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Atrazine and Breast Cancer: A Framework Assessment of the Toxicological and Epidemiological Evidence

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The causal relationship between atrazine exposure and the occurrence of breast cancer in women was evaluated using the framework developed by Adami *et al.* (2011) wherein biological plausibility and epidemiological evidence were combined to conclude that a causal relationship between atrazine exposure and breast cancer is "unlikely". Carcinogenicity studies in female Sprague-Dawley (SD) but not Fischer-344 rats indicate that high doses of atrazine caused a decreased latency and an increased incidence of combined adenocarcinoma and fibroadenoma mammary tumors. There were no effects of atrazine on any other tumor type in male or female SD or Fischer-344 rats or in three strains of mice. Seven key events that precede tumor expression in female SD rats were identified. Atrazine induces mammary tumors in aging female SD rats by suppressing the luteinizing hormone surge, thereby supporting a state of persistent estrus and prolonged exposure to endogenous estrogen and prolactin. This endocrine mode of action has low biological plausibility for women because women who undergo reproductive senescence have low rather than elevated levels of estrogen and prolactin. Four alternative modes of action (genotoxicity, estrogenicity, upregulation of aromatase gene expression or delayed mammary gland development) were considered and none could account for the tumor response in SD rats. Epidemiological studies provide no support for a causal relationship between atrazine exposure and breast cancer. This conclusion is consistent with International Agency for Research on Cancer's classification of atrazine as "unclassifiable as to carcinogenicity" and the United States Environmental Protection Agency's classification of atrazine as "not likely to be carcinogenic."

Key Words: **atrazine; mode of action; endocrine; breast cancer; weight-of-the-evidence; framework.**

Toxicology is rapidly being transformed from a descriptive science to one capable of prediction. Central to this progress is the understanding of key genomic, proteomic, biochemical, physiological, and pathological events on the pathway from chemical exposure to the expression of toxicity. The succession of key events following initial exposure, through intermediate states and ultimately to measurable adverse outcomes has been called a "mode of action" (Meek *et al.*, 2003; Sonich-Mullin *et al.*, 2001; USEPA, 2005). When the sequence of events is understood at a fundamental level of chemical-cell molecular interaction, the mode of action becomes a mechanism of toxicity. Mechanisms of toxicity are often postulated but in fact are rarely established. In the expression of toxicity, it is not uncommon for different facets of toxicity to be triggered at different dose levels (Slikker *et al.*, 2004a,b) or through different mechanisms. Furthermore, differences between species with respect to absorption, distribution, metabolism, elimination, and target-specific susceptibility may render the extrapolation from *in vitro* models or even from *in vivo* animal data to humans difficult.

To accommodate the complexity of evaluating a mode of action, systematic approaches have been developed to evaluate the relevance of findings in animal studies to humans (Meek *et al.*, 2003). Three key questions are: (1) Is there sufficient evidence in animal studies to propose a mode of action? (2) Is that mode of action operative in humans? and (3) Is the mode of action relevant to humans after considering differences between species with respect to pharmacokinetic and toxicodynamic factors operative at expected levels of human exposures?

Ultimately, the question of whether humans display toxicity following exposure to the chemical through ingestion, inhalation, or dermal contact can best be ascertained from observational epidemiology. Although there is a long history of interpreting epidemiology studies, only recently have methods

been developed to systematically integrate animal data with data from observational epidemiology (Adami *et al*., 2011; European Center for Ecotoxicology and Toxicology of Chemicals, 2009; Swaen, 2006). This paper presents a case study utilizing the methodology described by Adami *et al*. (2011) wherein toxicological and epidemiological evidence were combined in a systematic framework to conclude whether a causal relationship exists between atrazine exposure and breast cancer in humans. Mode of action research using animal models (Brusick, 1994; Cooper *et al*., 2007; Eldridge and Wetzel, 2008; Stevens *et al*., 1994; Yi, Simpkins, and Breckenridge, in preparation) and epidemiology studies on breast cancer in women were evaluated. Breast cancer was selected for this case study because there were many mode of action research studies and a number of epidemiological studies on breast cancer that have been previously evaluated by regulatory authorities as part of a comprehensive cancer risk assessment (Australian Pesticides and Veterinary Medicines Authority [APVMA], 2004, 2008; Food and Agricultural Organization of the United Nations and the World Health Organization [FAO/WHO], 2009; USEPA, 2003b, 2006). A detailed review of the association between triazine exposure and any cancer has also just recently been published (Sathiakumar *et al*., 2011).

MATERIALS AND METHODS

The five-step method outlined by Adami *et al*. (2011) was followed including (1) collection of all relevant studies, (2) assessment of quality, (3) evaluation of the weight of evidence, (4) assignment of a scalable conclusion, and (5) placement on a causal relationship grid. All relevant toxicological and epidemiological studies were identified and study quality was assessed according to guidelines for evaluating toxicological (USEPA, 1993, 2001, 2003a) and epidemiology studies (London Principles, 1995: von Elm, 2007). Studies that characterize the effect of atrazine on the latency and the incidence of adenocarcinoma and fibroadenoma in the mammary glands of female Sprague-Dawley (SD) rats were summarized (Cooper *et al*., 2007; Eldridge and Wetzel, 2008; Stevens *et al*., 1994, 1999).

Studies relevant to the proposed mode of action underlying the effect of atrazine on mammary tumors were evaluated and a concordance analysis was conducted. The framework analysis also examined and rejected alternative modes of action including hypotheses relating to

- Genotoxicity (Brusick, 1994);
- Direct estrogenicity (Eldridge *et al*., 2008);
- Induction of aromatase expression *in vivo* resulting in excess formation of estrogen (Sanderson *et al*., 2000, 2001; Yi, Simpkins, and Breckenridge, in preparation); and
- Delayed mammary gland development leading to an extended period in which the mammary gland remains undifferentiated and vulnerable to genotoxic or viral carcinogens (Hovey *et al*., 2010; Russo *et al*., 2006).

Once the weight of the evidence assessment was completed, the relevance of the mode of action to humans was considered. This analysis included a consideration of the dose at which humans are likely to be exposed to atrazine. The most probable mode of action underlying the occurrence of mammary tumors in animal models was identified and whether it was plausible that the mode of action underlying the mammary tumor response in rats would be operative in humans.

Epidemiological studies that evaluated the association between human exposure to atrazine and the incidence of breast cancer were identified. The quality of the studies was characterized and whether collectively the results supported an inference of causality. The overall assessment was completed by using a causal inference grid and assigning atrazine to one of the four categories described by Adami *et al*. (2011).

RESULTS AND DISCUSSION

Effects of Atrazine on Mammary Tumors in Female SD Rats

Stevens *et al*. (1999) summarized the results from nine carcinogenicity studies on atrazine in male and/or female SD rats (five studies), female Fischer 344 rats (two studies), and CD-1 mice (two studies). Additional non-guideline studies were conducted in SD and Fischer-344 rats and in two cross-bred mouse strains (C57Bl6 x C3H/Anf or AKR) (Table 1). In female SD rats, atrazine caused a decreased latency and/or increased combined incidence of mammary adenocarcinoma and fibroadenoma tumors (Figs. 1a and 1b) with no effect on the incidence of any other tumor type. There were no effects of atrazine at the maximum tolerated dose on the incidence of mammary tumors, on the incidence of any other tumor type in the female Fischer-344 rats (Fig. 1c) or on tumor incidence in three strains of mice (Table 1). Overall, the weight of the evidence indicates that the effect of atrazine on tumor incidence is specific to mammary tumors in female SD rats.

