. 4
3. Test animals: Species: female rat  
Strain: Sprague-Dawley  
Age and weight at study initiation: 23 days old; weight not given  
Source: Harlan Industries, Indianapolis, Ind.  
Housing: "group housed in controlled facilities"  
Diet: Purina rodent chow (no. 5002). Given ad libitum  
Water: tap water. Given ad libitum  
Environmental conditions: Temperature:  $73 \pm 2$  °F  
Humidity:  $50 \pm 10\%$   
Air changes: Not given  
Photoperiod: 12 hours light/12 hours dark  
Acclimation period: 7 days.

## B. STUDY DESIGN:

1. In life dates - Not given
2. Animal assignment: Animals were assigned randomly. Method of animal assignment was not given. There were 6-7 animals per dose group plus 3 for the positive and negative control groups. Each compound was tested 3-4 times.

TABLE 4. STUDY DESIGN FOR THYMIDINE INCORPORATION EXPERIMENTS

Test Group	Dose to animal (mg/kg/day)	Number of Rats
Vehicle Control	--	30
Atrazine	300	8
DACT	300	8
Simazine	300	13
2 $\mu$ g Estradiol + Vehicle	--	57
2 $\mu$ g Estradiol + Atrazine	1	7
2 $\mu$ g Estradiol + Atrazine	10	14
2 $\mu$ g Estradiol + Atrazine	50	14
2 $\mu$ g Estradiol + Atrazine	100	21
2 $\mu$ g Estradiol + Atrazine	300	18
2 $\mu$ g Estradiol + DACT	1	21
2 $\mu$ g Estradiol + DACT	10	22
2 $\mu$ g Estradiol + DACT	50	17
2 $\mu$ g Estradiol + DACT	100	22
2 $\mu$ g Estradiol + DACT	300	8
2 $\mu$ g Estradiol + Simazine	1	14
2 $\mu$ g Estradiol + Simazine	10	14
2 $\mu$ g Estradiol + Simazine	50	14
2 $\mu$ g Estradiol + Simazine	100	28
2 $\mu$ g Estradiol + Simazine	300	8



3. Method:

Animals were dosed by oral gavage using 0.5% carboxymethylcellulose as the vehicle. Concentrations were prepared such that 1 mL suspension/100 g body weight resulted in the following doses: 1, 10, 50, 100, and 300 mg/kg/day. Animals were weighed at pretest and at the end of the treatment (body weight data not given in study report). Animals were treated with triazines for 2 consecutive days. On day 2, each animal was injected subcutaneously 0.15  $\mu$ g estradiol in 0.25 mL 10% ethanol/90% saline vehicle. Additional dose groups included 300 mg/kg/day triazine for 2 days without estradiol.

Animals were euthanized by carbon dioxide asphyxiation 24 hours after the 2<sup>nd</sup> estradiol injection. The animals were dissected and the uteri removed, cleansed of connective tissue, and weighed (uterine weights not given).

The method of determining the thymidine incorporation rate into uterine DNA was similar to Stormshak *et al.* (1976). A tissue chopper was used to cut the uteri into 150  $\mu$ m slices. The uteri were into polypropylene tubes containing a minimal media supplemented with antibiotics. Radiolabeled thymidine was added at 1  $\mu$ Ci/mL. Following an incubation the media was decanted and 0.5 M perchloric acid was added. The suspensions were then homogenized, centrifuged, and washed. The pellet was resuspended in 2 mL perchloric acid and hydrolyzed at 90°C. Following centrifugation, the supernatant was analyzed using a liquid scintillation counter. Aliquots of the supernatant were analyzed for DNA content.

5. Dose Selection: Not given.

6. Statistical methods: See p. 10

## C. RESULTS

Raw data and/or means and standard deviations are not reported in this report. Data was only presented as a figure. Statistical significance was calculated by authors.

Thymidine incorporation was measured to assess the effect triazines on DNA synthesis in the uterus of exposed rats. Exposure to the positive control, 0.15  $\mu\text{g}$  estradiol, resulted in approximately 2.5 fold increase ( $p < 0.05$ ) in thymidine incorporation relative to the vehicle control. Exposure to triazines at 300 mg/kg/day alone resulted in a significant ( $p < 0.05$ ) reduction in thymidine incorporation relative to the vehicle control.

For all three triazines tested in co-exposure with 0.15  $\mu\text{g}$  estradiol, thymidine incorporation was similar to positive control at 1 and 10 mg/kg/day. At the doses 50, 100, and 300 mg/kg/day thymidine incorporation was suppressed significantly ( $p < 0.05$ ) when exposed to triazine plus 0.15  $\mu\text{g}$  estradiol compared to the positive control.

## VI. Discussion and Deficiencies

### A. DISCUSSION:

**Based on uterine weight, progesterone binding capacity, and thymidine incorporation in the female rat, the results from this study indicate that atrazine, DACT, and simazine up to 300 mg/kg/day do not exhibit estrogenic activity.**

*The study report does not contain raw data or mean data for uterine weight, thymidine incorporation and progesterone receptor binding capacity; only figures are provided. Therefore, quantitative comparisons between dose groups is not possible. Comparative estimations were made by the reviewer from the plots provided in the study report. Statistics were performed by author.*

Uterine weight is a sensitive assay for estrogenic activity of xenobiotics. Increases in uterine weight result from the proliferation and thickening of the uterine wall and can be caused by estrogen or chemicals with estrogen-like activity. Decreases in uterine weight are assumed to indicate antiestrogenic activity. The uterine weights of rats exposed to triazines were similar to vehicle control uterine weights. In co-exposure experiments with estradiol, exposure to atrazine and DACT at 100 and 300 mg/kg/day resulted in a dose-dependant and statistically significant decrease in uterine weight relative to the positive control. In co-exposure experiments with estradiol, uteri of rats exposed to simazine at 100 and 300 were significantly decreased relative to the positive control.

Thymidine incorporation was measured to assess the effect triazines on DNA synthesis

in the uterus of exposed rats. Exposure to the positive control, 0.15  $\mu\text{g}$  estradiol, resulted in approximately 2.5 fold increase in thymidine incorporation relative to the vehicle control. Exposure to triazines alone at 300 mg/kg/day alone or at 50, 100, and 300 mg/kg/day in co-exposure with 0.15  $\mu\text{g}$  estradiol resulted in a significant reduction in thymidine incorporation relative to the vehicle control and positive control, respectively.

The PR binding capacity indirectly evaluates PR levels by measuring binding to radioactive R5020. The binding capacity in uteri of rats exposed to atrazine, DACT, and simazine at 300 mg/kg/day alone was statistically decreased relative to vehicle control. As indicated above, PR binding capacity decreased significantly with exposure to atrazine, DACT, and simazine at 300 mg/kg/day in co-exposure experiments with 1  $\mu\text{g}$  estradiol.

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**B. DEFICIENCIES:**

1. As part of the public scientific literature, this study is not under GLP regulations. Based on the descriptions and references of methods given, it seems that the authors have utilized good animal husbandry and laboratory practice.
2. Although not required for journal publication, from a regulatory perspective, it would be valuable to have the following data:
  - Concentration analysis of the dilutions
  - Analysis and mass balance of radioactivity used
  - Specific experimental conditions used in animal care
3. Raw data or mean data are not reported in the assays; data for the is only reported on a graph.
4. In the PR binding capacity and thymidine incorporation assay, triazines were only in single exposure (i.e., not co-exposure with estradiol) tested at 300 mg/kg/day which is above the maximum tolerated dose (based on weight gain). Because an antiestrogenic response was observed, more doses would have improved the quality of these experiments.
5. The same number of animals should be used for each dose group. The variation in animal number within each experiment should be explained.

**VII. References**

- Dickerson, R., and Safe, S. 1992. The effects of 6-nitro-1,3,8-trichlorodibenzofuran as a partial estrogen in the female rat. *Toxicol. Appl. Pharmacol.* 113: 55-63.
- Lamb, D.J. and Bullock, D.W. 1983. Hydrophobic interaction chromatography of the rabbit uterine progesterone receptor. *J. Steroid Biochem.* 19: 1039-1045.
- Stormshak, F., Leake, R., Wertz, N., and Gorski, J. 1976. Stimulatory and inhibitory effects of estrogen on uterine DNA synthesis. *Endocrinology* 99: 1501-1511.
- Tennant, M.K.; Hill, D.S.; Elderidge, J.C.; Wetzel, L.T.; Breckenridge, C.B.; and Stevens, J.T. 1994. Possible antiestrogenic properties of chloro-S-triazines in rat uterus. *J. Toxicol. Environ. Health.* 43:183-186.

**Atrazine, Simazine, and DACT**

***In vivo* estrogenicity. Special Study**

SignOff Date: 3/24/99  
DP Barcode: D253661  
HED DOC Number: 013281  
Toxicology Branch: RCAB

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EPA Reviewer: Anna Bearden, PhD. \_\_\_\_\_, Date \_\_\_\_\_

EPA Secondary Reviewer: Roger Hawks, PhD. \_\_\_\_\_, Date \_\_\_\_\_

DATA EVALUATION RECORD
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STUDY TYPE: *In vivo and in vitro* endocrine effects in rats. Special study.

DP BARCODE: 253661

SUBMISSION CODE: S557393

P.C. CODE: 080803 (Atrazine)

TOX. CHEM. NO.: 063 Atrazine

080807 (Simazine)

740 Simazine

121301 (DACT)

TEST MATERIAL (PURITY): Atrazine, diaminochlorotriazine (DACT), and Simazine (>96%)

SYNONYMS: G-30027 (Atrazine); G-28273 (DACT); G-27692 (Simazine)

CITATION: Tennant, M.K. *et al.* (1994) Chloro-s-triazine antagonism of estrogen action: limited interaction with estrogen receptor binding. Department of Physiology and Pharmacology, Wake Forest University and Department of Toxicology, Ciba-Geigy Corporation, Greensboro, NC. Laboratory report number: N/A. MRID 43598618. Published in *Journal of Toxicology and Environmental Health*. 43: 197-211.

SPONSOR: Ciba Crop Protection, Ciba-Geigy Corporation (presently Novartis), PO BOX 18300, Greensboro, NC 27419.

EXECUTIVE SUMMARY:

In a special study (MRID 43598618) on *in vitro and in vivo* competitive binding of atrazine, diaminochlorotriazine (DACT, a mammalian metabolite of both atrazine and simazine) and simazine (>96 % a.i. for all three compounds) to the estrogen receptor (ER), several different experiments were performed utilizing extracted cytosolic ER from adult female Sprague-Dawley rats. Experiments observed the displacement of radiolabeled estradiol by atrazine, simazine, and DACT under both equilibrium (simultaneous exposure to triazine and radiolabeled estradiol at 4°C) and at disequilibrium conditions (pre-exposure to triazine at 25°C for 30 min. prior to exposure to radiolabeled estradiol). Results of equilibrium experiments indicated that atrazine, simazine, and DACT did not displace any radiolabeled estradiol whereas the positive control exhibited a dose-dependant displacement of the radiolabeled estradiol. Under disequilibrium conditions in a time-course experiment, uterine cytosolic extracts were first incubated with triazine at 100 µM for 30 min. prior to exposure to radiolabeled estradiol for 5-150 minutes. With time radiolabeled estradiol displaced triazine that had bound during the initial exposure. In a dose-response experiment under disequilibrium conditions, uterine cytosolic extracts were first incubated with triazine at concentrations 10<sup>-9</sup> to 10<sup>-3</sup> M or unlabeled estradiol at 10<sup>-11</sup> to 10<sup>-7</sup> M prior to exposure to radiolabeled estradiol. The IC<sub>50</sub> for unlabeled estradiol was approximately

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$10^{-9}$  M whereas the  $IC_{50}$ 's for the triazine were 20  $\mu$ M for atrazine and 100  $\mu$ M for simazine and DACT.

An additional experiment under disequilibrium conditions was performed to mimic a Scatchard type analysis using constant molar excess of triazine (100X estradiol, 10,000X atrazine, or 10,000X simazine) relative to the radiolabeled estradiol (0.2 nM, 0.5 nM, 1.5 nM, and 5.0 nM). Results indicated the triazines competed with the radioligand better at lower concentrations of tracer. For example, there was 60% displacement of 0.5 nM radiolabeled estradiol when co-exposed with 5  $\mu$ M atrazine whereas there was 11% displacement of 5 nM radiolabeled estradiol when co-exposed with 50  $\mu$ M atrazine. In the Scatchard-type plot, dissociation constants were produced; 0.5 nM in the presence of 100-fold molar excess of estradiol and 1 nM in the presence of 10,000 fold molar excess of atrazine and simazine. The x-intercepts of the plot were approximately equal (210 fmol/mg) in the presence of estradiol and simazine indicating competitive binding. The x-intercept for the atrazine plot was slightly less (158 fmol/mg) indicating the potential for competitive binding but also some noncompetitive binding under the disequilibrium conditions of this study.

Experiments using uteri extracted with KCl to extract the total ER and without KCl to extract the ER not bound or only loosely bound to chromatin were performed using cytosol fractions incubated with 50  $\mu$ M triazine or 500 nM estradiol under disequilibrium conditions followed by separation of the sample by sucrose density centrifugation. One hundred twenty five mL fractions were removed and counted for radioactivity. The authors generated plots representing the fraction number versus the radioactivity in the given fraction in order to observe which fraction exhibited the binding. In the extractions without KCl, the receptor binding peaks were detected in the 7-8S fraction for both estradiol and atrazine. Atrazine displaced an average of 10.7% of the radiolabeled estradiol whereas estradiol displaced all of the radiolabeled tracer. In the conditions favoring the extraction of total ER, activated and unactivated, binding was detected in the 4-5S fraction. Atrazine displaced an average of 29.6% of the radiolabeled estradiol whereas estradiol displaced all of the radiolabeled tracer.

Making the assumption that living rats contain the activated form of the ER, in order to test whether triazines competed more effectively against the transformed ER, ovariectomized rats were dosed with triazines prior to uterine dissection and competitive binding experiments. The binding of radiolabeled estradiol was reduced by an average of 33%, 39%, and 24% for atrazine, simazine, and DACT, respectively ( $p < 0.05$  vs. vehicle controls) in the 300 mg/kg/day group. In the 50 mg/kg/day group, binding of radiolabeled estradiol was reduced (not statistically significant) by 18, 21, and 13%.

**Overall the results indicate that atrazine, triazine, and DACT do exhibit some competitive binding with estradiol but only under conditions which favor triazine binding.**

This special study on *in vitro and in vivo* competitive binding of atrazine, DACT and simazine binding to the estrogen receptor in the rat is **Acceptable-nonguideline**.

COMPLIANCE: Signed and dated GLP and Data Confidentiality were provided. Quality Assurance and Flagging Statements were not provided. This study was published in the public literature and therefore was not subject to these requirements.

## I. Organization of this DER

This study entails several *in vitro* and *in vivo* competitive binding assays. Because of the diversity in experiment design, this DER is separated into specific sections describing different assays. Each section has a separate materials and methods, and results. There is one discussion at the end.

## II. Description of different assays.

Extractions of the estrogen receptor was performed according to Eldridge, *et al.* (1986). All the experiments performed in this study are based on the competitive binding of triazines with radiolabeled estradiol for estrogen receptor isolated from the uterine tissue of female adult rats. Each experiment consists of several test tubes each containing a different binding scenario using different chemicals or different concentrations of test chemical. The uterine samples in this study are processed in two different ways based on the specific binding scenario is the test tube. Some uterine extracts were extracted with TEG buffer and were pooled from approximately 18 rats and freeze-dried (*i.e.*, lyophilized). The function of TEG buffer which contains chelating agents and glycerol is to decrease the strength of the outer membrane of the uterine cells to improve the homogenization and extraction steps. This was done in order to achieve a stock of estrogen receptor with consistent activity between experiments. TEG only extractions contain estrogen receptor loosely or unattached to chromatin and/or estrogen at the time of extraction. Some uterine extracts were extracted with KCl and TEG, particularly. These extracts are assumed to contain the complete estrogen receptor content in the tissue which includes estrogen receptor bound to estrogen and/or chromatin material at the time of extraction.

In each experiment, estrogen receptor was incubated with both test compound and radiolabeled estradiol followed by the addition of a dextran-charcoal suspension and centrifugation. The dextran-charcoal suspension was used to precipitate unbound and excess radioligand. The amount of radioactivity in the supernatant is indirectly related to the degree of triazine binding (*i.e.*, more radioactive estradiol in the supernatant correlates with less triazine bound to estrogen receptor; p. 7 MRID 43598618).



1. Receptor binding assays: In these assays, uteri from rats not previously exposed to triazines were extracted and pooled to yield a freeze-dried sample of cytoplasmic protein. Several binding scenarios were performed including "equilibrium" conditions where exposure entailed simultaneous exposure to both the test compound (*i.e.*, triazine or unlabeled estradiol) and radiolabeled estradiol and "disequilibrium" conditions where exposure entailed first exposure to the test compound under relatively warm temperature conditions followed by exposure to radiolabeled estradiol at cold conditions. The "disequilibrium" conditions favor binding of the triazine to the estrogen receptor. Several experiments using "disequilibrium conditions" were performed which observed dose-response relationships of receptor binding of test chemical. Additionally, experiments were performed where a constant molar excess of test compound over the radiolabeled estradiol (called a "tracer") at different concentrations of tracer were performed in order to do a Scatchard-type analysis.

A Scatchard plot graphs the ratio of bound/free radiolabeled tracer (y-axis) versus the bound tracer (x-axis). The slope of this plot is equal to  $-1/k_a$  where  $k_a$  is the association constant. The x-intercept represents the number of binding sites for a given receptor. In this study, Scatchard plots of the triazines are used to assess the potential for competitive binding by observing both the  $k_a$  and the x-intercept.

2. Sucrose Density Gradients: The purpose of performing the sucrose density gradients was to separate two different subtypes of the estrogen receptor. By separating these subtypes, it is possible to determine if differences in binding ability exist between them. First, cytosolic uterine extracts with and without KCl were freshly prepared. These uterine extracts were incubated under disequilibrium conditions with test compounds and radiolabeled estradiol. The incubation sample was then run on a sucrose density gradient using sucrose solutions of 5-20% and centrifuged to separate the sample into fractions of variable density or molecular weight. The radioactivity of these fractions is counted by liquid scintillation. The authors generated x-y plots of radioactivity versus fraction number. The position (representing different fractions) and height of the peaks (representing the competitive binding to estrogen receptor) in these plots have been compared by the authors.
3. Estrogen binding in triazine-dose rats. In this assay, ovariectomized rats were exposed to atrazine, simazine, and DACT for 2 days. Following sacrifice, the uteri of the rats were extracted with TEG plus KCl and incubated with radiolabeled estradiol.

## I. Receptor binding assays.

### A. MATERIALS:

#### 1. Test Material: Atrazine, Simazine, and DACT

Description: Not given

Lot/Batch #: Obtained by author from Ciba Plant Protection Division (Greensboro, NC).

Purity: Atrazine: 97.7%; Simazine: 96.9% a.i.; DACT: 98.2%

Stability of compounds: Not given

CAS #: 1912-24-9 (Atrazine); 122-34-9 (Simazine); 3397-62-4 (DACT)

Radiolabeled estradiol: Purchased from Amersham Corp. (Arlington Heights, Ill.).

Specific activity of individual batches ranged from 90 to 110 Ci/mmol. At 1 mCi/mL, the concentration of the stock ranged from 9.0 to 11.1 x 10<sup>6</sup> M.

#### 2. Vehicle and/or positive control:

Two vehicle controls were used: tap water and 0.5% carboxymethyl cellulose.

Positive control: unlabeled estradiol.

#### 3. Test animals:

Species: female rat

Strain: Sprague-Dawley

Age and weight at study initiation: "adult"; weight not given

Source: Charles River Corporation, Raleigh, NC

Housing: 2 per cage

Diet: Purina rodent chow (no. 5002) lab feed provided *ad libitum*

Water: provided *ad libitum*

Environmental conditions: Temperature: 73 ± 2

Humidity: 50 ± 10

Air changes: Not given

Photoperiod: 12 hours light/ 12 hours dark

Acclimation period: Not given

**B. METHODS:****1. Uterine tissue and estrogen receptor preparation:**

Eighteen rats were sacrificed in a CO<sub>2</sub> chamber and uteri were dissected, cleaned of fat, and placed in ice cold TEG buffer. The uterine tissue was homogenized using a commercial homogenizer. The homogenates were then centrifuged to separate the cell cytosol from the remainder of the cell. The supernatants containing the cytosol were pooled and 2 mL aliquots were put into scintillation vials. The vials were frozen with liquid nitrogen. The frozen scintillation vials were put in lyophilizing bottles which were attached to a commercial freeze-drying apparatus. Freeze-drying was conducted for 72 hours. One vial reconstituted with 2 mL water was used for each experiment and separated into 40 assay tubes of 50  $\mu$ L cytosol per tube containing 2.5-3.0 mg/mL of cytosolic protein each.

**2. Receptor binding assays.**

Binding of the competitor to the estrogen receptor was evaluated by incubating cytosolic protein, [<sup>3</sup>H]-Estradiol, and competitor (atrazine, simazine, DACT, estradiol, or blank) in a total volume of 150  $\mu$ L in 8 x 150 mm flat-bottom glass culture vials. The temperature of incubation varied with the experiment (see below). Following incubation(s), 150  $\mu$ L of dextran-charcoal was added to the vials followed by a 10 min incubation at 4°C. After centrifugation of the vials, the supernatant which contained the protein-bound compound was removed and counted on a liquid scintillation counter. The purpose of the dextran-charcoal mixture was to precipitate unbound radioligand. Therefore, a decrease in radioactivity in the supernatant correlates with more competitive binding by the non-labeled test compounds.

**3. Conditions of receptor binding assays:**

- a. Equilibrium conditions: Cytosol was incubated with 5 nM [<sup>3</sup>H]-Estradiol plus competitor (estradiol, atrazine, simazine, or DACT) for 18 hours at 4°C. Concentrations of competitor tested were 10<sup>-10</sup>, 10<sup>-9</sup>, 10<sup>-8</sup>, 10<sup>-7</sup>, 10<sup>-6</sup>, 10<sup>-5</sup>, 10<sup>-4</sup>, and 10<sup>-3</sup> M.
- b. Disequilibrium conditions—time course experiment: Cytosol (constant protein concentration in each sample) was incubated with competitor for 30 min at 25°C with 100  $\mu$ M atrazine, simazine, or DACT. After this incubation, 5 nM [<sup>3</sup>H]-Estradiol was added to sample. The vial was incubated at 4°C for 5-150 min. Samples were taken at 5, 15, 30, 45, 60, 90, 120, and 150 min.

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- c. Disequilibrium conditions–dose response experiment: The dose-inhibition of radiolabeled estradiol binding by pre-incubated triazines. Cytosol samples (constant protein concentration in each sample) were incubated at 25°C for 30 min. with  $10^{-11}$  to  $10^{-7}$  M estradiol or  $10^{-9}$  to  $10^{-3}$  M triazine. Five nM of [ $^3$ H]-Estradiol was added at 4°C for 60 min. The  $IC_{50}$  (concentration resulting in 50% inhibition in binding of [ $^3$ H]-estradiol). Three test tubes per test chemical were performed.
  - d. Disequilibrium conditions (Scatchard type analysis; Constant molar excess of test compound using different concentrations of radiolabeled tracer): Test tubes were incubated with 100X estradiol, 10,000X atrazine, or 10,000X simazine relative to the radiolabeled estradiol. Concentrations of radiolabeled estradiol were 0.2 nM, 0.5 nM, 1.5 nM, and 5.0 nM. Binding between triazine and cytosol groups was compared. A Scatchard analysis was performed using 8 concentrations of radiolabeled tracer plus excess estradiol, atrazine, or simazine. Eight test tubes/dose/tracer concentration were performed.
4. Dose selection rationale: Not given
  5. Dose analysis : Not given.