Key Events Underlying the Occurrence of Mammary Tumors in Female SD Rats

Key events (Fig. 2) leading to a decreased latency and an increased incidence of mammary tumors in atrazine-treated female SD rats have been proposed (Stevens *et al*., 1994) such that:

- Atrazine causes a dose-dependent effect on the synchronized activity of gonadotrophin-releasing hormone (GnRH) neurons during the estrogen-induced luteinizing hormone (LH) surge so that the amplitude of the LH surge is reduced (Foradori *et al*., 2009b).
- The reduction in the LH surge amplitude and a reduced area under the LH curve causes a failure of the maturing ovarian follicles to ovulate their eggs.
- The failure of ovulation results in continuous secretion of estrogen over successive days until the unovulated follicles undergo atresia.
- Repeated failure of ovulation over successive days of the estrous cycle produces a persistent state of estrus resulting in prolonged exposure of the mammary gland and the pituitary to endogenous estrogen. Estrogen is known to be mitogenic and a metabolite of estradiol, 4-hydroxy estradiol, is possibly directly carcinogenic by redox recycling between a semiquinone and quinine form liberating supraoxide radicals (Jefcoate *et al*., 2000).
- Under the influence of estrogen, proliferative changes are observed earlier in the mammary gland and in the

TABLE 1
Effect of Oral Administration of Atrazine on the Incidence and/or Latency of Tumors in Rodents

Study reference	Species	Strain	Sex	Levels tested (mg/kg/day)	Duration (months)	Results
Innes *et al.* (1969) (NTP Study)	Mouse	C57BL/6 X C3H/Anf; C57BL/6 X AKR	M and F	21.5[a]	18	Negative
Stevens *et al.* (1999) (M1)	Mouse	CD-1	M and F	0, 10, 300, and 1000[b]	M 21, F 22	Negative
Stevens *et al.* (1999) (M2)	Mouse	CD-1	M and F	0, 1.6, 47.9, 246.9, and 482.7	21	Negative
Pintér *et al.* (1990)	Rat	Fischer-344	M and F	0, 375, and 750[b]	29	Inconclusive
Thakur *et al.* (1998)	Rat	Fischer-344	F	0, 0.7, 4.7, 13.6, and 27.4	24	Negative
Thakur *et al.* (1998)	Rat	Fischer-344	M and F	0, 0.7, 4.7, 13.6, and 27.4	24	Negative
Stevens *et al.* (1994) (Study 1)	Rat	SD	M and F	0, 0.5, 5.5, and 60	24	Male: negative Female: positive (0.5, 60 mg/kg/day) Increased incidence
Stevens *et al.* (1999) (SDR1)	Rat	SD	M and F	0, 0.5, 3.5, 29.5, and 64.7	24	Male: negative; Female: positive (3.5, 29.5, 64.7 mg/kg/day) (Increased incidence)
Stevens *et al.* (1994) (Study 3)	Rat	SD	F	0, 0.7, 3.5, and 37.6	24	Negative
Stevens *et al.* (1999) (SDR2)	Rat	SD	F	0, 4.3, and 25.6	24	Positive: (early onset) at 25.6 mg/kg/day No effect on incidence
Stevens *et al.* (1999) (SDR3)	Rat	SD	F	0, 4.3, and 25.6	24	Positive: (early onset) at 25.6 mg/kg/day No effect on incidence
Stevens *et al.* (1999) (SDR5)	Rat	SD	F (intact)	0, 1.5, 3.1, 4.2, and 24.4	12, 24	Positive: (early onset) at 24.4 mg/kg/day No effect on incidence
Stevens *et al.* (1999) (SDR4)	Rat	SD	F (ovex)	0, 1.2, 2.5, 3.5, and 20.9	12, 24	Negative
Pettersen and Turnier (1995)[c]	Rat	SD	F	0, 0.8, 1.7, 2.8, 4.1, and 23.9	12	Positive: (early onset) at 23.9 mg/kg/day No effect on incidence

[a]Only female doses given.
[b]Presented in ppm only, as conversion data were unavailable.
[c]Study has not been published.

pituitary. Prolonged stimulation of ductal tissue in the mammary gland by estrogen causes an increased incidence and a decreased latency of adenocarcinomas (Sielken *et al.*, 2005).

- Proliferative changes in the pituitary in response to increased exposure to endogenous estrogens are postulated to result in an earlier development of pituitary tumors resulting in hyperprolactinemia (O'Connor *et al.*, 2000).
- Prolonged exposure of ductal tissue in the mammary gland to endogenous prolactin is postulated to cause an earlier occurrence of mammary fibroadenoma (Eldridge *et al.*, 1999a,b; Eldridge and Wetzel, 2008; Sielken *et al.*, 2005; Stevens *et al.*, 1994).

Experimental Evidence Supporting the Proposed Mode of Action

Figure 3 shows the number of estrous days in female control SD rats (Fig. 3a) and in those exposed to 400 ppm atrazine (Fig. 3b) after 26 weeks of treatment. High-dose atrazine-treated female SD rats displayed an increased proportion of days in constant estrus commencing after three months of treatment (week 13–14) in the 400 ppm dose group (Fig. 3b) compared with untreated controls (Fig. 3a). A dose-dependent relationship existed, with a modest increase in the percent days in estrus noted in the 70 ppm group after nine months of treatment (Fig. 4b); no effect was observed at feeding levels less than or equal to 50 ppm (Fig. 4a). The earliest appearance of disrupted estrous cycles in the 400 ppm treated groups occurred prior to the earliest appearance of the increased incidence of mammary tumors (week 26; Fig. 1b). There was no effect of 400 ppm atrazine on either mammary tumor incidence (Fig. 1c) or on the estrous cycle in female Fischer-344 rats (Fig. 4c).

Studies investigating the effect of atrazine on the estrogen-induced LH surge in female SD and Fischer-344 rats indicate that doses of atrazine that disrupted the estrous cycle and caused a decreased latency and/or increased incidence of mammary

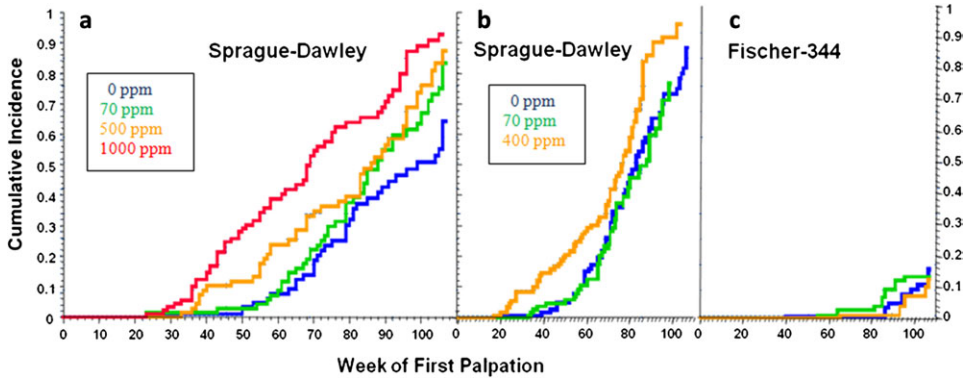

FIG. 1. (a) Atrazine administered at dietary concentrations of 500 or 1000 ppm caused a decreased latency of the combined incidence of adenocarcinoma and fibroadenoma mammary tumors in female SD rats. The 500 ppm had no effect on the terminal incidence of mammary tumors; 70 ppm was the no observed effect level (NOEL) for both latency and incidence (Study SDR1, Stevens *et al.*, 1999). (b) Atrazine administered at dietary concentration of 400 ppm caused a decreased latency of the combined incidence of adenocarinoma and fibroadenoma mammary tumors in female SD. The NOEL was 70 ppm (Studies SDR3; Stevens *et al.*, 1999). (c) Atrazine administered at dietary concentration of 70 or 400 ppm had no effect on the latency or the combined incidence of adenocarinoma and fibroadenoma mammary tumors in female Fischer-344 rats (Study FR4; Stevens *et al.*, 1999).

tumors in female SD rats also suppressed the LH surge (Fig. 5a). In contrast, there was no effect of dietary concentrations of atrazine up to 400 ppm on the estrogen-induced LH surge in female Fischer-344 rats (Fig. 5b). This latter finding is consistent with the absence of an effect of atrazine on the estrous cycle (Fig. 4c) or on the latency or incidence of mammary tumors in female Fischer-344 rats (Fig. 1c).

The weight of evidence indicates that atrazine induces mammary tumors in female SD rats by suppressing the LH surge, thereby supporting a state of persistent estrous and prolonged exposure to endogenous estrogen and prolactin. The results are consistent, within and across studies as discussed above, with respect to dose and duration of exposure needed to produce the neuroendocrine-mediated effects. The results are highly specific to the female SD rat because another strain of rat, the Fischer-344, is insensitive to this mode of action. Neither the LH surge, the estrous cycle, nor mammary tumor incidence are altered in atrazine-treated female Fischer-344 rats compared with untreated controls. The Fischer-344 rat is non-responsive because it maintains a normal estrous cycle through a greater portion of its life span, followed by a decline in ovarian estrogens similar to the human female (Table 2).

Structure-activity assessments indicate that the chlorotriazines share this common mechanism of action. When other functional groups are substituted for the chlorine atom on the triazine nucleus (S-methyl, O-methyl, and hydroxy), there was no effect of treatment on mammary tumor incidence in chronic bioassays in SD rats (Supplementary Figure S1). Terbumeton, which has an O-methyl group substitution, was the only exception (Stevens *et al.*, 1994).

Relevance of the Proposed Mode of Action for Women

Folliculogenesis during the mammalian ovarian cycle is regulated by relatively low levels of circulating LH and follicule-stimulating hormone (FSH) produced by a "basal" mode of gonadotropin secretion, which is controlled by the negative feedback action of ovarian steroids, primarily estradiol. Ovulation, which occurs at the end of the follicular phase, is achieved by a massive discharge of LH that is generated by a "surge" mode of gonadotropin secretion. Both modes of gonadotropin secretion are dependent on hypophysiotropic stimulation from the hypothalamus in the form of GnRH. Basal gonadotropin secretion is dependent on the intermittent release of brief "pulses" of GnRH from the hypothalamus into the hypophysial portal circulation, and this pulsatile secretion occurs approximately every 20–60 min depending on species (Freeman, 2006; Zeleznik and Pohl, 2006). The neural mechanism that generates pulsatile GnRH release is resident in the medial-basal hypothalamus (MBH) (Blake and Sawyer, 1974; Krey *et al.*, 1975) and is termed the GnRH pulse generator (Knobil, 1980). While incompletely understood, emerging models of GnRH pulse generation suggest similarity across species (Lehman *et al.*, 2010; Wakabayashi *et al.*, 2010). Intermittent GnRH stimulation of the gonadotropin-secreting cells of the pituitary, which is

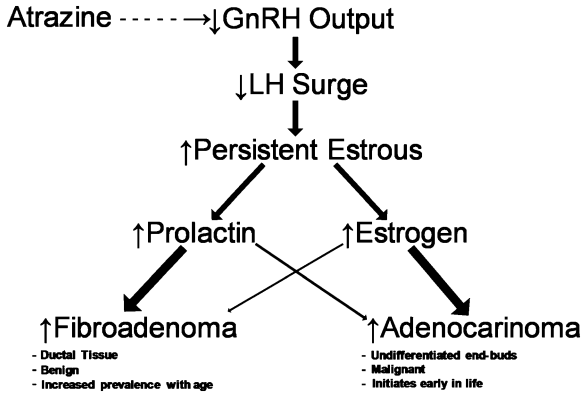

FIG. 2. Key events associated with the earlier appearance and increased incidence of mammary tumors in atrazine-treated female SD rats.

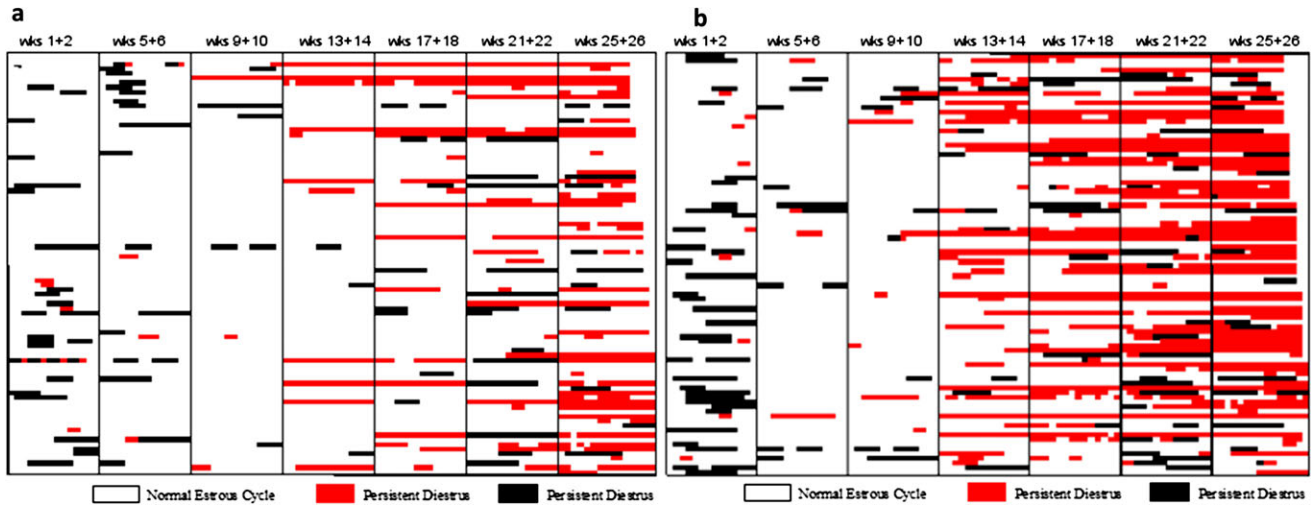

FIG. 3. (a) Characterization of the estrous cycle in individual control female SD rats (Study SDR3 Stevens *et al*., 1999) during treatment weeks 1 and 2, 9 and 10, 13 and 14, 17 and 18, 21 and 22, and weeks 25 and 26. A normal estrous cycle was defined as a cycle lasting four or five days. Persistent diestrus was defined as occurring when there were more than two successive days of diestrus; persistent estrus was defined as occurring when there were two or more successive days when the vaginal smear indicated that the rat was in estrus. Each row represents the result from 1 of 90 rats evaluated in each group. (b) Characterization of the estrous cycle in individual 400 ppm atrazine-treated female SD rats (Study SDR3, Stevens *et al*., 1999).

obligatory for sustained gonadotropin secretion (Belchetz *et al*., 1978), induces a corresponding pulsatile pattern of LH secretion into the systemic circulation. Thus, the pulsatile pattern of LH concentration in peripheral blood serves as a surrogate for the GnRH pulse generator (Plant, 1986). Surge gonadotropin secretion is achieved either by amplification of pituitary gonadotropin responsiveness to GnRH pulses or by activation of a hypothalamic GnRH surge generator that results in a large and relatively sustained discharge of GnRH into the hypophysial portal circulation. The relative importance of these mechanisms for generating gonadotropin surges in mammals is species dependent.

The estrous cycle in rodents is short, and the pre-ovulatory LH surge is brief, governed by the light-dark cycle, with the hypothalamus playing a key role in timing the pre-ovulatory LH surge (Freeman, 2006). Every afternoon during a critical

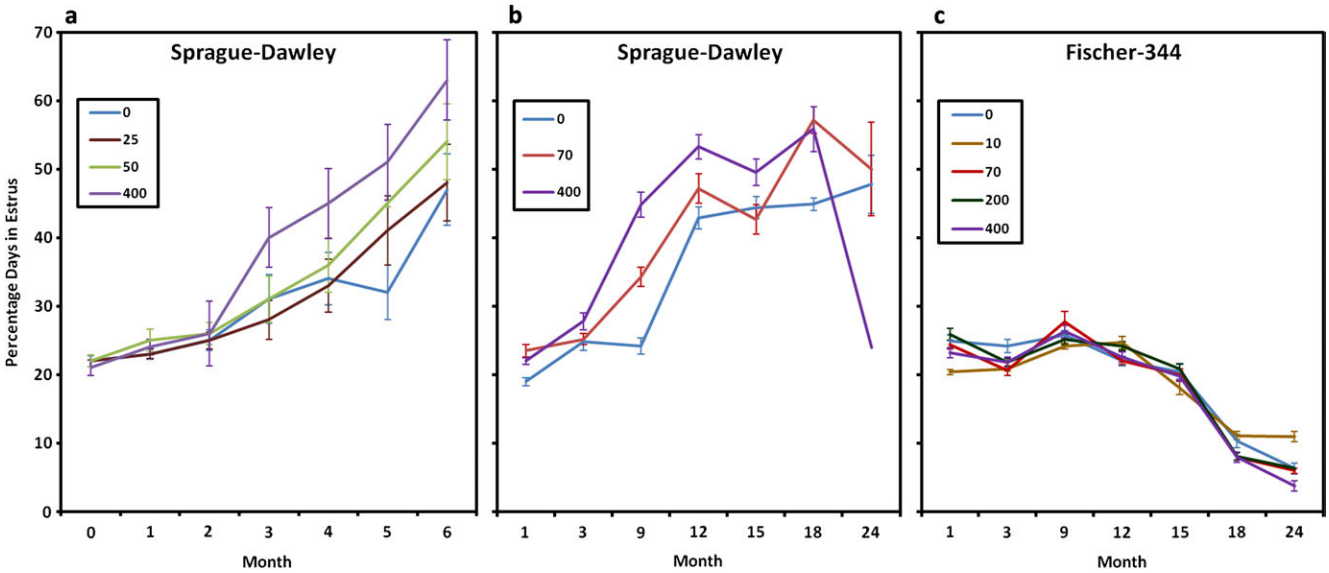

FIG. 4. (a) Dose- and time-dependent effect of 0, 25, 50, or 400 ppm atrazine administered in the diet on the percent days in estrus in female SD rat; 50 ppm was the no observed effect level (Study SDR3, Stevens *et al*., 1999). (b) Comparison of the dose- and the time-dependent effects of atrazine administered at dietary concentrations of 0, 70, or 400 ppm on the percent days in estrus in female SD rats; 70 ppm was the no observed effect level (Study SDR3, Stevens *et al*., 1999). (c) Atrazine administered at dietary concentrations of 70 or 400 ppm had no effect on the percent days in estrus in female Fischer 344 rats (Study FR2, Stevens *et al*., 1999).