Results : Homogeneity, Stability, and Concentration Analysis: Not evaluated

6. Statistics - Means and standard deviations were calculated by the author. A 2-way ANOVA was performed between groups.

### C. RESULTS

1. Equilibrium conditions: When uterine cytosolic extracts were incubated with radiolabeled estradiol simultaneously with test chemical, no displacement of radiolabeled estradiol binding occurred at triazine concentrations up to  $10^{-3}$ M (10,000 fold excess to radiolabeled estradiol). Positive control (non-labeled estradiol) resulted in a dose-dependant displacement of the radiolabeled estradiol. (MRID 43598618, p. 9, figure 1)

2. Disequilibrium conditions–time course experiment: Uterine cytosolic extracts were first incubated with atrazine at 100  $\mu\text{M}$  for 30 min. prior to exposure to radiolabeled estradiol for 5-150 minutes. Results indicated that the triazines did interact with the estrogen receptor during the preincubation with test chemical. As indicated by a plot of time versus radioactivity, with time radiolabeled estradiol displaced bound triazine (that had bound during the initial exposure without estradiol present). Based on the plot provided for this experiment atrazine appeared to compete with estradiol more than simazine or DACT. (MRID 43598618, p. 9, figure 2)
3. Disequilibrium conditions–dose-response experiment: Uterine cytosolic extracts were first incubated with triazine at concentrations  $10^{-9}$  to  $10^{-3}$  M or unlabeled estradiol at  $10^{-11}$  to  $10^{-7}$  M prior to exposure to radiolabeled estradiol. The  $\text{IC}_{50}$  for unlabeled estradiol was approximately  $10^{-9}$  M whereas the  $\text{IC}_{50}$  's for the triazine were 20  $\mu\text{M}$  for atrazine and 100  $\mu\text{M}$  for simazine and DACT. (MRID 43598618, p. 10, figure 3)
4. Disequilibrium conditions (Scatchard type analysis; Constant molar excess using different concentrations of radiolabeled tracer). In these experiments varying concentrations of both radiolabeled estradiol and also test compound although the ratio of test compound to radiolabeled estradiol was constant. Results indicated the triazines competed with the radioligand better at lower concentrations of tracer. There was an indirect relationship between the concentration of radiolabeled estradiol and the competitive binding of the triazines. For example, there was 60% displacement of 0.5 nM radiolabeled estradiol when co-exposed with 5  $\mu\text{M}$  atrazine whereas there was 11% displacement of 5 nM radiolabeled estradiol when co-exposed with 50  $\mu\text{M}$  atrazine. (MRID 43598618, p. 11, figure 4)

In the Scatchard-type plot for competitive binding, similar dissociation constants were produced; 0.5 nM in the presence of 100-fold molar excess of estradiol and 1 nM in the presence of 10,000 fold molar excess of atrazine and simazine. The x-intercepts of the plot were approximately equal (210 fmol/mg) in the presence of estradiol and simazine indicating competitive binding. The x-intercept for the atrazine plot was slightly less (158 fmol/mg) indicating the potential for competitive binding but also some noncompetitive binding under the disequilibrium conditions of this study. (MRID 43598618, p. 12, figure 5)

## II. Sucrose Density Gradient.

### A. MATERIALS:

1. Test Material: See p. 5.
2. Vehicle and/or positive control: See p. 5.
3. Test animals: See p. 5

### B. METHODS:

1. Uterine tissue and estrogen receptor preparation:  
In the sucrose-gradient experiments, fresh uterine cytosolic extracts were prepared. Following homogenization from p. 6, tissue was extracted in TEG buffer and 0.4 M KCl. The function of this extraction is solubilize estrogen receptor tightly bound to chromatin.
2. Sucrose density gradients.  
Extraction of uteri either in TEG or TEG plus KCL (protein concentration of 6-8 mg/mL) were performed. The following incubations were run simultaneously at 25°C for 30 min. 1) cytosol plus 50  $\mu$ M triazine competitor; 2) cytosol plus 500 nM estradiol; 3) cytosol alone. Five nM of [<sup>3</sup>H]-Estradiol was added for 60 min on ice. The samples "were then added to an ice-cold dextran charcoal pellet and centrifuged for 10 min." (MRID 43598618 p. 7) Samples were then loaded onto a 4 mL sucrose gradient of 5-20% sucrose in TEG with or without KCl but without molybdate. Gradients were centrifuged for 18 hours at 280,000 g. Fractions of 0.125 mL were collected dropwise and counted by liquid scintillation. Experiments were performed 4 or 5 times.
3. Dose selection rationale: Not given
4. Dose analysis : Not given.  
  
Results : Homogeneity, Stability, and Concentration Analysis: Not evaluated
5. Statistics - Means were calculated by the study author.

### C. RESULTS:

The authors generated plots representing the fraction number (x-axis) versus the radioactivity (y-axis) of the given fraction. Two experimental conditions were tested with each chemical; with KCL to extract the total estrogen receptor (including the activated receptor) and without KCL to extract only the estrogen receptor not bound or loosely bound to chromatin and/or estrogen. In the TEG only conditions, the peaks representing the fractions with the receptor binding were detected in the 7-8S fraction for both estradiol and atrazine. Averaging the results for four experiments, atrazine displaced 10.7% of the radiolabeled estradiol whereas estradiol displaced all of the radiolabeled tracer. Results for simazine and DACT were not given in the study report.

In the conditions favoring the extraction of total estrogen receptor, activated and unactivated, binding was detected in the 4-5S fraction. Averaging the results for five experiments, atrazine displaced 29.6% of the radiolabeled estradiol whereas estradiol displaced all of the radiolabeled tracer.

## **IV. Estrogen binding in triazine dosed rats.**

### A. MATERIALS:

1. Test Material: See p. 5
2. Vehicle and/or positive control: See p. 5
3. Test animals: See p. 5; Ovariectomized rats were used.

### B. STUDY DESIGN:

1. In life dates - Not given
2. Animal assignment-- Not given
3. Dose selection rationale: Not given.
4. Diet preparation and analysis : Not given.

Results : Homogeneity, Stability, and Concentration Analysis: Not evaluated

5. Statistics - Means were calculated by the study author.

### C. METHODS:

Animals were administered 1 mL/100 g of body weight of either 10 or 30 mg/mL triazine suspension. Suspensions were made using 0.5% carboxymethylcellulose in tap water as the vehicle. Doses were administered daily by oral gavage. Ovariectomized rats were dosed with atrazine, simazine, or DACT at 50 and 300 mg/kg/day for 2 days. At sacrifice, the uteri were dissected, cleansed, sliced, and incubated with 10 nM [<sup>3</sup>H]-Estradiol for 30 min at 37°C in 95% O<sub>2</sub>/5% CO<sub>2</sub>. The tissue was then homogenized and cytosol prepared as described on p. X in TEG containing 0.4 M KCL.

### C. RESULTS

In order to test whether triazines competed more effectively against activated estrogen receptor assumed by the authors to be the 4S form, ovariectomized rats were dosed with triazines prior to uterine dissection and competitive binding experiments. The binding of radiolabeled estradiol was reduced by an average of 33%, 39%, and 24% for atrazine, simazine, and DACT, respectively ( $p < 0.05$  vs. vehicle controls) in the 300 mg/kg/day group. In the 50 mg/kg/day group, binding of radiolabeled estradiol was reduced (not statistically significant) by 18, 21, and 13% .



### III. DISCUSSION

- A. Previous studies submitted by Novartis have indicated the potential for antiestrogenic activity by atrazine, simazine, and DACT (MRID no. 43598617, 43598619, 4394403 and 43598614). This study investigates the hypothesis that antiestrogenic activity results from competitive binding with estradiol for the estrogen receptor. To this end, the authors have performed several different competitive binding experiments using radiolabeled estradiol and extracted estrogen receptor. **Overall the results indicate that atrazine, triazine, and DACT do exhibit some competitive binding with estradiol but only under conditions which favor triazine binding.**

The ability of triazines to competitively bind to the estrogen receptor depends on the conditions of the experiment. At 4° C and simultaneous exposure, no displacement of estradiol by the triazines occurred. Whereas at 25° C and pre-incubation with triazine resulted in some interaction between the triazine and estrogen receptor. Estradiol was a better competitor in all experiments performed including the dose-response experiment (IC<sub>50</sub> for unlabeled estradiol was approximately 10<sup>-9</sup> M whereas the IC<sub>50</sub>'s of atrazine was 20 μM and 100 μM for simazine and DACT) and constant molar excess experiments which estimated disassociation constants (0.5 nM in the presence of 100-fold molar excess of estradiol and 1 nM in the presence of 10,000 fold molar excess of atrazine and simazine).

Additionally, the form of the estrogen receptor may be an important determinant of triazine binding. As indicated by the sucrose density centrifugation experiments followed by experiments using total estrogen receptor from triazine exposed rats, atrazine bound more estrogen receptor when extracted with 0.4 M KCL than under conditions where only the presumed inactive form of the receptor was present. Results also indicate that between the two extracted forms of the receptor, unactivated or 7-8S and transformed, or 4-5S, that triazines displace more estradiol in the activated form. The concentration of estradiol present also effected the degree to which triazines bound to the estrogen receptor. At lower concentrations of radiolabeled estradiol, triazines were better competitors than when concentrations of radiolabeled estradiol were present. The quantitative example given by the authors is the following: 60% of 0.5 nM radiolabeled estradiol was displaced when co-exposed with 5 μM atrazine whereas 11% of 5 nM radiolabeled estradiol was displaced when co-exposed with 50 μM atrazine.

B. Study deficiencies: The results given in this DER are only qualitative in nature since no raw data and only limited means and measure of variations were given. The following deficiencies were present but did not prevent the reviewer from evaluating the study:

1. This study did not follow GLP regulations but this study is not required for FIFRA. Based on the descriptions and references of methods given, it seems that the authors have utilized good animal husbandry and laboratory practice.
2. Although not required for journal publication, from a regulatory perspective, it would be valuable to have the following data:
  - Concentration analysis of the dilutions
  - Analysis and mass balance of radioactivity used
  - Raw data and/or means and deviations; data is reported only as figures

#### IV. REFERENCES

Eldridge, J.C., Cidlowski, J.A., and Muldoon, T. G. 1986. Correlation between LH and estrogen receptor turnover in pituitary and hypothalamus of castrate rats following estrogen agonists and antagonists. *J. Steroid Biochem.* 24: 623-628.

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Wetzel, L.T.; Luempert, L.G.; Breckenridge, C.B.; Tisdell, C.B.; Stevens, M.O.; Thakur, J.T.; Extrom, A.J.; and Elderidge, J.C.. 1994. Chronic effects of atrazine on estrus and mammary formation in female Sprague-Dawley and Fischer-344 rats. *J. Toxicol. Environ. Health.* 43:169-182. (MRID no. 43598615).

**Atrazine, DACT, and Simazine**

*In vivo and in vitro* endocrine effects. Special study.

SignOff Date: 3/24/99  
DP Barcode: D253661  
HED DOC Number: 013281  
Toxicology Branch: RCAB

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EPA Reviewer: Anna Bearden, PhD  
EPA Secondary Reviewer: Roger Hawks, Ph.D.

\_\_\_\_\_, Date \_\_\_\_  
\_\_\_\_\_, Date \_\_\_\_

DATA EVALUATION RECORD

STUDY TYPE: *In vitro* estrogenicity. Special study.

DP BARCODE: D253661

SUBMISSION CODE: S557393

P.C. CODE: 080803 Atrazine; 080807 Simazine

TOX. CHEM. NO.: 063 Atrazine  
740 Simazine

TEST MATERIAL (PURITY): Atrazine (> 97%) and Simazine (> 97%)

SYNONYMS: G-30027 (Atrazine); G-27692 (Simazine)

CITATION: Failure of atrazine and simazine to induce estrogenic responses in MCF-7 human breast cancer cells. 1995. Veterinary Physiology and Pharmacology Texas A&M University, College Station, TX; Department of Pharmacology and Toxicology, University of Western Ontario, London, Ontario. Laboratory number not given. Completed March 13, 1995. Safe, S, *et al.* MRID 43598619. Not published..

SPONSOR: Ciba Crop Protection. Ciba-Geigy Corporation (presently Novartis). PO Box 18300, Greensboro, NC 27419.

EXECUTIVE SUMMARY:

This study (MRID no. 43598619) combines the following *in vitro* assays to investigate the estrogenic and antiestrogenic activity of atrazine and simazine: binding to hepatocyte Ah receptor; proliferation of MCF-7 (human breast cancer cell line) cells; gel electrophoresis mobility shift assay to measure levels of progesterone receptor; and MCF-7 transfection using a luciferase reporter gene.

Competitive binding to the aryl hydrocarbon (Ah) receptor was measured using TCDD as the competitor. This assay is performed because some dioxins which are strong Ah receptor agonists have also been shown to exhibit antiestrogenic activity. Cytosol from male rat hepatocytes containing Ah receptor was isolated and incubated with the positive control, radiolabeled 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), plus either atrazine or simazine. With addition of hydroxylapatite to this mixture, high molecular weight compounds, such as test compound bound to the Ah receptor will precipitate out of solution. Subsequent centrifugation of this solution will result in the Ah receptor, and anything bound to it, being spun out into a pellet. By counting the radioactivity in the pellet, it is possible to measure the amount of radiolabeled TCDD displaced indicating competitive inhibition with the triazine. Competitive binding was measured as the decrease in radioactivity versus the positive control. At concentrations up to 10,000 nM, atrazine and simazine did not displace TCDD from the Ah receptor.

A cell proliferation assay using the MCF-7 cell line was performed using the following doses: 10

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$\mu\text{M}$ ,  $1.0 \mu\text{M}$ ,  $0.1 \mu\text{M}$ , and  $0.01 \mu\text{M}$  for atrazine and simazine and  $1 \text{ nM}$  for estradiol. Experiments using individual chemicals plus triazine were performed. Results indicated that the positive control increased the density of MCF-7 cells two fold relative to the negative control after 11 days. Exposure to atrazine and simazine alone neither increased nor decreased cell number at day 11 relative to the negative control. Results of co-exposure experiments indicate that cell number did not change relative to the positive control.