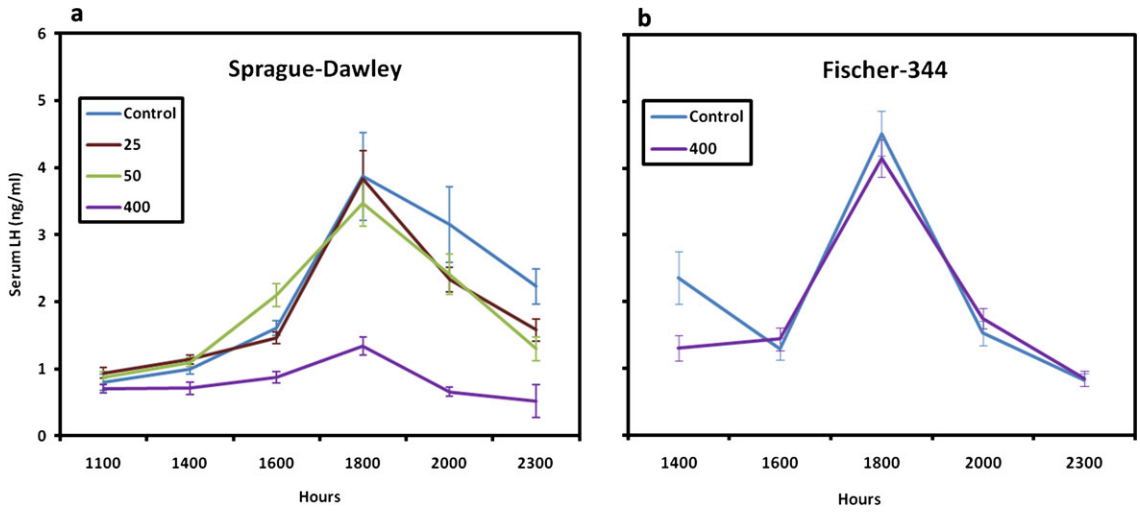

FIG. 5. (a) Atrazine administered for 6 months at dietary concentration of 400 ppm significantly suppressed the estrogen-induced LH surge in female SD rats; 50 ppm was the no observed effect level (Study SDR3, Stevens *et al*., 1999). (b) Atrazine administered for 6 months at a dietary concentration of 400 ppm had no effect on the estrogen-induced LH surge in female Fischer-344 rats (Study FR2, Stevens *et al*., 1999).

period spanning ~2 h, the rodent brain generates a circadian signal, which in combination with the positive feedback action exerted by the elevated levels of circulating estradiol on proestrus, activates the GnRH surge generator that triggers the LH surge. Thus, the role of the rodent brain in controlling the timing of ovulation may be viewed as deterministic. The hypothalamic neurons synthesizing GnRH in the rodent brain are located primarily in the pre-optic area (POA), with few in the more caudal MBH (Silverman *et al*., 1994). Release of GnRH into the portal circulation is dependent on increased activity of norepinephrine neurons located in the brainstem (Sawyer, 1995; Simpkins *et al*., 1979a,b; Herbison, 1997; Wise *et al*., 1997, 1999) and on kisspeptin neurons located in the anteroventral periventricular nucleus of the POA (Oakley *et al*., 2009), the site of positive feedback of ovarian estradiol (Goodman, 1978). As such, inhibition of neural activity early in the afternoon of proestrus by administration of barbiturate or other centrally-acting drugs blocks the pre-ovulatory LH surge in rats (Freeman, 2006, Goodman and Knobil, 1981). Another fundamental characteristic of the GnRH surge mechanism in the rodent is its susceptibility to perinatal programming by testicular androgens such that the hypothalamus of the adult male rat is unable to respond to the positive feedback of estradiol (Feder, 1981).

The human menstrual cycle is long and exhibits a protracted pre-ovulatory LH surge spanning 2–3 days and ends with menses, due to the involution of the corpus luteum and the resulting decline in estrogens and progesterone (Hall, 2009; Zeleznik and Pohl, 2006). In contrast to the rodent, the role of the primate brain, although obligatory in driving the menstrual cycle, is permissive rather than deterministic. In the human female, the preovulatory LH surge appears to occur in the

TABLE 2
Species Differences in Reproductive Senescence (Adapted from Chapin *et al*., 1996)

Parameter	SD rat	Fischer-344 rat	Women
Start of Senescence (% of normal lifespan)	30–40%	60–70%	60–70%
Principal cause of senescence	Hypothalmic failure to stimulate LH/FSH	Hypothalmic failure to control prolactin surges	Depletion of ovarian follicle content
LH surge capability	Lost	Maintained	Maintained
Predominant cycle pattern	Persistent estrus	Pseudopregnancy episodes	Menopause
Estrogen/progesterone ratio	Elevated/prolonged	Reduced	Reduced
Prolactin secretion	Persistently elevated	Episodically elevated	Reduced
Spontaneous mammary tumor incidence (lifetime)	30–40%	2–5%	8–10%
Principal known factors that increase MT Risk	Prolactin, estrogen, chemical mutagens	Prolactin, estrogen, chemical mutagens	Estrogen, nulliparity, family history
Prolactin dependence	High	Medium	None

absence of a GnRH surge (Hall *et al*., 1994; Martin *et al*., 1998; Ottowitz *et al*., 2008) and unfolds in the face of an unchanging frequency of the GnRH pulse generator, as reflected by pulsatile LH release (Adams *et al*., 1994; Martin *et al*., 1998). Thus, it must be concluded that the LH surge is timed and elicited by a positive feedback action of estradiol at the level of the pituitary to dramatically enhance responsiveness to pulsatile GnRH stimulation in women. Indeed, the spontaneous menstrual cycle can be recapitulated in women deficient in GnRH, simply by the exogenous administration of a series of pulses of GnRH (Hall, 2009; Filicori *et al*., 1986; Martin *et al*., 1998; Santoro *et al*., 1986). These findings indicate that the entire pattern of gonadotropin secretion throughout the human menstrual cycle is governed by the negative and positive feedback actions of ovarian estradiol at the level of the pituitary. Similar observations regarding pulsatile GnRH replacement have been made in female rhesus monkey following hypothalamic lesions that abolish endogenous GnRH release (Knobil *et al*., 1980; Zeleznik and Pohl, 2006). In the monkey, however, the preovulatory LH surge is associated with a GnRH surge (Pau *et al*., 1993), which, in contrast to the rat, the GnRH surge is timed solely by the positive feedback action of estradiol acting at the level of the MBH-pituitary unit (Karsch *et al*., 1973). Unlike the rodent, many GnRH neurons in primates are located in the MBH (Silverman *et al*., 1994).

In contrast to rodents (Simpkins *et al*., 1985), inhibition of neural activity by administration of barbiturates or other centrally acting drugs does not affect the estrogen induced pre-ovulatory LH surge in women or the female monkey (Knobil, 1974; Weiss *et al*., 1977). The hypothalamic control of the pre-ovulatory LH surge in primates is emancipated from perinatal programming by testicular androgen secretion, as reflected by the ability of estradiol to elicit LH surges in adult male rhesus monkeys castrated postpubertally, and either bearing a subcutaneous ovarian transplant (Norman and Spies, 1986) or receiving estradiol to mimic a late follicular phase pattern of circulating estradiol (Karsch *et al*., 1973).

Reproductive aging in rodents and women is also different. In female SD rats, reproductive senescence occurs as a result of a breakdown of the brain regulation of the LH surge, while the ovaries remain functional very late into life (Aschheim, 1976; Meites *et al*., 1978). The decline in reproductive function is primarily a result of (1) the inability of brain norepinephrine neurons to transmit the estrogen signal to GnRH neurons (Meites *et al*., 1978; Simpkins *et al*., 1979a,b; Weiss *et al*., 1977), (2) the inability to stimulate a pre-ovulatory LH surge resulting in the maintenance of ovarian follicles, and (3) the persistent secretion of estrogens. Increased secretion of estrogens causes a persistent state of hyperprolactinemia (Sarkar *et al*., 1982). Thus in the SD rat, reproductive senescence is characterized by persistent hyperestrogenemia and hyperprolactinemia with low levels of LH and FSH.

In contrast to female SD rats, which begin to display episodes of persistent estrus as early as six months of age, female Fischer-344 rats maintain normal 4–5 day estrous cycles through 18 months of age (Estes et al., 1982; Estes and Simpkins, 1984; Lu *et al*., 1979, 1980). At two years of age, Fischer-344 rats display normal estrous cycles, interspersed with periods where corpus lutea (CLs) are maintained for extended periods, accompanied by secretion of ovarian progesterone (Estes and Simpkins, 1984; Lu et al., 1980). Thus, in the aging Fischer-344 rat, the hypothalamic-pituitary-gonadal (HPG) axis maintains the ability to mediate an estrogen-induced LH surge although it cannot inhibit episodic prolactin surges after the LH surge has occurred (Estes and Simpkins, 1982). This creates an endocrine state called pseudopregnancy (Beach *et al*., 1975; Estes *et al*., 1982) because prolactin secretion is prolonged and CLs are maintained (Estes *et al*., 1982).

In women, reproductive aging and the occurrence of menopause is due to the exhaustion of ovarian follicles and a diminution of ovarian estrogen (Burger *et al*., 2007; Hale and Burger, 2005; Hale and Burger, 2009; Rance, 2009; Schiff and Wilson, 1978). During the menopause, however, the ability of exogenous estrogens to induce a pre-ovulatory LH surge is maintained, albeit slightly diminished (Santoro, 2005). In the absence of endogenous estrogen secretion, the feedback signal from the ovary governing the cyclic pattern of gonadotropin secretion is lost, and a constant hypergonadotropic state is produced as a result of GnRH pulse generator activity that is robustly maintained in the postmenopausal women. Postmenopausal estrogens and prolactin are very low, but LH and FSH secretions are greatly elevated (Burger *et al*., 2007; Hale and Burger, 2005; Hale and Burger, 2009; Rance, 2009; Schiff and Wilson, 1978). The major differences between aging of the human menstrual cycle and rodent estrous cycles are summarized in Table 2 and discussed specifically for atrazine in Chapin *et al*. (1996).

Possible Alternative Modes of Actions

Genotoxicity. Atrazine is one of the most extensively tested chemicals for genotoxicity. Published studies include *in vitro* and *in vivo* experiments with mammalian cells, microbial systems, invertebrates, fish, and several plant species (Supplementary Tables S1 and S2; Brusick, 1994). Utilizing a weight of the evidence approach developed by the International Commission for Protection against Environmental Mutagens and Carcinogens (Brusick *et al*., 1992; Lohman *et al*., 1992), Brusick (1994) evaluated all published and registrant studies on the genotoxicity of atrazine. The evaluation was later expanded to include all studies published through December 2010. Seventeen of the 23 gene mutation studies following *in vitro* exposure of mammalian cell lines to atrazine were negative (Supplementary Table S1); gene mutation studies on atrazine metabolites (hydroxyatrazine, deethyl atrazine [DEA], deisopropylatrazine [DIA], and diaminochlorotriazine [DACT]) were also negative (Supplementary Table S3). With the exception of studies conducted in

plant-based systems (Supplementary Table S4), the majority of the studies show that atrazine or its metabolites do not directly interact with DNA resulting in mutation. Hence, governmental agencies responsible for reviewing and interpreting toxicology data have concluded that atrazine is not genotoxic (APVMA, 2004, 2008; FAO/WHO, 2009; International Agency for Research on Cancer (IARC), 1999; USEPA, 2003a).

While the results shown in Supplementary Tables S1–S3 are predominately negative, there are a few isolated positive results. Isolated conflicting responses are not uncommon when a chemical is evaluated in a large number of studies. Such isolated occurrences are considered spurious responses due to normal variability or technical inadequacies (Brusick *et al*., 1998). Multiple positive responses tended to occur for those methods that detect chromosomal damage (structural or numerical changes) or DNA strand breakage (Gebel *et al*., 1997; Pino *et al*., 1988; Ribas *et al*., 1995; Singh *et al*., 2008). Unlike tests measuring gene mutation, these types of studies can give positive responses due to cytotoxic or other secondary effects that are not true indicators of a genotoxic mode of action. Atrazine has been reported to cause lipid peroxidation *in vivo* at high doses (Singh *et al*., 2011), and oxidative peroxidation is known to cause cell death and necrosis, which will yield false-positive effects in tests such as alkaline elution techniques and the comet assay (Choucroun *et al*., 2001; Pool-Zobel *et al*., 1999). A study by Singh *et al.* (2008) reported increased micronuclei and comet tail lengths in liver cells obtained from rats exposed to 300 mg/kg of atrazine for 7, 14, and 21 days. This dose level appeared to be high enough to induce oxidative damage in the target tissue, as the reported positive effects were minimized by the co-administration of an antioxidant. Consequently, the increased levels of DNA strand breakage in this and other elution assays may have been secondary to toxicity-induced apoptosis and not from direct effects of atrazine on liver cell DNA.

Some studies report chromosomal changes in atrazine-exposed cells evaluated using flow-cytometry (Biradar and Rayburn, 1995a,b; Rayburn *et al*., 2001; Taets *et al*., 1998). Flow cytometry quantifies changes in whole-cell DNA or individual chromosomes through analysis of coefficients of variation (CVs) in flow histograms. Biradar and Rayburn reported that atrazine caused a statistically significant increase in the CV of DNA content of G1 cells at concentrations as low as 0.005μM. In an assessment of the Biradar and Rayburn flow studies, Kligerman *et al.* (2000a) concluded that flow cytometry and the use of changes in CV is not a reliable method for the determination of clastogenicity. Application of flow analysis to chromosomal CV is generally subjected to computer-generated analysis of the results. Biradar and Rayburn (1995a) were unable to find statistically significant increases in the atrazine results using computer-based analysis and instead applied a unique manual approach to their studies to generate positive effects. When utilizing methods that permit the direct visualization of chromosomal damage, the vast majority of atrazine studies were negative (Adler, 1980; Basler and Rohrborn, 1978; Meisner *et al*., 1992; Kligerman *et al*., 2000a,b; Ribas *et al*., 1998).

Supplementary Table S4 gives the results of genotoxicity studies in plants and fungi. Atrazine is active in most plant-based tests as well as in a number of tests employing fungi. Compared with the data from tests in animals or animal cells, these results indicate that atrazine is handled differently in plant-based systems. Although there may be multiple reasons for the differences, clearly unique attributes of animal metabolism and detoxification processes not present in plant-based systems are likely most relevant. Thus, these studies are of minimal value in evaluating the genotoxicity potential of atrazine.

Except for studies on genotoxicity in plants and fungi, there is no reason to give more weight to *in vivo* studies versus *in vitro* studies because the chloro- and hydroxyl- metabolites of atrazine tested for mutagenic potential in *in vitro* are the predominant metabolites formed *in vivo* (Kim *et al*., 2010; McMullin *et al*., 2003). Oxidative metabolism of atrazine (Supplementary Figure S1) has been well characterized *in vitro* and *in vivo* in rodents (Hanoika *et al*., 1999), nonhuman primates (Hui *et al*., 2011; Maibach *et al*., 2001) and humans (Ademola *et al*., 1993; Buchholtz *et al*., 1999; Joo *et al*., 2010; Maibach *et al*., 2001). Although phase II metabolites such as glutathione conjugates and mercapturates (Supplementary Figure S2) are unique to *in vivo* systems and their formation *in vivo* might be a basis for assigning more weight to *in vivo* studies, these metabolites appear to be toxicologically less active (USEPA, 2002; Yi, Simpkins, and Breckenridge, in preparation) and are rapidly eliminated (Hui *et al*., 2011; Maibach *et al*., 2001). Overall, considering the results from both *in vitro* and *in vivo* models, the weight-of-evidence supports the conclusion that atrazine and its phase I and phase II metabolites are unlikely to be genotoxic.

Estrogen receptor agonist/antagonist. Eldridge *et al.* (2008) recently summarized the results of more than 40 studies that evaluated the potential of atrazine to bind to or interact with estrogen receptors (ERs) in 17 estrogen-dependent systems *in vivo* or in more than a dozen different ER reporter or binding systems *in vitro*. None of the 24 studies that evaluated the *in vivo* expression of estrogen following atrazine exposure showed any estrogen-agonist effects, irrespective of the species or the strain of rat evaluated (Supplementary Table S5). In 10 of 15 studies, atrazine inhibited the action of estrogen *in vivo*, but only when the administered dose of atrazine was at least 100,000 times greater than the dose of estradiol. Four other studies showed no effect of atrazine at high concentrations and one study was inconclusive. None of the 18 studies reported any stimulatory effect of atrazine on estrogenic expression systems *in vitro*; weak inhibition of estrogen was reported in 3 of 10 studies (Supplementary Table S6). Weak competition between atrazine and estrogen for ERα

or ERβ was noted in 10 of 17 studies that used donor tissues from a variety of species including two strains of rats (Supplementary Table S7). In some studies, weak competition was seen only when atrazine was pre-incubated with the receptor before estradiol was added to the media.