Estradiol has been shown to increase the level of progesterone receptor in mammalian cells. This assay measures the affect of estradiol and/or triazine on the binding capacity of a high affinity ligand (R5020) to the progesterone receptor which an indirect measure of progesterone level. The gel electrophoresis mobility shift assay utilized indirectly measures progesterone receptor (PR) levels in MCF-7 cells following exposure to atrazine, simazine, or estradiol for 3 days. Polyacrylamide gel electrophoresis can separate molecules, such as DNA, or complexes of molecules, such as protein-DNA complexes, based on size. The DNA used in this study is a radioactive oligonucleotide of a specific sequence which binds to the PR. This sequence is called a progesterone response element (PRE). PR isolated from MCF-7 cells is first incubated with R5020. PR-R5020 complex was then incubated with radiolabeled PRE. The R5020-PR-PRE complex was then run on a polyacrylamide gel. The unbound PRE was separated from the bound complex based on differences in mobility through the gel. Doses used were  $1 \mu\text{M}$  for atrazine and simazine and  $10 \text{ nM}$  for estradiol. Exposure to estradiol resulted in a 3.5-fold increase in reactivity of the R5020-PR-PRE complex relative to the negative control. The radioactivity in the R5020-PR-PRE band following exposure to atrazine and simazine was similar to negative control. The radioactivity in the band representing the R5020-PR-PRE complex was measured to get an indirect measure of PR levels in MCF-7 cells. Results indirectly indicate that the levels of PR did not increase or decrease with exposure to atrazine or simazine.

Transfection experiments were performed where the human estrogen receptor along with the luciferase expression system were inserted into MCF-7. The MCF-7 cell assay measured luciferase activity (*i.e.*, light production) and is quantitative in nature (*i.e.*, concentration of estrogenic chemical is directly related to light production). Doses used in the luciferase experiments were  $10^{-13}\text{M}$  to  $10^{-8}\text{M}$  estradiol and  $10^{-9}\text{M}$  to  $10^{-5}\text{M}$  atrazine and simazine. Atrazine and simazine did not induce luciferase activity above background at concentrations as high as  $10^{-5}\text{M}$  (concentrations above  $10^{-5}\text{M}$  are toxic to MCF-7 cells). Exposure to the positive control, estradiol, resulted in significant luciferase activity beginning at  $10^{-12}\text{M}$ . Light production increased exponentially up to  $10^{-8}\text{M}$  where light production plateaued.

**Neither atrazine nor simazine displayed estrogenic activity or interacted with the Ah receptor in the set of experiments described in this paper.**

This study is classified as **Acceptable-nonguideline** as a special study on *in vitro* estrogenic effects.

COMPLIANCE: Signed and dated Data Confidentiality and GLP statements were provided. Signed and dated Quality Assurance and Flagging statements were not provided.

## I. Organization of this DER

This study entails several different *in vitro* assays. Because of the diversity in experiment design and assay complexity, this DER is separated into specific sections describing different assays. Each section has a separate materials and methods, and results. There is one discussion at the end.

## II. Description of different assays.

### 1. Binding to the hepatocyte Ah receptor

This is a competitive binding assay using the aryl hydrocarbon (Ah) receptor isolated from male rat hepatocytes. The Ah receptor is a receptor which binds aromatic compounds such as PCBs and dioxins. Some dioxins which are Ah receptor agonists have been shown to exhibit antiestrogenic activity (such as decreased uterine weight, uterine peroxidase activity, and estrogen and progesterone receptor binding).

The purpose of this assay is to examine whether atrazine or simazine competitively bind to the Ah receptor. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is used as the positive control and triazine competitor.

Cytosol from male rat hepatocytes which contains Ah receptor is isolated and incubated with radiolabeled TCDD plus either atrazine or simazine. Next, there is a precipitation step using hydroxylapatite and two wash steps. Test compound, either TCDD or triazine, bound to the Ah receptor will precipitate and remain in the pellet. Unbound chemical will not precipitate out and will remain in the supernatant. By counting the radioactivity in the pellet versus the supernatant, it is possible to measure the amount of radiolabeled TCDD displaced indicating competitive inhibition with the triazine. Competitive binding is demonstrated by a decrease in radioactivity of the pellet versus the positive control. Although not definitive, competitive binding with TCDD *could* indicate that atrazine and simazine exhibit antiestrogenicity via a similar molecular mechanism as TCDD.

### 2. MCF-7 cell proliferation assay

This assay measures the number of MCF-7 cells which proliferate following 11 days of exposure to estradiol, atrazine, and simazine alone or in combination. MCF-7 is a cell line derived from human breast cancer cells which naturally have the estrogen receptor. MCF-7 cells are considered estrogen-responsive in that supplementing media with estrogen induces proliferation.

### 3. Gel electrophoresis mobility shift assay

Estradiol has been shown to increase the binding capacity of the progesterone receptor. Binding capacity is an indirect measure of receptor level. This assay measures the affect of estradiol and/or triazine on the capacity of the progesterone receptor to bind to a high affinity ligand (R5020). Polyacrylamide gel electrophoresis (PAGE) can separate biological molecules, such as DNA, or complexes of molecules, such as a protein-DNA complex, based on size. R5020 is a ligand for the PR which has high affinity for the PR. The DNA utilized in this assay is a radioactive oligonucleotide which contains a progesterone response element (PRE; PRE is a specific sequence of DNA which can bind to the PR).

In this study, MCF-7 cells exposed to atrazine, simazine, and /or estradiol for 3 days will be induced to express PR. The cells will then be incubated with radiolabeled R5020 which will bind to the PR. The R5020-PR complex is then isolated from the cells and incubated with radiolabeled PRE. The R5020-PR-PRE complex is then run on a polyacrylamide gel. The unbound PRE will separate from the bound complex based on differences in mobility through the gel. Differences in mobility result from different size molecules which move through the gel at variable rates. The radioactivity in the band representing the R5020-PR-PRE complex can be measured to get an indirect measure of PR levels in MCF-7 cells. The relative radioactivity between dose groups is compared.

### 4. Transfection and luciferase reporter gene assay

This assay uses a hybrid genetic system (*i.e.*, DNA from more than one species) to measure binding to the estrogen receptor *in vitro* using the luciferase reporter gene. Luciferase is an enzyme found in fireflies and light producing fish which produces measurable quantities of light.

Briefly MCF-7 cells are transfected (foreign DNA is inserted into MCF-7 cells) with DNA for a chimeric (from more than one species) receptor which contains the ligand binding region of the human estrogen receptor (HEGO) and the DNA binding region of the Gal4 gene. (Gal4 is a yeast gene which in its entirety codes for  $\beta$ -galactosidase; only a portion of the Gal4 gene is used in this study). In this assay, the HEGO-Gal4 chimeric enzyme is transcribed by the MCF-7 cells. An estrogenic chemical binds to the ligand binding portion of the HEGO causing an allosteric change in the Gal4 region of the chimeric protein. The Gal4 portion of the chimeric receptor then binds to the DNA upstream from the luciferase gene. The region where Gal4 binds is a repeated sequence of 17 bases which is recognized by the Gal4. Binding to this repeated sequence results in transcription of the luciferase reporter gene thus generating light. Theoretically there is a direct relationship between the binding of the estrogenic chemical to the estrogen receptor and light production such that light production can

used as a surrogate measurement for estrogen receptor binding.



III. *In vitro* assays

1) BINDING TO HEPATIC AH RECEPTOR

A. MATERIALS

1. Test Material: Atrazine and Simazine

Description: Not given

Lot/Batch #: Obtained by author from Chem-Service (West Chester, PA) and from Ciba-Geigy (Greensboro, NC).

Purity: > 97% a.i.

Stability of compounds: Not given

CAS #: 1912-24-9 (Atrazine); 122-34-9 (Simazine)

2. Vehicle and/or positive control:

[<sup>3</sup>H]TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) was synthesized by the authors and purified to > 95%.

3. Test animals: Species: male rat

Strain: Long Evans

Age and weight at study initiation: "immature"; age not given, weight not given

Source: Harlan-Sprague-Dawley; Houston, TX

Housing: 4 per cage

Diet: not specified. Given ad libitum

Water: not specified. Given ad libitum

Environmental conditions: Temperature: Not given

Humidity: Not given

Air changes: Not given

Photoperiod: Not given

Acclimation period: Not given

B. STUDY DESIGN:

1. In life dates - Not given

2. Animal assignment -N/A

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3. Method:

Animals were euthanized by cervical dislocation. Livers were perfused *in situ* with buffer via the inferior vena cava. Livers were homogenized using a commercial homogenizer. Homogenates were centrifuged at 10,000 g and the resulting supernatant was saved. This supernatant was ultracentrifuged. The resulting supernatant containing the liver cytosol was incubated with [<sup>3</sup>H]TCDD and either atrazine or simazine at 20°C for 2 hours. Hydroxyapatite was added to precipitate high molecular weight compounds including the Ah receptor with bound compound. The resulting pellet was washed in ice cold buffer containing 1% Triton-X and recentrifuged. The final pellet was rinsed with 1 mL of ethanol and radioactivity was determined by liquid scintillation counting. Atrazine and simazine were tested in the range of 0 nM to 10,000 nM.

5. Dose Selection: N/A6. Statistical methods:

Statistical significance was determined by ANOVA and student's t-test. Means and standard deviations were calculated for at least 3 samples per concentration.

## C. RESULTS:

At concentrations up to 10,000 nM, atrazine and simazine did not displace TCDD from the Ah receptor. One hundred-120% of radioactivity was precipitated in the pellet at all concentrations of triazine tested indicating that triazine was not bound to the Ah receptor.

## 2) MCF-7 CELL PROLIFERATION ASSAY

## A. MATERIALS

1. Test Material: Atrazine and Simazine

Description: not given

Lot/Batch #: Obtained by author from Chem-Service (West Chester, PA) and from Ciba-Geigy (Greensboro, NC).

Purity: > 97% a.i.

Stability of compounds: Not given

CAS #: 1912-24-9 (Atrazine); 122-34-9 (Simazine)

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2. In vitro cell line :

Strain: MCF-7; human breast cancer cell line

Source: American Type Culture Collection

Growth conditions of cell line:

MCF-7 cells were seeded (initial inoculation on a growth plate) at 50,000 cells/well in 6 well plates with a standard mammalian cell culture growth media. Components of media include DME/F12, NaHCO<sub>3</sub>, apotransferrin, fetal bovine serum (FBS), and bovine serum albumin. The cells were allowed to stabilize for 16 hours in serum free media followed by 24 hours in 5% DCC-FBS media before use in assays.

Temperature: Not given. Humidity: Not given

B. STUDY DESIGN:

1. Method:

Cells in growth media were dosed with estradiol, atrazine, simazine dissolved in DMSO as shown in Table 1. Levels of DMSO in growth media did not exceed 0.1 % to prevent toxic effects from DMSO. Cells were exposed to the compounds for 11 days. Growth media and test compounds were changed every other day. Cell viability and cell attachment were monitored with a light microscope; viability and attachment did not change during the study. After 11 days, cells were washed and counted electronically using a Coulter particle counter. Three assays were performed twice per dose group.

TABLE 1. STUDY DESIGN FOR MCF-7 CELL PROLIFERATION ASSAY.

Dose groups for MCF-7 proliferation assay	
Experiment 1	Experiment 2
Vehicle control (DMSO)	Vehicle control (DMSO)
100 $\mu$ M Atrazine	Positive control (1nM Estradiol)
10 $\mu$ M Atrazine	100 $\mu$ M Atrazine + 1nM Estradiol
1.0 $\mu$ M Atrazine	10 $\mu$ M Atrazine + 1nM Estradiol
0.1 $\mu$ M Atrazine	1.0 $\mu$ M Atrazine + 1nM Estradiol
0.01 $\mu$ M Atrazine	0.1 $\mu$ M Atrazine + 1nM Estradiol
100 $\mu$ M Simazine	0.01 $\mu$ M Atrazine + 1nM Estradiol
10 $\mu$ M Simazine	100 $\mu$ M Simazine + 1nM Estradiol
1.0 $\mu$ M Simazine	10 $\mu$ M Simazine + 1nM Estradiol
0.1 $\mu$ M Simazine	1.0 $\mu$ M Simazine + 1nM Estradiol
0.01 $\mu$ M Simazine	0.1 $\mu$ M Simazine + 1nM Estradiol
	0.01 $\mu$ M Simazine + 1nM Estradiol

2. Dose rationale. Not given

3. Statistical methods:

Statistical significance was determined by ANOVA and student's t-test. Means and standard deviations were calculated for at least 3 samples per concentration.