Overall, the weight of evidence indicates that atrazine does not act as an estrogen agonist. Atrazine may act as a weak ER antagonist if present in tissues at concentrations that are more than 100,000-fold greater than the concentration of estradiol. This is unlikely to occur under conditions of human exposure. For example, a 60 kg person consuming 2 l of water containing atrazine at the Maximum Contaminant Level (MCL) of 3 ppb would receive a dose of 0.1 μg atrazine/kg. Assuming the human plasma concentration scaled 1:1 to the plasma concentration determined in the rat (See Supplementary Figs. S4 and S5), then the expected steady state plasma concentration of total triazine in humans exposed at the MCL would be 0.1 ppb (4.8×10^{-10}M). Total chlorotriazine concentration in plasma is expected to be approximately fivefold lower in humans because the concentration of total chlorotriazine eliminated in urine of men comprised only 14.4% of the administered dose (Maibach *et al*., 2001). Tissue concentration of atrazine is likely to be at parity with plasma at steady state because plasma:tissue partition coefficients are near 1.0 (P. S. Coder, unpublished data). The K_d for estradiol binding to the estrogen receptor is 7.7×10^{-10}M (Laws *et al*., 2006; Rider *et al*., 2009). Thus, under conditions of human exposure, the concentration of atrazine equivalents in tissue is approximately the same as the K_d for estradiol. Therefore, it is unlikely that atrazine will antagonize the binding of estradiol to its receptor *in vivo*. Even if atrazine was a weak estrogen receptor antagonist *in vivo*, the consequence would likely be a diminution of estrogenic activity rather than an increase called for by the results of the carcinogenicity studies on atrazine.

Induction of messenger RNA for aromatase and/or aromatase enzyme activity. *In vitro* studies (Sanderson *et al*., 2000, 2001) indicate that high concentrations of atrazine in 0.1% dimethyl sulfoxide increases aromatase activity and gene expression in the H295R adrenocorticocarcinoma cell line and in JEG-3 placental choriocarcinoma cells but not in the MCF-7 breast cancer cells or the R2C rat Leydig cell cancer cells (Heneweer *et al*., 2004; Supplementary Table S8). Carp hepatocytes treated with atrazine *in vitro* did not show altered vitellogenin synthesis (Sanderson *et al*., 2001). In the majority of these studies, statistically significant induction of aromatase activity or gene expression was observed only at the concentration near the solubility limit of atrazine (30μM; 6.5 ppm) although effects on aromatase have been reported in some studies at 10μM but not at 1μM of atrazine (Yi, Kim, Breckenridge, and Simpkins, in preparation). Ohno *et al*. (2004) reported that aromatase activity was unaffected by atrazine in KGN ovarian granulosa carcinoma cells and Keller and McClellan-Green (2004) found no effect of atrazine in an immortalized sea turtle cell line. The results from primary ovarian granulosa and endometrial stromal cells are mixed. Tinfo *et al*. (2011) reported a 2- to 2.5-fold increase in aromatase activity with atrazine but no change in aromatase message or protein in rat granulosa cells. Holloway *et al*. (2008) did not see a dose-response in human granulosa cells exposed *ex vivo* to atrazine and found no effect of atrazine on human endometrial stromal cells (Supplementary Table S8). Crain *et al*. (1997) and Spiteri *et al*. (1999) reported increased aromatase activity in alligator eggs painted with 14 ppm atrazine dissolved in ethanol. Even under these extreme conditions, not a single male was converted to a female. In contrast, when the male eggs were treated with 14 ppb of estradiol, 100% of the males were feminized. This indicates that the reported changes in aromatase in alligator eggs were of no functional consequence. A study in chicken eggs failed to show any effect on atrazine on aromatase activity (Matsushita *et al*., 2006).

The effect of other chlorotriazines such as simazine, as well as primary (Supplementary Figure S1) and secondary metabolites of atrazine (Supplementary Figure S2) on aromatase activity has been investigated (Supplementary Table S8). Exposure to DEA and DIA resulted in weaker responses than did exposure to atrazine, whereas DACT, the major animal chlorometabolite of atrazine, had no effect (Sanderson *et al*., 2000; Tinfo *et al*., 2011; Yi, Kim, Breckenridge, and Simpkins, in preparation). The glutathione and mercapturate conjugates of atrazine, which are secondary metabolites of atrazine, as well as hydroxyatrazine (HA) and ammeline, which are major plant metabolites, had no effect on aromatase messenger RNA (mRNA) in H295R or JEG3 cells (Yi, Kim, Breckenridge, and Simpkins, in preparation).

The weight of the evidence indicates that high micromolar concentrations of atrazine and to a lesser extent, its mono-dealkylated metabolites at concentrations above the solubility limit in aqueous media, cause an approximately twofold increase in expression or activity of aromatase in sensitive cell lines capable of steroidogenesis. Neither the major atrazine chlorometabolite, DACT, nor the glutathione or mecapturates of atrazine had any effect; HA or ammeline, the predominant plant metabolites of atrazine also were negative (Yi, Kim, Breckenridge, and Simpkins, in preparation). Because atrazine and the mono-dealkylated metabolites of atrazine are rapidly metabolized after ingestion, these chemicals are not expected to persist long enough to have any effect on aromatase activity *in vivo* (APVMA, 2010; FAO/WHO, 2009; Yi, Simpkins, and Breckenridge, in preparation). As discussed in the previous section, tissues concentration of atrazine equivalents will not be greater than 10^{-10}M for humans exposed to atrazine at the MCL. In the rat, a repeated oral dose of 0.25 mg/kg for 5 days would be needed to produce a tissue concentration of 1μM atrazine (Supplementary Fig. S5), a concentration which clearly had no effect on aromatase activity or expression when evaluated in any *in vitro* system. However, atrazine is rapidly metabolized to less active metabolites, most notably DACT and the concentration of atrazine in plasma is short lived (*t*max

= 30 min; half-life of clearance from plasma is 30–45 min; Kim *et al*., 2010). In contrast, in the *in vitro* aromatase models, atrazine had to be present in media for at least 2 h at a concentration above 1μM to significantly affect aromatase mRNA expression (Yi, Kim, Breckenridge, and Simpkins, in preparation).

Studies on intact animals (Supplementary Table S9) indicate that neither the induction of mRNA aromatase gene expression nor the increased aromatase activity is observed in atrazine-treated animals. Hayes *et al*. (2002) argued that changes in circulating or whole body testosterone reflect an alteration in aromatase activity in atrazine-exposed *Xenopus laevis*. However, this cannot be taken as evidence that atrazine is altering aromatase expression because plasma hormone levels change dynamically from moment to moment and are subject to a host of variables (age, sex, season, time of day, and stress level) that are uncontrolled in environmental studies (Kang *et al*., 1995). Other studies have failed to observe a relationship between triazine exposure and testosterone levels in wild-caught *Xenopus laevis* (Hecker *et al*., 2004). Furthermore, the reported "feminization" effect of atrazine on amphibian gonadal development (Hayes *et al*., 2002), which has been attributed to an effect on aromatase, has not been replicated by other laboratories (Kloas *et al*., 2009a). Lastly, when changes in aromatase mRNA were evaluated in amphibians, no effects of atrazine exposure were found (Hecker *et al*., 2004, 2005a,b; Kloas *et al*., 2009b).

High doses of atrazine (50 or 200 mg/kg/day) administered daily to 60-day-old male Wistar rats for 1, 2, 3, 4, or 21 days resulted in slight and highly variable increases in serum androstenedione, testosterone, estradiol, estrone, corticosterone, and progesterone, as quantified by radio-immuno-assay (Modic *et al*., 2004). Although the acute effect of high doses of atrazine on corticosterone and progesterone have been confirmed in subsequent studies (Fraites *et al*., 2009; Laws *et al*., 2009), the effect on androstenedione testosterone, estradiol, and estrone has not been confirmed by liquid chromatography with tandem-mass spectrometry (LC-MS-MS) using internal ^{13}C-stable label for identification and unlabeled standards for quantification (Handa, 2010).