### C. RESULTS

No changes in numbers of MCF-7 cells were seen relative to the negative control with exposure to either atrazine or simazine alone. The positive control, estradiol, caused a 2 fold increase in number of MCF-7 cells after 11 days exposure. No changes in cell density of MCF-7 cells were seen relative to the positive control with exposure to estradiol plus either atrazine or simazine.

### 3) GEL ELECTROPHORETIC MOBILITY SHIFT ASSAY

A. MATERIALS: see previous section (p. 6).

B. STUDY DESIGN:

1. Method:

MCF-7 cells in growth media were exposed for 3 days with estradiol, atrazine, simazine dissolved in DMSO as shown in Table 2. Levels of DMSO in growth media did not exceed 0.1 % to prevent toxic effects from DMSO. (Three days is the optimal time for progesterone receptor induction.) One hour before harvesting cells, 2 nM of [<sup>3</sup>H]R5020 was added to the growth media. MCF-7 cells were harvested, washed, and pelleted according to a standard procedure. The cells were resuspended in an isotonic buffer and homogenized using a Teflon pestle/drill apparatus. The nucleus was separated from the remainder of the cell by centrifugation.

PRE-oligonucleotide (DNA containing the progesterone receptor element) was end labeled with <sup>32</sup>P-ATP at the 5' end using a commercial T4-polynucleotide kinase end labeling kit. Five to 10 μg of nuclear extract was incubated with 100,000 cpm (counts per minute) of radiolabeled PRE for 15 minutes at 20°C. The reaction mixture plus 2 μL loading buffer was loaded onto a 5% nondenaturing polyacrylamide gel and electrophoresed at 110V (time not given). The gels were dried and protein-DNA complexes were visualized using autoradiography and quantified with a Betagen Betascope 603 blot analyzer.

TABLE 2. STUDY DESIGN FOR THE GEL ELECTROPHORESIS.

Dose groups
Negative control
Positive control (1 nM Estradiol)
1 μM Simazine
1 μM Atrazine
1 μM Simazine + 1 nM Estradiol
1 μM Atrazine + 1 nM Estradiol

2. Dose rationale:  
Not given.

3. Statistics :

Means and standard deviations were calculated by the authors but not given in data.

C. RESULTS:

In this assay, the intensity of radioactivity in the band representing the R5020-PR-PRE complex is measured. Because gel electrophoresis separates molecules based on size, this band representing the R5020-PR-PRE complex migrates more slowly than does the oligonucleotide alone. The intensity of this band represents the amount of radioactive PRE-oligonucleotide bound to the PR-R5020 complex. By definition, the negative control is defined as 100% relative mean intensity. The treatment groups are compared to the negative control group. The positive control (estradiol alone) caused a 3.5 fold increase in band intensity. Simazine or atrazine alone at 1  $\mu$ M do not change band intensity relative to the negative control. When exposed to estradiol plus atrazine or simazine, the intensity of the band representing bound PR complex was similar to that of the positive control.

**4) TRANSFECTION AND LUCIFERASE REPORTER ASSAY USING MCF-7**

A. MATERIALS: See p. 6.

B. STUDY DESIGN1. Method:

MCF-7 cells were transiently transfected according to the procedure of Zacharewski *et al.* (1995) with 5  $\mu$ g pCH110 (commercially purchased  $\beta$ -galactosidase expression vector), 5  $\mu$ g 17m5-G-Luc (17 base oligonucleotide-regulated luciferase reporter gene), 1  $\mu$ g Gal4-HEGO (Gal4 is the gene for  $\beta$ -galactosidase; HEGO is the gene for the estrogen receptor) and 4  $\mu$ g pBS (carrier DNA, commercially purchased). Transfections were performed according to the procedure of Sambrook *et al.* (1989) which entails a calcium phosphate coprecipitation. Twenty-four hours following transfection, plates with cells were washed and media replaced. Transfection efficiency was determined using the standard procedure of Sambrook *et al.* (1989) by cotransfection of a reference plasmid, pCH110. Transfected cells were exposed to estradiol, atrazine, and simazine for 24 hours. Five  $\mu$ L of compound dilution (in DMSO) was dissolved in 5 mL of media. Exposure concentrations are given in Table 3. Following exposure, cells were harvested. The luciferase assay was performed according to Brasier *et al.* (1989). Light was measured using a flourometer.

TABLE 3. STUDY DESIGN OF LUCIFERASE ASSAYS.

Treatment	Concentrations of test chemical (M)
Untreated control	0
Estradiol	$10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}$
Atrazine	$10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5*}$
Simazine	$10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5*}$

\*Concentrations of test chemicals  $>10^{-5}$  resulted in loss of cell attachment to plate.

2. Dose selection.: Not given

3. Statistics.

Each chemical exposure was performed in duplicate from two samples for one transfection to yield four values per exposure concentration. Means and standard deviations were calculated by the authors but not given in study report.

### C. RESULTS:

Atrazine and simazine did not induce luciferase activity above background at concentrations as high as  $10^{-5}$  M. Estradiol resulted in significant luciferase activity beginning at  $10^{-12}$  M and increased exponentially up to  $10^{-8}$  M where light production plateaued. According to these results, exposure to atrazine and simazine did not induce DNA binding to the estrogen receptor.

In order to test the antiestrogenic activity of atrazine and simazine, co-exposure experiments were performed. The luciferase activity measured following exposure to estradiol plus atrazine or simazine was similar relative to the positive control.

#### IV. Discussion and deficiencies:

##### A. DISCUSSION:

The results from the assays performed in this study indicate that atrazine and simazine do not have estrogenic or antiestrogenic activity. Atrazine, simazine, and estradiol were tested both individually and in combination in the assays used here. Experiments using individual chemicals are testing for positive estrogenic effects. Co-exposure experiments are testing for synergism and/or anti-estrogenic activity of atrazine and simazine. Four *in vitro* assays were performed: binding to the hepatocyte Ah receptor, cell proliferation of MCF-7 cells, gel electrophoresis mobility shift assay, and luciferase activity of transformed MCF-7 cells.

Ah receptor binding assay used here is a competitive binding assay using a triazine and TCDD. Some dioxins, such as TCDD, which are Ah receptor agonists have been shown to exhibit antiestrogenic activity (such as decreased uterine weight, uterine peroxidase activity, and estrogen and progesterone receptor binding). Competitive binding would have been shown as the decrease in radioactivity of the precipitated pellet versus the positive control. Atrazine and simazine did not out compete TCDD from the Ah receptor since 100-120% of radioactive TCDD in the pellet was measured in the triazine treated sample. These results only indicate that atrazine and simazine may not exhibit the same mechanism of action for antiestrogenicity as TCDD.

In the cell proliferation assays, estradiol significantly increased the number of cell numbers of MCF-7 at day 11. No changes in cell density of MCF-7 cells were seen relative to the negative control with exposure to either atrazine or simazine alone or relative to the positive control in co-exposure experiments.

The level of the progesterone receptor *in vitro* was measured indirectly by the amount of radioactivity in a retarded band of a polyacrylamide gel. Exposure to estradiol significantly increased the amount of radioactivity in this band but atrazine and simazine at 1  $\mu$ M did not. In co-exposure experiments, the amount of radioactivity was similar to the positive control alone indicating that atrazine and simazine at 1  $\mu$ M did not exhibit any antiestrogenic activity.

Transfection experiments were performed where the human estrogen receptor was inserted into MCF-7. This assay measures luciferase activity (i.e., light production) which is a surrogate measure of estrogen receptor binding. Exposure to estradiol resulted in strong light production. Atrazine and simazine did not exhibit any estrogenic or antiestrogenic activity as shown by no light production.



B. DEFICIENCIES:

1. This study did not follow GLP regulations but this study is not required for FIFRA. Based on the descriptions and references of methods given, it seems that the authors have utilized good animal husbandry and laboratory practice.
2. Although not required for journal publication, from a regulatory perspective, it would be valuable to have the following data:
  - Concentration analysis of the dilutions
  - Analysis and mass balance of radioactivity used
  - Specific experimental conditions used in animal care and cell growth chambers
  - Calibration criteria for Coulter counter and flourometer
  - Raw data and/or means and deviations; data is reported only as figures
3. The active ingredient came from two different sources.
4. Because the estrogenic effect of estradiol is so strong relative to the antiestrogenic effect of atrazine or simazine, it would be valuable scientifically to observe the effects of co-exposure using a lower estradiol concentration.
5. Transfection efficiency is not addressed in the study.

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**Atrazine and Simazine**

*SignOff Date:*

3/24/99

*DP Barcode:*

D253661

*HED DOC Number:*

013281

*Toxicology Branch:*

RCAB

**Estrogenicity *in vitro*. Special Study**

EPA Reviewer: Anna Bearden, PhD

\_\_\_\_\_, Date \_\_\_\_

EPA Secondary Reviewer: Roger Hawks, PhD

\_\_\_\_\_, Date \_\_\_\_

DATA EVALUATION RECORD
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STUDY TYPE: *In vitro* and *in vivo* estrogenicity. Special study.

DP BARCODE: 253661

SUBMISSION CODE: S557393

P.C. CODE: 080803 (Atrazine); 080807 (Simazine)

TOX. CHEM. NO.: 063 Atrazine  
074 Simazine

TEST MATERIAL (PURITY): Atrazine (> 97%) and Simazine (> 97%)

SYNONYMS: G-30027 (Atrazine); G-27692 (Simazine)

CITATION: Conner, K.; J. Howell; I. Chem; *et al.* (1996) Failure of chloro-S-triazine-derived compounds to induce estrogen receptor-mediated responses *in vivo* and *in vitro*. Texas A&M University, College Station, Texas and University of Western Ontario, London, Ontario, Canada. MRID 4394403. Published in *Fundamental and Applied Toxicology*. 30:93-101.

SPONSOR: N/A

EXECUTIVE SUMMARY:

This study (MRID no. 4394403) combines three *in vivo* and four *in vitro* assays to investigate the estrogenic and antiestrogenic activity of atrazine and simazine. *In vivo* experiments, 4 or 5 female Sprague-Dawley rats/dose were dosed orally or by intraperitoneal injection. In order to test for estrogenic activity, rats were exposed to atrazine (> 97% a.i.) or simazine (> 97% a.i.) at 50, 150, and 300 mg/kg/day or estradiol (positive control) at 10  $\mu$ g/kg/day. In order to test for antiestrogenic activity, co-exposure experiments using atrazine or simazine plus estradiol were performed using the same concentrations. Following a three day exposure, the following *in vivo* endpoints were measured: uterine weight, progesterone receptor levels and uterine peroxidase.

Exposure to estradiol results statistically significant increased uterine weights, increased levels of the progesterone receptor, and increased activity of uterine peroxidase. Exposure to atrazine and simazine individually resulted in negative effects indicating antiestrogenic activity are shown in slightly reduced uterine weights, statistically significantly reduced levels of the progesterone receptor, and statistically significantly reduced activity of uterine peroxidase.

The following *in vitro* assays were performed: proliferation of MCF-7 (human breast cancer cell line) cells; gel electrophoresis mobility shift assay to measure levels of progesterone receptor; MCF-7 transfection using the luciferase reporter gene; and yeast transfection using a selective media expression system.

Doses for the cell proliferation assay when tested individually and in combination were 10  $\mu$ M,

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1.0  $\mu\text{M}$ , 0.1  $\mu\text{M}$ , and 0.01  $\mu\text{M}$  for atrazine and simazine and 1 nM for estradiol. Results indicated that the positive control significantly increased the density of MCF-7 cells three fold relative to the negative control after 11 days. Exposure to atrazine and simazine alone neither increased nor decreased cell number at day 11 relative to the negative control. Results of co-exposure experiments indicate that cell number did not change relative to the positive control.

The gel electrophoresis mobility shift assay used in this study indirectly measures progesterone receptor (PR) levels in MCF-7 cells following exposure for 3 days. The oligonucleotide used contains progesterone response element (PRE) which binds to the PR. PR from MCF-7 cells was isolated and incubated with radiolabeled PRE. The PR-PRE complex was then run on a polyacrylamide gel to separate bound and unbound PRE based on differences in mobility through the gel. The radioactivity in the band representing the PR-DNA complex was measured as an indirect measure of PR levels. Doses used were 1  $\mu\text{M}$  for atrazine and simazine and 10 nM for estradiol. Results indicated that exposure to estradiol resulted in a 3.5-fold increase in reactivity of the DNA-PR complex relative to the negative control. The radioactivity in the DNA-PR band following exposure to atrazine and simazine was similar to negative control. These results indicate indirectly that the levels of PR did not increase or decrease with exposure to atrazine or simazine.