Overall, the weight of the evidence indicates that high micromolar concentrations of atrazine and its mono-dealkylated metabolites, but not DACT, caused an approximate twofold increase in aromatase mRNA and activity in immortalized cell cultures *in vitro*. The absence of effects *in vivo* is likely attributed to the rapid metabolism to inactive metabolites (Kim *et al*., 2010). Hence, the *in vitro* mode of action is not likely relevant to intact mammals.

Delayed development of the mammary gland. It has been proposed that a delay in the development and differentiation of mammary gland increases susceptibility to physical, chemical, or viral agents that initiate cancer (Russo *et al*., 1992; Trichopoulos *et al*., 2008), whereas factors that delay the onset of menarche are protective (Russo *et al*., 2001, 2006). *In utero* exposure of female Long-Evans rats to 100 mg/kg/day atrazine (Rayner *et al*., 2005) or a mixture of atrazine and its metabolites (Enoch *et al*., 2007) has been reported to delay postnatal mammary gland development using, for the most part, subjective rating scales. When an unbiased, blinded quantitative assessment of mammary glands (ductal length, ductal area, epithelial area, epithelial density, and cell proliferation) was used, there was no evidence of a delay in mammary gland development at doses of atrazine up to 100 mg/kg/day (Hovey *et al*., 2010). Hovey and colleagues controlled for the effect of treatment-related reductions in body weight gain in the pregnant dams on subsequent pup development, as well as for atrazine-induced delay in sexual maturation, which has been reported in atrazine-treated females (Ashby *et al*., 2002; Laws *et al*., 2000). Delay in sexual maturation is known to affect mammary gland development because growth during puberty is allometric and sensitive to hormonal changes during the estrous cycle (Hovey *et al*., 2002). The results from a second laboratory confirmed the absence of an atrazine effect on postnatal mammary gland development at doses as high as 100 mg/kg/day (Davis *et al*., 2011). In addition, female SD rats exposed to atrazine *in utero* and throughout life did not have an increased incidence of mammary tumors compared with controls, suggesting that *in utero* exposure to atrazine did not contribute to an increased risk of developing mammary tumors later in life (Stevens *et al*., 1999). However, this study was underpowered ($N = 30$ per group); thus small effects may not have been detectable.

Overall, there is no compelling evidence to indicate that *in utero* exposure to atrazine increases the incidence of mammary tumors in rodents. There is a clear trend among females in developed countries to experience an earlier onset of puberty, leading to an earlier rather than a delayed development of breasts in young girls. The prolongation of exposure to endogenous estrogen increases breast cancer risk later in life (Russo *et al*., 2006; Trichopoulos *et al*., 2008).

Conclusions of Animal Mode of Action Research

The hypothesis that decreased latency and increased incidence of mammary tumors in female SD rats is mediated through the key events shown in Figure 2, is strongly supported by the data. This conclusion is consistent with a detailed analysis conducted by FAO/WHO (2009; Appendix 1, pp 118–124).

The mechanism of action underlying the effect of atrazine on the HPG axis is unknown because the molecular target(s) have yet to be identified. Some hypotheses have been ruled out but none has been established (Cooper *et al*., 2007). Recent work has allowed for a comparison of the effect of atrazine on the HPG and HPA axes in female Wistar, Long-Evans, and SD rats. Although the Wistar and inbred strains of Long-Evans rats have not been evaluated in traditional carcinogenicity studies, both SD and Long-Evans rats were derived from the original

Wistar albino rat (Lindsey, 1979) and both Wistar and Long-Evans rats respond to high doses of atrazine with reduced LH surges (Cooper *et al*., 2000; Foradori *et al*., 2009a,b). New mechanistic studies suggest that the effect of atrazine on the LH surge may be secondary to an activation of corticosterone release in the rat (Fraites *et al*., 2009; Laws *et al*., 2009). However, the effect of atrazine on the estrogen plus progesterone induced LH surge in female Wistar rats was unaffected by adrenalectomy, whereas the suppressive effect of atrazine on pulsatile LH release was abolished (Foradori *et al*., 2011). This suggests that the effect of atrazine on the final common hypothalamic pathway (i.e., suppression of pulsatile versus surge release of GnRH/LH) may be mediated via distinct mechanisms in rodents. This dichotomy in the response to atrazine is intriguing because the control mechanism of pulsatile GnRH release in nonhuman primate and women is similar to the rodent control mechanisms. In contrast, major differences exist in the regulation of the LH surge in rodents compared with nonhuman primates and women. In the human female, the preovulatory LH surge appears to occur in the absence of a GnRH surge (Hall *et al*., 1994; Martin *et al*., 1998; Ottowitz *et al*., 2008).

The proposed mode of action underlying the decreased latency and increased incidence of mammary tumor response in the female SD rat is unlikely to be relevant to women due to the differences in reproductive aging of rodents and women. Although it is plausible that high doses of atrazine could suppress the LH surge in women by interfering with GnRH pulse generation, the consequence of that suppression would not result in an increased incidence of breast cancer in women. Therefore, atrazine was placed in the lower half of the biological plausibility scale described by Adami *et al.* (2011). There is little uncertainty in this conclusion because of the quality and the breadth of the studies that underlie the assessment. This conclusion is consistent with independent reviews by regulatory authorities around the world (AVMPA, 2004, 2008; FAO/WHO, 2009; IARC, 1999; USEPA, 2003b, 2006).

The evidence supporting alternative modes of action evaluated in this paper (genotoxicity, estrogenicity, aromatase, and delayed mammary gland development) is not compelling and do not indicate that these alternate modes of action play a role in the mammary tumor response observed in atrazine-treated female SD rats. These alternate modes of action are also unlikely to contribute to the occurrence of breast cancer in women, especially at doses to which women could conceivably be exposed.

Atrazine exposure and breast cancer: epidemiological evidence. The potential association between atrazine exposure and the risk of breast cancer has been evaluated in epidemiological studies of incidence or mortality among women involved in triazine manufacture (MacLennan *et al*., 2002, 2003); women who lived on farms (Engel *et al*., 2005) or in proximity to farms and/or were associated with agricultural work (Reynolds *et al*., 2004); and women potentially exposed to atrazine via drinking water (McElroy *et al*., 2007). Ecological studies have also been conducted in which breast cancer incidence was examined across geographic areas defined by triazine exposure (Hopenhayn-Rich *et al*., 2002; Kettles *et al*., 1997; Mills and Yang, 2006; Muir *et al*., 2004; Reynolds *et al*., 2005).

Cohort studies on women involved with triazine manufacture. Breast cancer incidence was examined in women employed in the manufacture and formulation of triazines (primarily atrazine). Among 184 women employed in a Louisiana plant who were followed from 1985 to 1997, one was diagnosed with breast cancer (expected = 1.5) (MacLennan *et al*., 2002). In a parallel study of mortality at this same plant, there was one death from breast cancer during 1970–97 among 211 female employees (expected = 0.6) (MacLennan *et al*., 2003).

The quality of these studies was classified as acceptable. One strength was that exposure to atrazine at this production facility could be quantified using job histories in a fashion similar to that employed by Hessel *et al.* (2004) in a study of prostate cancer at this facility. The major weakness was that there were only a few breast cancer cases and thus the study did not have adequate statistical power.

Cohort studies on women with potential exposure to atrazine or triazines on farms. Spouses of professional pesticide applicators, recruited to the Agricultural Health Study in Iowa and North Carolina from 1993 through 1997, provided information about their own herbicide exposures and potential risk factors for breast cancer (Engel *et al*., 2005). Of the 309 women who went on to be diagnosed with breast cancer through the year 2000, only 11 (3.6%) had directly used atrazine; this was similar to the percentage for controls diagnosed with breast cancer (4.4%, relative risk [RR] = 0.7, 95% confidence interval [CI] 0.4–1.2). Among women who did not apply any pesticide, the percentage of their husbands who had done so was similar for cases and controls (74.8 vs. 73.8%, respectively, RR 1.1, 95% CI 0.7–1.6). Similar results were obtained when the assessment was based on 13 women with breast cancer who reported using triazines (Supplementary Table S10).

The study was classified as acceptable. The strength of the study was its prospective design with exposure assessment prior to disease diagnosis. The weakness was that there were too few cases through the end of 2000 to provide adequate statistical power.