Two transfection experiments were performed where the human estrogen receptor was inserted into the genome of MCF-7 and yeast cells. The MCF-7 cell assay measured luciferase activity as a surrogate measure of estrogen receptor activity. Doses used were  $10^{-13}\text{M}$  to  $10^{-8}\text{M}$  estradiol and  $10^{-9}\text{M}$  to  $10^{-5}\text{M}$  atrazine and simazine. Atrazine and simazine did not induce luciferase activity above background at concentrations as high as  $10^{-5}\text{M}$  (concentrations above  $10^{-5}\text{M}$  are toxic to MCF-7 cells). Exposure to the positive control, estradiol, resulted in significant luciferase activity beginning at  $10^{-12}\text{M}$  and increased exponentially up to  $10^{-8}\text{M}$  where light production plateaued. Atrazine and simazine did not exhibit estrogenic or antiestrogenic activity and did not interact directly with the estrogen receptor.

In the yeast transfection assay, the human estrogen receptor linked to a necessary amino acid was inserted into the yeast. Growth on selective media is a qualitative measure of activity of estrogen receptor. Yeast were dosed with 1 nM estradiol or 10  $\mu\text{M}$  atrazine or simazine. Yeast proliferated when exposed to estradiol but did not proliferate when exposed to atrazine or simazine. These results indicate that atrazine and simazine did not exhibit estrogenic activity and did not directly interact with the estrogen receptor.

**In conclusion, the results of these experiments indicate that *in vivo* atrazine and simazine exhibited some antiestrogenic activity but no estrogenic activity. Based on the *in vitro* results, this antiestrogenic activity is not the result of *direct* interaction with the estrogen receptor.**

This study is classified as **acceptable-nonguideline** as a special study on *in vivo* and *in vitro* estrogenic effects.

COMPLIANCE: Signed and dated GLP, Quality Assurance, Data Confidentiality, and Flagging statements were not provided. This study was published in the public literature at a university and therefore was not subject to these requirements.

## II. Organization of this DER

This study entails several different assays including *in vivo* and *in vitro* endpoints. Because of the diversity in experiment design and assay complexity, this DER is separated into specific sections describing different assays. Each section has a separate materials and methods, and results. There is one discussion at the end.

## **III. Description of different assays.**

### A. *In vivo* studies using rat uterine tissue

#### 1. Uterine weight assay

This assay measures the weight of uteri taken from young female rats which have been exposed to a test chemical for 3 days. As an *in vivo* assay, it incorporates important factors such as toxicokinetics and metabolism which are ignored in typical *in vitro* studies. Increases in uterine weight result from proliferation and thickening of the uterine wall induced by estrogen or chemicals with estrogenic activity.

#### 2. Progesterone receptor assay

Progesterone receptor levels (PR) were measured using the methods described by Lamb and Bullock (1983) and Dickerson and Safe (1992).

This assay indirectly measures levels of PR in uteri of exposed rats. Activity of the estrogen receptor in exposed uteri causes an increase in the level of the PR in the uterine nuclear cytoplasm. In this assay the dissected uteri are homogenized and then separated into cytosol and membrane fractions by ultracentrifugation. The cytosolic fraction contains the PR. A radiolabeled compound called R5020 is used to measure the concentration of PR in the sample. R5020 is a protein ligand for the PR which has high affinity for the PR. Radiolabeled R5020 will bind PR in the uterine tissue sample. After separating R5020-PR complex from unbound R5020, scintillation counts can be used to measure PR levels. It is assumed that one molecule of R5020 binds to one molecule of PR.

### 3. Uterine peroxidase assay

It has been shown that as the activity of the estrogen receptor increases, there is a direct increase in activity of the enzyme uterine peroxidase. Uterine peroxidase activity can therefore be used a surrogate measure for activity of the estrogen receptor. This assay was initially described by Lyttle and DeSombre (1977).

Uterine homogenates are suspended in an isotonic buffer containing  $\text{CaCl}_2$ .  $\text{CaCl}_2$  is used to reduce the strength of the cellular membrane in order to make the contents of the cell available for the assay. Following a centrifugation wash step, hydrogen peroxide and guaiacol are added to the supernatant. Guaiacol is a compound that turns red following an oxidation reaction with peroxide. The uterine peroxidase oxidizes guaiacol. The oxidized product changes to red in color; the red color can then be measured using a spectrophotometer. Therefore, the increasing red color in solution directly corresponds to more uterine peroxidase activity which in turn is directly related to the activity of the estrogen receptor.

## B. *In vitro* studies

### 1. MCF-7 cell proliferation assay

This assay measures the number of MCF-7 cells which proliferate following 11 days of exposure to estradiol, atrazine, and simazine alone or in combination. MCF-7 is a cell line derived from human breast cancer cells which naturally have the estrogen receptor. MCF-7 cells are considered estrogen-responsive in that supplementing media with estrogen induces proliferation.

### 2. Gel electrophoresis mobility shift assay

As indicated above, levels of the progesterone receptor *in vivo* are measured by the amount of radioactive R5020 bound to the PR. Because the level of PR in MCF-7 cells is much lower than uterine tissue, a more sensitive assay is required.

A polyacrylamide gel can separate biological molecules, such as DNA, or complexes of molecules, such as a protein-DNA complex, based on size. As performed in this study, there are two proteins utilized: the PR and the ligand, R5020. The DNA utilized in this assay is a radioactive oligonucleotide which contains a progesterone response element (PRE; PRE is a specific sequence of DNA which can bind to the PR).



In this study, the PR will be induced in MCF-7 cells. The cells will then be incubated with radiolabeled R5020. R5020 will bind to the PR. The R5020-PR complex is then isolated from the cells and incubated with radiolabeled PRE. The R5020-PR-PRE complex is then run on a polyacrylamide gel. The unbound PRE will separate from the bound complex based on differences in mobility through the gel. The difference in mobility results from difference size molecules which move through the gel at different rates. The radioactivity in the band representing the R5020-PR-PRE complex can be measured to get an indirect measure of PR levels in MCF-7 cells.

### 3. Transfection and luciferase reporter gene assay

This assay uses a hybrid genetic system (*i.e.*, DNA from more than one species) to measure binding to the estrogen receptor *in vitro* using the luciferase reporter gene. Luciferase is an enzyme found in fireflies and light producing fish which produces measurable quantities of light.

Briefly MCF-7 cells are transfected (foreign DNA is inserted into MCF-7 cells) with DNA for a chimeric (from more than one species) receptor which contains the ligand binding region of the human estrogen receptor (HEGO) and the DNA binding region of the Gal4 gene. (Gal4 is a yeast gene which in its entirety codes for  $\beta$ -galactosidase; only a portion of the Gal4 gene is used in this study). In this assay, the HEGO-Gal4 chimeric enzyme is transcribed by the MCF-7 cells. An estrogenic chemical binds to the ligand binding portion of the HEGO causing an allosteric change in the Gal4 region of the chimeric protein. The Gal4 portion of the chimeric receptor then binds to the DNA upstream from the luciferase gene. The region where Gal4 binds is a repeated sequence of 17 bases which is recognized by the Gal4. Binding to this repeated sequence results in transcription of the luciferase reporter gene thus generating light. Theoretically there is a direct relationship between the binding of the estrogenic chemical to the estrogen receptor and light production such that light production can be used as a surrogate measurement for activity of the estrogen receptor.

### 4. Estrogen-receptor mediated growth assay in yeast

This assay uses a hybrid genetic system (*i.e.*, DNA from more than one species) to determine activity of the estrogen receptor *in vitro*. Briefly yeast cells are transfected (foreign DNA is inserted into yeast cells) with the human estrogen receptor (HEGO) and an expression system. An expression system acts as a link between the activity of interest (*i.e.*, estrogenicity) and a measurable endpoint. In this study the measured endpoint is growth/non-growth on selective media. Similar to mammalian cells, yeast normally have the ability to produce most of the amino acids they need for survival. The genetically engineered strain of yeast used in this assay (*Saccharomyces cerevisiae* PL3) does not contain the complete genes for production of the following necessary amino acids: histidine, leucine, and uracil. Therefore for this strain to grow, growth

media must be supplemented with histidine, leucine, and uracil. This assay takes advantage of this need.

The yeast are transfected with DNA which contain both the human estrogen receptor and also the complete genes for uracil. This DNA has been constructed such that uracil is expressed only when the estrogen receptor is expressed. Therefore, if an external stimulus (*i.e.*, an estrogenic chemical) induces the expression of the estrogen receptor, uracil is also produced and the yeast colonies proliferate. Conversely, if an estrogenic chemical is not present, the receptor is not produced and therefore the amino acids are also not produced and the yeast does not survive.

This assay is a qualitative measure of estrogenicity. In this study the yeast assay is used to verify the results of the previous assays, particularly the MCF-7 luciferase assay. MCF-7 cells naturally contain the estrogen receptor. Basal activity of this estrogen receptor can interact with the inserted HEGO. Based on the status and husbandry of the cells in culture, this background can significantly interfere with the experimental results. Yeast do not naturally contain estrogen or an estrogen receptor. Therefore the activity inserted HEGO gene should result only from interaction with the test chemical(s).

**IV. IN VIVO ASSAYS USING UTERINE MATERIAL****A. MATERIALS****1. Test Material: Atrazine and Simazine**

Description: Not given

Lot/Batch #: Obtained by author from Chem-Service (West Chester, PA) and from Ciba-Geigy (Greensboro, NC).

Purity: &gt; 97% a.i.

Stability of compounds: Not given

CAS #: 1912-24-9 (Atrazine); 122-34-9 (Simazine)

**2. Vehicle and/or positive control:**

Two vehicle controls were used. One was made by the following dilution: 1 mL of DMSO added to appropriate volumes of water and hydroxypropyl cellulose to yield 5% hydroxypropyl cellulose. The second vehicle control was corn oil. In both experiments 0.5 mL of test substance was administered once a day.

The positive control was estradiol dissolved in DMSO and hydroxypropyl cellulose as above or estradiol dissolved in corn oil.

**3. Test animals: Species: female rat**

Strain: Sprague-Dawley

Age and weight at study initiation: 21 days old, weight not given

Source: Harlan-Sprague-Dawley, Houston, TX

Housing: 4 per cage

Diet: not specified. Given ad libitumWater: not specified. Given ad libitum

Environmental conditions: Temperature: Not given

Humidity: Not given

Air changes: Not given

Photoperiod: Not given

Acclimation period: Not given

**B. STUDY DESIGN:****1. In life dates - Not given****2. Animal assignment**

Method of animal assignment was not given.

### 3. Method:

Due to logistical reasons, two separate groups of animals were used to perform the *in vivo* experiments. The first group of animals were used to test atrazine, simazine, and estradiol individually. The second group was exposed to estradiol plus atrazine or simazine. In both experiments 0.5 mL of test substance was administered once a day for 3 days.

The routes of administration and solvent vehicles varied for the experiments. In the single chemical experiments, rats were dosed orally using DMSO and adequate water to yield 5% hydroxypropyl cellulose. In the second co-exposure experiments, rats were dosed by intraperitoneal injection using corn oil as the vehicle.

Animals were euthanized by carbon dioxide asphyxiation 20 hours following the last treatment. The animals were dissected and the uteri removed, cleansed of connective tissue, weighed, blotted, bisected into two uterine horns and placed on ice. Uterine horns for each dose group were pooled in 1 mL/50 mg tissue of ice cold TESH-Mo buffer. (TESH-Mo buffer contains Tris, EDTA, thioglycerol, and sodium molybdate. The function of this buffer is to reduce the strength of the outer membrane of the cells in the uterine tissue samples to improve the effectiveness of the homogenation procedure.)

The uterine tissue was then homogenated using a tissue grinder. This homogenate was used for two different assays: measurement of progesterone receptor (PR) levels and the uterine peroxidase assay.

#### i. *Progesterone receptor measurement*

The cytosol was incubated with [<sup>3</sup>H]R5020 in the presence and absence of progesterone. Assays performed in the presence of progesterone act as a control for the presence and functionality of the PR. Following 18 hour incubation at 4°C, samples were treated with dextran coated charcoal and centrifuged. The supernatant which contained radiolabeled R5020 bound to PR was counted using a scintillation counter. Data in Table 2 represent the mean of three assays.

ii. *Uterine peroxidase assay*

It has been shown that as the activity of the estrogen receptor increases, there is a direct increase in activity of the enzyme uterine peroxidase. Thus, uterine peroxidase activity can be used a surrogate measure for activity of the estrogen receptor.

Uterine homogenates were centrifuged and the resultant pellet washed. The washed pellet was then resuspended in an isotonic buffer containing  $\text{CaCl}_2$  ( $\text{CaCl}_2$  is used to reduce the strength of the cellular membrane in order to make the contents of the cell available for the assay). The sample was then centrifuged and the supernatant saved for analysis. Thirteen mM Guaiacol and 0.3 mM hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) were added to the supernatant. (Guaiacol is a compound that turns red following a oxidation reaction with peroxide.) The uterine peroxidase oxidizes guaiacol. The oxidized product changes to red in color; the red color can then be measured using a spectrophometer at 470 nM. Therefore, the increasing red color in solution directly corresponds to more uterine peroxidase activity which in turn is directly related to the activity of the estrogen receptor. Three assays per dose were performed.

5. Dose Selection:

Based on published studies (Eldridge, *et al.*, 1994; Stevens, *et al.*, 1994; Tennant *et al.*, 1994a, b; Wetzel, *et al.*, 1994) which have reported estrogenic and antiestrogenic responses resulting from exposure to atrazine and simazine.

6. Statistical methods:

The mean and standard deviation were reported for four or five uterine weights per dose group and for three data points per dose group for the progesterone receptor measurements and the uterine peroxidase activity assay. Statistical differences were determined by Duncan's multiple range test by ANOVA analysis. The level of significance was set a  $p < 0.05$ .

C. RESULTS: *IN VIVO* STUDIES1. Uterine weights:

Changes in uterine weight following a 3 day exposure is considered a sensitive *in vivo* endpoint for estrogenicity. As shown in Table 2, exposure to estradiol results in a 6 fold increases in uterine weight relative to untreated controls. The uterine weights of rats exposed to atrazine and simazine are statistically similar to control uterine weights. There is a slight trend for the uterine weight to decrease in the dose groups treated with 150 and 300 mg/kg/day atrazine.

A separate experiment studied the effects of co-exposure with estradiol and atrazine or simazine. Increases in uterine weight, with the exception noted below, do not change with co-exposure to either atrazine or simazine relative to the positive control. The uterine weight of rats exposed to both estradiol and 300 mg/kg/day of simazine are reduced ( not statistically significant) relative to positive control weights.

2. Progesterone receptor levels:

The amount of radiolabeled R5020 bound to PR is assumed to be directly related the concentration of the PR in the cells. As shown in Table 2, levels of the PR in rats, as measured by radioactivity of bound R5020, exposed to atrazine at 150 and 300 mg/kg/day and simazine at 50, 150, and 300 mg/kg/day are statistically decreased relative to control uterine weights.

Exposure to estradiol results in a 5 to10 fold increase in PR levels relative to untreated controls. In dose groups co-exposed to estradiol and either atrazine or simazine, increases in levels of PR are similar to the positive control. The exception to this is the estradiol + simazine at 150 mg/kg/day dose group which is statistically decreased relative to the positive control.

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TABLE 1: STUDY DESIGN FOR *IN VIVO* STUDIES

Test Group	Dose to animal (units)	Number of animals per group
Vehicle Control (DMSO and hydroxyl cellulose)	--	5
Positive control (estradiol)	10 $\mu\text{g}/\text{kg}/\text{day}$	5
Atrazine	50 mg/kg/day	4
Atrazine	150 mg/kg/day	4
Atrazine	300 mg/kg/day	4
Simazine	50 mg/kg/day	4
Simazine	150 mg/kg/day	4
Simazine	300 mg/kg/day	4
Vehicle control <sup>a</sup> (corn oil)	--	5
Positive control (estradiol)	10 $\mu\text{g}/\text{kg}/\text{day}$	5
Atrazine + Estradiol	50 mg/kg/day	4
Atrazine + Estradiol	150 mg/kg/day	4
Atrazine + Estradiol	300 mg/kg/day	4
Simazine + Estradiol	50 mg/kg/day	4
Simazine + Estradiol	150 mg/kg/day	4
Simazine + Estradiol	300 mg/kg/day	4

<sup>a</sup> Due to logistical reasons, two experiments were run. Each experiment had separate positive and negative controls.

3. Uterine peroxidase:

Because cellular levels of the enzyme uterine peroxidase increase with the activity of the estrogen receptor, this enzyme can be used as a biomarker for estrogen receptor activity. In this study, exposure to estradiol resulted in a 10 fold increase in levels of uterine peroxidase relative to the untreated control. Exposure to atrazine alone resulted in a dose dependant and statistically significant decrease in uterine peroxidase levels. Exposure to simazine alone resulted in statistically significant 6 fold decrease in uterine peroxidase levels in all dose levels.

Experiments were performed with both estradiol plus atrazine or simazine. Exposure to estradiol alone resulted in a 10 fold increase in levels of uterine peroxidase relative to the untreated control. Although several dose groups exhibited significant differences relative to the positive control, these differences do not exhibit a trend and result from inherent variability in the assay.

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TABLE 2. RESULTS OF *IN VIVO* STUDIES.

Test Group	Dose to animal (units)	Uterine Weight (mg)	Progesterone Receptor (fmol/uterus)	Uterine peroxidase (units/mg prot)
Vehicle Control	--	50 ± 6.1	744 ± 74	0.62 ± 0.019
Positive control (estradiol)	10 µg/kg/day	308 ± 71*	6640 ± 638*	5.99 ± 0.019*
Atrazine	50 mg/kg/day	53 ± 20	769 ± 107	0.37 ± 0.023*
Atrazine	150 mg/kg/day	35 ± 2.2	425 ± 104*	0.26 ± 0.008*
Atrazine	300 mg/kg/day	36 ± 2.2	419 ± 37*	0.03 ± 0.002*
Simazine	50 mg/kg/day	42 ± 10	376 ± 24*	0.09 ± 0.004*
Simazine	150 mg/kg/day	36 ± 8.5	320 ± 215*	0.12 ± 0.011*
Simazine	300 mg/kg/day	47 ± 7.0	266 ± 18*	0.02 ± 0.007*
Vehicle control (corn oil)	--	54 ± 21	889 ± 457	0.377 ± 0.03
Positive control (estradiol)	10 µg/kg/day	198 ± 57*	4084 ± 540*	4.13 ± 0.06*
Estradiol + Atrazine	50 mg/kg/day	185 ± 61*	3230 ± 968*	4.25 ± 0.12*
Estradiol + Atrazine	150 mg/kg/day	164 ± 49*	3002 ± 430*	4.49 ± 0.10 <sup>aa</sup>
Estradiol + Atrazine	300 mg/kg/day	166 ± 71*	4445 ± 2171*	4.34 ± 0.22*
Estradiol + Simazine	50 mg/kg/day	212 ± 31*	2841 ± 185*	3.61 ± 0.05 <sup>aa</sup>
Estradiol + Simazine	150 mg/kg/day	155 ± 69*	2074 ± 345 <sup>aa</sup>	5.23 ± 0.20 <sup>aa</sup>
Estradiol + Simazine	300 mg/kg/day	116 ± 37*	3146 ± 1189*	3.73 ± 0.08 <sup>aa</sup>

\*Significantly different from vehicle control ( $p < 0.05$ )<sup>a</sup> Significantly different from positive control

## V. IN VITRO STUDIES

### 1) MCF-7 Cell proliferation assay

#### A. MATERIALS

1. Test Material: Atrazine and Simazine

Description: not given

Lot/Batch #: Obtained by author from Chem-Service (West Chester, PA) and from Ciba-Geigy (Greensboro, NC).

Purity: > 97% a.i.

Stability of compounds: Not given

CAS #: 1912-24-9 (Atrazine); 122-34-9 (Simazine)

2. In vitro cell line:

Strain: MCF-7; human breast cancer cell line

Source: American Type Culture Collection

Growth conditions of cell line:

MCF-7 cells were seeded (initial inoculation on a growth plate) at 50,000 cells/well in 6 well plates with a standard mammalian cell culture growth media. Components of media include DME/F12, NaHCO<sub>3</sub>, apotransferrin, and bovine serum albumin. The cells were allowed to stabilize for 24 hours before use in assays.

Temperature: Not given. Humidity: Not given

#### B. STUDY DESIGN:

1. Method:

Following the 24 hour stabilization period described above, cells in growth media were dosed with estradiol, atrazine, simazine dissolved in DMSO as shown in Table 3. Levels of DMSO in growth media did not exceed 0.1 % to prevent toxic effects from DMSO. Cells were exposed to the compounds for 11 days. Growth media and test compounds were changed every other day. Cell viability and cell attachment were monitored with a light microscope; viability and attachment did not change during the study. After 11 days, cell were washed and counted electronically using a Coulter particle counter. Three assays were performed twice per dose group.

TABLE 3. STUDY DESIGN FOR MCF-7 CELL PROLIFERATION.

Dose groups for MCF-7 proliferation assay	
Experiment 1	Experiment 2
Vehicle control (DMSO)	Vehicle control (DMSO)
10 $\mu$ M Atrazine	Positive control (1nM E2)
1.0 $\mu$ M Atrazine	10 $\mu$ M Atrazine + 1nM Estradiol
0.1 $\mu$ M Atrazine	1.0 $\mu$ M Atrazine + 1nM Estradiol
0.01 $\mu$ M Atrazine	0.1 $\mu$ M Atrazine + 1nM Estradiol
10 $\mu$ M Simazine	0.01 $\mu$ M Atrazine + 1nM Estradiol
1.0 $\mu$ M Simazine	10 $\mu$ M Simazine + 1nM Estradiol
0.1 $\mu$ M Simazine	1.0 $\mu$ M Simazine + 1nM Estradiol
0.01 $\mu$ M Simazine	0.1 $\mu$ M Simazine + 1nM Estradiol
	0.01 $\mu$ M Simazine + 1nM Estradiol

2. Dose rationale.

Not given

3. Statistical methods:

Means and standard deviations were calculated by the authors but not given in the study report.

## C. RESULTS

No changes in cell number of MCF-7 cells were seen relative to the negative control with exposure to either atrazine or simazine alone.

The positive control, estradiol, causes a 3 fold increase in number of MCF-7 cells after 11 days exposure. No changes in cell density of MCF-7 cells were seen relative to the positive control with exposure to estradiol plus either atrazine or simazine.

## 2) GEL ELECTROPHORETIC MOBILITY SHIFT ASSAY

### A. MATERIALS

1. Test Material: Atrazine and Simazine

Description: not given

Lot/Batch #: Obtained by author from Chem-Service (West Chester, PA) and from Ciba-Geigy (Greensboro, NC).

Purity: > 97% a.i.

Stability of compounds: Not given

CAS #: 1912-24-9 (Atrazine); 122-34-9 (Simazine)

2. In vitro cell line:

Strain: MCF-7; human breast cancer cell line

Source: American Type Culture Collection

Growth conditions of cell line:

MCF-7 cells were seeded (initial inoculation on a growth plate) at 50,000 cells/well in 6 well plates with a common mammalian cell culture growth media. Components of media include DME/F12, NaHCO<sub>3</sub>, apotransferrin, and bovine serum albumin. The cells were allowed to stabilize for 24 hours before use in assays.

Temperature: Not given. Humidity: Not given

### B. STUDY DESIGN:

1. Method:

Following the 24 hour stabilization period described above, cells in growth media were dosed with estradiol, atrazine, simazine dissolved in DMSO as shown in Table 4. Levels of DMSO in growth media did not exceed 0.1 % to prevent toxic effects from DMSO. MCF-7 were exposed for 3 days to estradiol, atrazine, and simazine as indicated in Table 3. (Three days is the optimal time for progesterone receptor induction.) One hour before harvesting cells, 2 nM of [<sup>3</sup>H]R5020 was added to the growth media. MCF-7 cells were harvested, washed, and pelleted according to a standard procedure. The cells were resuspended in an isotonic buffer and homogenized. The nucleus was separated from the rest of the cell by centrifugation.

PRE-oligonucleotide (DNA containing the progesterone receptor element) was end labeled with  $^{32}$  P-ATP at the 5' end using a commercial T4-polynucleotide kinase end labeling kit. 5 to 10  $\mu$ g of nuclear extract was incubated with 100,000 cpm (counts per minute) of radiolabeled PRE for 15 minutes at 20°C. The reaction mixture plus 2  $\mu$ L loading buffer was loaded onto a 5% nondenaturing polyacrylamide gel and electrophoresed at 110V (time not given). The gels were dried and protein-DNA complexes were visualized using autoradiography and quantified with a Betagen Betascope 603 blot analyzer.

TABLE 4. STUDY DESIGN FOR THE GEL ELECTROPHORETIC MOBILITY SHIFT ASSAY.

Dose groups
Negative control
Positive control (10 nM Estradiol)
1 $\mu$ M Simazine
1 $\mu$ M Atrazine
1 $\mu$ M Simazine + 10 nM Estradiol
1 $\mu$ M Atrazine + 10 nM Estradiol

2. Dose rationale:  
Not given.
3. Statistics :  
Means and standard deviations were calculated by the authors but not given in data.