The California Teachers study evaluated residential proximity to areas of simazine application and incidence of breast cancer in these regions (Reynolds *et al*., 2004). In this study, 1552 participants were diagnosed with breast cancer during 1996 through 1999. Using data obtained in the California

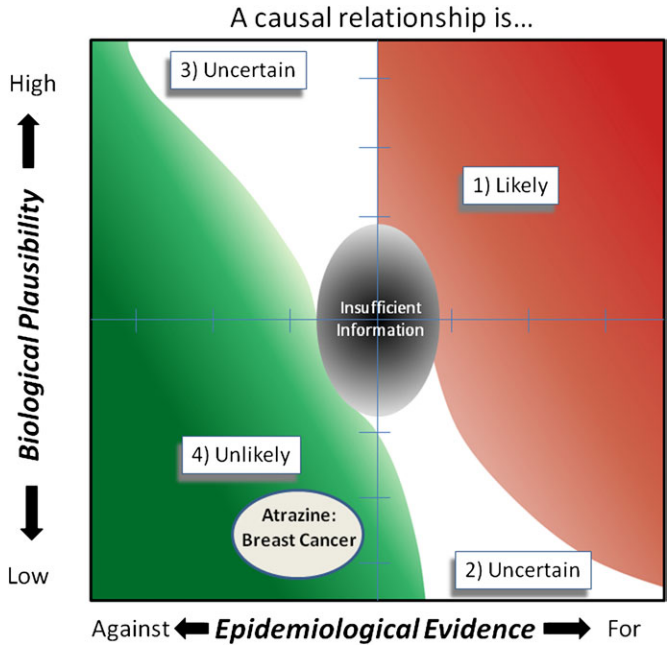

FIG. 6. Summary of the weight of evidence of the mode of action (biological plausibility) and the epidemiological evidence for a causal relationship between atrazine exposure and the occurrence of breast cancer in women.

TABLE 3
Framework Assessment of the Animal Mode of Action, the Relevance of the MOA to Breast Cancer in Women and the Epidemiology Evidence

EPA human cancer classification	Carcinogenic	Likely	Suggestive	Not likely
Human evidence				
Causation established				No
Association established				No
Animal evidence				
Multi versus single (sex, species, strain, and site)			Yes	
Mode of action				
Key events proposed				Yes
Concordance of dose response relationships				Yes
Temporal association of key events				Yes
Strength, consistency, and specificity of association				Yes
Biological plausibility and coherence				Yes
Alternative MOAs evaluated				Yes
Human relevance				
MOA operative at any dose				No
MOA operative at plausible human dose				No
Framework: unlikely (category 4)				

Pesticide Use Reporting System for 1993 through 1995, the incidence of breast cancer was examined in relation to the amount of various pesticides and herbicides applied in the area within one-half mile of the residence of each participant. Breast cancer incidence did not vary appreciably across levels of simazine application compared with women living in an area in which less than one pound of simazine per square mile had been applied; the relative incidence was 0.9, 0.9, and 1.1 for women living in areas of simazine application of 1–13 pounds per square mile, 14–40 pounds per square mile and 41 or more pounds per square mile, respectively. A subset analysis of women who lived within 0.5 mile radius of the section of land sprayed or who lived within the section of land sprayed, yielded hazard ratios of 1.17 (CI = 0.82–1.67) and 1.44 (CI = 1.01–2.05), respectively, based upon an unspecified numbers of cases.

This study was categorized as acceptable. Strengths of the study included a large number of breast cancer cases and the control for suspected risk factors for breast cancer. However, the relationship between residential proximity to application of simazine and actual exposure was not quantified. Furthermore, confounding for co-exposure to other pesticides, which were used in proximity to the residence of women with breast cancer, was not controlled.

Case-control study on women with potential exposure to atrazine via groundwater. This study evaluated the amount of atrazine in the groundwater near residences of women with and without breast cancer (McElroy *et al*., 2007). Female residents of rural Wisconsin (ages 20 through 79 years) who had been diagnosed with breast cancer during 1987 through 2000 were identified (n = 3275), as were 3669 demographically-similar control women. All women were interviewed with respect to exposure and characteristics relevant to the risk of breast cancer. Using data from surveys of atrazine levels in well water in 1994, 1996, and 2001, the investigators assigned an atrazine level to each woman's own well water, based on the proximity of the well to those wells for which atrazine concentration had been determined. The distribution of estimated levels of atrazine in well water was nearly identical between the wells of cases and those of the controls. For women whose water was estimated to contain less than 0.15 ppb of atrazine, the relative risk of breast cancer in those whose water had 1–3 ppb atrazine was 1.1 (95% CI 0.9–1.4), adjusted for other breast cancer risk factors. Only nine women (five cases and four controls) had wells with an atrazine concentration estimated at greater than 3 ppb, too small a number to draw any conclusion.

Study quality was classified as acceptable. Strengths included the fact that 83 percent (11,001) of eligible case women and 83.1% (11,494) of eligible age-matched control

women participated in the study. A non-biased approach was used to assign residence and well water concentration of atrazine or atrazine plus its chlorometabolites, but there was no control for duration of time in residence. The authors controlled for confounding by potential breast cancer risk factors (Supplementary Table S11). Weaknesses in the study included the fact that atrazine exposure was not assessed during a period of time that was etiologically relevant to development of breast cancer. In general, atrazine concentrations in groundwater were low (0.15–3 ppb) and varied over too narrow a concentration range to plausibly permit the documentation of a dose-response.

Ecological studies on breast cancer and triazines use in geographical regions. Because ecological studies are generally hypothesis generating rather than hypothesis testing, the quality of individual studies in this category are not discussed except for the study of Muir *et al.* (2004), which appeared to have major limitations.

Reynolds *et al.* (2005) characterized residences in each square mile of the state of California in terms of estimated pesticide application during 1990 through 1997. The incidence of breast cancer during 1988 through 1997 bore no relation to the quantity of simazine application: rates were identical among women living in areas in the upper-fourth of the distribution of simazine application and those living in areas where virtually no simazine had been applied (RR 1.0, 95% CI 0.95–1.07).

A county-level study in California among Latin women during 1988 through 2000 similarly found no association between the incidence of breast cancer and the quantity of application of either simazine or atrazine (Mills and Yang, 2006). After adjusting for age, socioeconomic status, and fertility rates, the RRs for low, mid, and high "exposed" groups ranged from 0.83 to 0.87, 0.94 to 0.91, and 0.86 to 0.87, respectively, for assessment periods from 1988 to 1993 and from 1994 to 1999.

Two studies in Kentucky correlated breast cancer incidence with county-level exposure to triazines. The latter was estimated by jointly considering triazine levels in water samples, the amount of corn crops grown and county-specific pesticide use data. The first of these studies (Kettles *et al.*, 1997) observed that in 1991 through 1992, the adjusted incidence of breast cancer in the 24 counties with the greatest estimated triazine exposure was nine percent higher than in the 54 counties with the lowest exposure. In 1993–1994, the corresponding figure was a 22% increase. In contrast, the second study (Hopenhayn-Rich *et al.*, 2002) observed a 2% lower breast cancer incidence during 1993 through 1997 in counties with the highest, compared with the lowest, estimated atrazine exposures.

One additional ecologic study of breast cancer and atrazine (and cyanazine), in groupings of electoral wards in two districts of England (Muir *et al.*, 2004), was not considered because the incidence rates varied across geographic groupings to such an implausibly large degree, more than 24-fold, that chance must have been exerting a strong influence. This variability was probably due to the evaluation of small geographic areas and the narrow window of time (1989 through 1991).

In summary, the association between exposure to atrazine or triazines exposure and breast cancer was largely null. These results are consistent with conclusions reached by various regulatory agencies that atrazine or the triazines are "unclassifiable as to carcinogenicity" (IARC, 1999) or that atrazine is not likely to be carcinogenic (USEPA, 2003b, 2006) with regard to any cancer.

CONCLUSIONS

Using the framework outlined by Adami *et al.* (2011), atrazine is placed within the lower left hand quadrant of the framework categorical diagram (Fig. 6), which classifies atrazine in Category 4 unlikely. This conclusion is based upon (1) epidemiological evidence suggesting that exposure to atrazine is not associated with the occurrence of breast cancer in women and (2) the lack of a plausible mode of action for the occurrence of atrazine-related breast cancer in women.

This classification took into account a substantial body of literature on the mode of action plus the absence of positive epidemiological evidence, recognizing that the majority of the relevant studies were underpowered and might not detect small changes in breast cancer incidence. This conclusion is consistent with previous schemes for the classification of the carcinogenic potential of atrazine in humans (Table 3). These assessments collectively have taken into account the animal and all the human epidemiology evidence (IARC, 1999; USEPA, 2003b, 2006). However, the integrated approach developed by Adami *et al.* (2011) and used here provides explicit guidance on how to weigh study quality and how to integrate toxicological and epidemiology evidence. This approach has the additional advantage of qualitatively characterizing the uncertainty associated with any inference of causality and identifying whether additional mechanistic studies or epidemiological research would be more effective in reducing uncertainty.

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