### C.. RESULTS:

In this assay, the intensity of radioactivity in the band representing the R5020-PR-PRE complex is measured. This band migrates more slowly through the polyacrylamide gel than does the oligonucleotide alone. The intensity of this band represents the amount of radioactive oligonucleotide which binds to the PR-R5020 complex. By definition, the negative control is defined as 100% relative mean intensity. The treatment groups are compared to the negative control group. As shown in Table 5, the positive control (estradiol alone) causes a 3.5 fold increase in band intensity. Levels of simazine and atrazine alone do not change band intensity relative to the negative control. When exposed to estradiol plus atrazine or simazine, the intensity of the band representing bound PR complex are the similar to that of the positive control.

TABLE 5. RESULTS OF GEL SHIFT PROLIFERATION ASSAY. (MEAN AND STANDARD DEVIATION RELATIVE TO NEGATIVE CONTROL.

Dose group	Relative mean intensity (%) of retarded band and the standard deviation
Negative control	100%
Positive control (10 nM Estradiol)	347 ± 53%
1 $\mu$ M Simazine	126 ± 48%
1 $\mu$ M Atrazine	124 ± 71%
1 $\mu$ M Simazine + 10 nM Estradiol	277 ± 47%
1 $\mu$ M Atrazine + 10 nM Estradiol	343 ± 16%

### 3) TRANSFECTION AND LUCIFERASE REPORTER ASSAY USING MCF-7

#### A. MATERIALS

##### 1. Test Material: Atrazine and Simazine

Description: not given

Lot/Batch #: Obtained by author from Chem-Service (West Chester, PA) and from Ciba-Geigy (Greensboro, NC).

Purity: > 97% a.i.

Stability of compounds: Not given

CAS #: 1912-24-9 (Atrazine); 122-34-9 (Simazine)

##### 2. In vitro cell line:

Strain: MCF-7; human breast cancer cell line

Source: American Type Culture Collection

Growth conditions of cell line:

MCF-7 cells were seeded (initial inoculation on a growth plate) at 50,000 cells/well in 6 well plates with a standard mammalian cell culture growth media. Components of media include DME/F12, NaHCO<sub>3</sub>, apotransferrin, and bovine serum albumin. The cells were allowed to stabilize for 24 hours before use in assays. Six hours prior to transfection, MCF-7 cells were plated on 60 mm dishes at 50% confluency in media supplemented with 5% DCC-FBS.

Temperature: Not given. Humidity: Not given

B. STUDY DESIGN1. Method:

MCF-7 cells were transiently transfected according to the procedure of Zacharewski *et al.* (1995) with 5  $\mu\text{g}$  pCH110 (commercially purchased  $\beta$ -galactosidase expression vector), 5  $\mu\text{g}$  17m5-G-Luc (17 base oligonucleotide-regulated luciferase reporter gene), 1  $\mu\text{g}$  Gal4-HEGO (Gal4 is the gene for  $\beta$ -galactosidase; HEGO is the gene for the estrogen receptor) and 4  $\mu\text{g}$  pBS (carrier DNA, commercially purchased).

Transfections were precipitated according to the procedure of Sambrook *et al.* (1989) which entails a calcium phosphate coprecipitation. Twenty-four hours following transfection, plates with cells were washed and media replaced. Transfection efficiency was determined using the standard procedure of Sambrook *et al.* (1989) by cotransfection of a reference plasmid, pCH110.

Transfected cells were exposed to estradiol, atrazine, and simazine for 24 hours. Five  $\mu\text{L}$  of compound dilution (in DMSO) was dissolved in 5 mL of media. Exposure concentrations are given in Table 6. Following exposure, cells were harvested. The luciferase assay was performed according to Brasier *et al.* (1989). Light was measured using a fluorometer.

TABLE 6. STUDY DESIGN OF LUCIFERASE ASSAYS.

Treatment	Concentrations of test chemical (M)
Untreated control	0
17 $\beta$ -Estradiol	$10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}$
Atrazine	$10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}^*$
Simazine	$10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}^*$

\*Concentrations of test chemicals  $>10^{-5}$  resulted in loss of cell attachment to plate.

2. Dose selection: Not given

3. Statistics.

Each chemical exposure was performed in duplicate from two samples for one transfection to yield four values per exposure concentration. Means and standard deviations were calculated by the authors but not given in study report.

**C. RESULTS:**

Atrazine and simazine did not induce luciferase activity above background at concentrations as high as  $10^{-5}$  M. Estradiol results in significant luciferase activity beginning at  $10^{-12}$  M and increased exponentially up to  $10^{-8}$  M where light production plateaued. According to these results, exposure to atrazine and simazine did not result in activity of the estrogen receptor.

In order to test the antiestrogenic activity of atrazine and simazine, co-exposure experiments were performed. The luciferase activity measured following exposure to estradiol plus atrazine or simazine was similar relative to the positive control.

**4) Estrogen receptor-mediated growth in yeast****A. MATERIALS:****1. Test Material: Atrazine and Simazine**

Description: not given

Lot/Batch #: Obtained by author from Chem-Service (West Chester, PA) and from Ciba-Geigy (Greensboro, NC).

Purity: > 97% a.i.

Stability of compounds: Not given

CAS #: 1912-24-9 (Atrazine); 122-34-9 (Simazine)

**2. In vitro cell line:**

Organism: Yeast

Strain: *Saccharomyces cerevisiae* PL3

Source: Dr. P. Chambon; described in Pierrat *et al.* (1992)

Growth conditions of cell line: This cell line does not contain the complete genes for production of the following necessary amino acids: histidine, leucine, and uracil. Yeasts are grown in a minimal media described by Pierrat *et al.* (1992) containing necessary salts and glucose supplemented with 20 mg/L L-histidine, 30 mg/L L-leucine, and 20 mg/L uracil. Cell density of stock not given. Temperature: 30°C.



B. STUDY DESIGN1. Methods:

The PL3 strain of *Saccharomyces cerevisiae* was transformed with the high copy 2 $\mu$  parent vector YEp10 (Parent vector acts a negative control for basal estrogen receptor activity.) or the YEp10-HEGO (HEGO is the gene for the human estrogen receptor). Positive transformants were selected for on 100-mm minimal media plates supplemented with 20 mg/L L-histidine, 30 mg/L L-leucine, and 20 mg/L uracil. Individual colonies were resuspended in water. Ten  $\mu$ L of suspension was spotted on minimal media plates supplemented as shown in Table 7. Plates were placed in an incubator at 30°C. Photos of the plates were taken every 24 hours for five days.

TABLE 7. STUDY DESIGN OF YEAST TRANSFORMATION ASSAYS.

Treatment	Components of selective yeast plates
Untreated control	20 mg/L histidine, 30 mg/L leucine, 20, mg/L uracil
Control for cell line	20 mg/L histidine, 30 mg/L leucine
Estradiol	20 mg/L histidine, 30 mg/L leucine, 1 nM estradiol
Atrazine	20 mg/L histidine, 30 mg/L leucine, 10 $\mu$ M atrazine
Simazine	20 mg/L histidine, 30 mg/L leucine, 10 $\mu$ M simazine

2. Dose selection.

Not given.

3. Statistics

The probability of differences between groups was determined by Duncan's new multiple ranges test by ANOVA.

C. RESULTS:

The yeast growth assay measures activity of the estrogen receptor inserted during the transfection. Theoretically, in this assay, proliferation on the selective plates indicates expression and activity of the estrogen receptor. There was no background proliferation of colonies without the estrogen receptor indicating successful construction of YEp10-HEGO. Controls supplemented with uracil grew from day 2 until day 4 indicating viability of the cell line. Colonies supplemented with estradiol began to divide at day 2 and increased in number until day 4. At day 5 colonies began to die. Colonies supplemented with atrazine and simazine did not proliferate during the experiment.

#### IV. DISCUSSION AND DEFICIENCIES:

##### A. DISCUSSION:

The results from this study indicate that atrazine and simazine do not act as estrogenic compounds by directly interacting with the estrogen receptor. Results of these assays indicate that atrazine and simazine *may* act as antiestrogens but not through direct interaction with the estrogen receptor. This conclusion has been demonstrated by a combination of diverse *in vivo* and *in vitro* estrogenic assays.

Atrazine, simazine, and estradiol were tested both individually and in combination in the assays used here. Experiments using individual chemicals are testing for positive estrogenic effects. Co-exposure experiments are testing for synergism and/or anti-estrogenic activity of atrazine and simazine.

Three *in vivo* assays were performed: uterine weight, progesterone receptor levels, and uterine peroxidase. All three assays demonstrated similar results. Exposure to estradiol resulted in a strong estrogenic response as measured by increased uterine weights, increased levels of the progesterone receptor, and increased activity of uterine peroxidase. Atrazine and simazine alone did not induce these effects. Instead, exposure to atrazine and simazine individually resulted in negative effects indicating antiestrogenic activity. These negative effects are shown in slightly reduced uterine weights, reduced levels of the progesterone receptor, and reduced activity of uterine peroxidase.

In co-exposure *in vivo* experiments, antiestrogenic activity was tested. There was only a slight trend for the levels of progesterone receptor to be reduced. Based on the results of these assays, the antiestrogenic activity of atrazine and simazine at the concentrations tested is weaker than the estrogenic activity of estradiol at the concentration tested. The antiestrogenic response may have been stronger if different concentrations of estradiol or pesticide had been used.


Four *in vitro* assays were performed: cell proliferation of MCF-7 cells, gel electrophoresis mobility shift assay, luciferase activity of transformed MCF-7 cells, and growth of transformed yeast on selective media.

In the cell proliferation assays, estradiol significantly increased the number of cell numbers of MCF-7 at day 11. No changes in cell density of MCF-7 cells were seen relative to the negative control with exposure to either atrazine or simazine alone or relative to the positive control in co-exposure experiments. Therefore, in the cell proliferation assay atrazine and simazine did not exhibit estrogenic or antiestrogenic activity.

As indicated above, levels of the progesterone receptor *in vivo* (as measured by binding to radioactive R5020) increased with exposure to estradiol but decreased with exposure to atrazine and simazine. *In vitro* the level of the progesterone receptor was measured indirectly by the amount of radioactivity in a retarded band of a polyacrylamide gel. As indicated in Table 5, exposure to estradiol significantly increased the amount of radioactivity in this band but atrazine and simazine did not. In co-exposure experiments, the amount of radioactivity was similar to the positive control alone indicating that atrazine and simazine did not exhibit any antiestrogenic activity.

Two transfection experiments were performed where the human estrogen receptor was inserted into the genome of MCF-7 and yeast cells. The MCF-7 cell assay measures luciferase activity (*i.e.*, light production) and is a quantitative assay (*i.e.*, concentration of estrogenic chemical is directly related to light production). The yeast assay measures growth on selective media and is qualitative only. As mentioned previously the yeast assay is performed to verify the results of the MCF-7 transformation experiments. In both transformation assays, exposure to estradiol resulted in strong activity of the estrogen receptor. Atrazine and simazine did not exhibit any estrogenic or antiestrogenic activity in either transformation experiment. These results indicate that atrazine and simazine do not directly interact with the estrogen receptor.

In conclusion, the results of these experiments indicate that *in vivo* atrazine and simazine exhibit some antiestrogenic activity but not estrogenic activity. Based on the *in vitro* results, this antiestrogenic activity is not the result of *direct* interaction with the estrogen receptor.

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## B. DEFICIENCIES:

This is a special study and therefore not does have a specific guideline. Although there are several deficiencies, overall this study was well designed and conducted.

1. As part of the public scientific literature, this study is not under GLP regulations. Based on the descriptions and references of methods given, it seems that the authors have utilized good animal husbandry and laboratory practice.
2. Although not required for journal publication, from a regulatory perspective, it would be valuable to have the following data:
  - Concentration analysis of the dilutions
  - Analysis and mass balance of radioactivity used
  - Specific experimental conditions used in animal care and cell growth chambers
  - Calibration criteria for Coulter counter and flourometer
3. The active ingredient came from two different sources.
4. Dosing and dilution regimen for the two *in vivo* experiments were different between experiments.
5. Because the estrogenic effect of estradiol is so strong relative to the antiestrogenic effect of atrazine or simazine, it would be valuable scientifically to observe the effects of co-exposure using a lower estradiol concentration.
6. Transfection efficiency is not addressed in the study.
7. Raw data or mean data are not reported in the cell proliferation and luciferase assays; data for the is only reported on a graph.

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**Atrazine and Simazine**

**Estrogenicity in vitro and in vivo. Special Study**

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