

Effects of Chloro-*s*-Triazine Herbicides and Metabolites on Aromatase Activity in Various Human Cell Lines and on Vitellogenin Production in Male Carp Hepatocytes

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We investigated a potential mechanism for the estrogenic properties of three chloro-*s*-triazine herbicides and six metabolites *in vitro* in several cell systems. We determined effects on human aromatase (CYP19), the enzyme that converts androgens to estrogens, in H295R (adrenocortical carcinoma), JEG-3 (placental choriocarcinoma), and MCF-7 (breast cancer) cells; we determined effects on estrogen receptor-mediated induction of vitellogenin in primary hepatocyte cultures of adult male carp (*Cyprinus carpio*). In addition to atrazine, simazine, and propazine, two metabolites—atrazine-desethyl and atrazine-desisopropyl—induced aromatase activity in H295R cells concentration-dependently (0.3–30 μ M) and with potencies similar to those of the parent triazines. After a 24-hr exposure to 30 μ M of the triazines, an apparent maximum induction of about 2- to 2.5-fold was achieved. The induction responses were confirmed by similar increases in CYP19 mRNA levels, determined by reverse-transcriptase polymerase chain reaction. In JEG-3 cells, where basal aromatase expression is about 15-fold greater than in H295R cells, the induction responses were similar but less pronounced; aromatase expression in MCF-7 cells was neither detectable nor inducible under our culture conditions. The fully dealkylated metabolite atrazine-desethyl-desisopropyl and the three hydroxylated metabolites (2-OH-atrazine-desethyl, -desisopropyl, and -desethyl-desisopropyl) did not induce aromatase activity. None of the triazine herbicides nor their metabolites induced vitellogenin production in male carp hepatocytes; nor did they antagonize the induction of vitellogenin by 100 nM (EC₅₀) 17 β -estradiol. These findings together with other reports indicate that the estrogenic effects associated with the triazine herbicides *in vivo* are not estrogen receptor-mediated, but may be explained partly by their ability to induce aromatase *in vitro*. **Key words:** antiestrogenic, aromatase, atrazine, carp, chloro-*s*-triazines, CYP19, estrogenic, H295R, hepatocytes, herbicides, JEG-3, MCF-7, vitellogenin. *Environ Health Perspect* 109:1027–1031 (2001). [Online 26 September 2001] <http://ehpnet1.niehs.nih.gov/docs/2001/109p1027-1031sanderson/abstract.html>

The 2-chloro-*s*-triazine family of herbicides, widely used to control broad-leaved and grassy weeds, includes the chemicals atrazine, simazine, and propazine. Triazine herbicides have been used increasingly since the 1960s, particularly on maize crops, in North America and Europe. The estimated use of atrazine alone in the United States was almost 35,000 tons in 1993 (1). As a result it is found in relatively high concentrations in surface waters in certain parts of the North American continent (2). Triazine herbicides are relatively persistent to abiotic and biotic breakdown (2,3) producing detectable levels in drinking water, foods, and fish (2).

Epidemiologic studies have associated long-term exposures to triazine herbicides with increased risk of ovarian cancer in female farm workers in Italy (4) and of breast cancer in the general population of Kentucky in the United States (5). In experiments with female F344 rats, atrazine induced tumors of the mammary gland and reproductive organs (6). In female Sprague-Dawley rats, atrazine caused lengthening of estrous cycle and a dose-dependent increase in plasma levels of 17 β -estradiol (7).

Atrazine also caused an earlier onset of the incidence of mammary and pituitary tumors in this rat strain (7), a response typical of exposure to exogenously administered estrogens (8,9). Recently, atrazine exposure during lactation has been shown to suppress suckling-induced prolactin release in female Wistar rats (10). Further, the lactationally exposed male offspring of the atrazine-exposed dams had an increased incidence of prostatitis (10), an effect also induced by exposure to exogenous 17 β -estradiol (11). A subsequent study in Long-Evans and Sprague-Dawley rats has attributed the effects of atrazine on serum prolactin levels to alterations in the hypothalamic control of the release of this hormone by the pituitary (12).

Investigations into the mechanism of these apparent estrogenic effects have not been able to demonstrate any consistent interactions of triazine herbicides with the estrogen receptor or effects on receptor-mediated responses (13–15). Effects on enzymes involved in steroid synthesis or metabolism have been limited to a study of the inhibition of testosterone metabolism in

the anterior pituitary of rats exposed *in vivo* or of whole anterior pituitaries exposed *in vitro* to atrazine (16). Weak inhibitory effects were observed on testosterone 5 α -reductase (20–37%) at an atrazine concentration of 0.5 mM; a similar observation was made for the deethylated metabolite atrazine-desethyl (16). Taken together, effects of atrazine and other triazine herbicides on estrogen receptor function or enzymes involved in sex hormone metabolism have been inconsistent and occurred at extremely high concentrations.

Triazine herbicides are known to be metabolized in various mammals (17–19) and chickens (3). In human liver microsomes, the major metabolites formed are the mono-dealkylated forms of atrazine: atrazine-desethyl and atrazine-desisopropyl; hydroxylation of the isopropyl groups present in atrazine and propazine also occurs, but to a lesser extent (for structures see Figure 1). Other metabolites formed *in vivo* and found in human urine are the fully dealkylated metabolite of the triazines (atrazine-desethyl-desisopropyl) and several 2-hydroxylated metabolites. Triazine metabolism is catalyzed primarily by cytochrome P450 (CYP) enzymes (19). The fully dealkylated metabolite of atrazine, like atrazine, has been shown to have little interaction with the estrogen receptor (14). Other than this, little or no toxicologic information is available for the metabolites of triazine herbicides.

Recently, we reported the ability of atrazine, simazine, and propazine to induce aromatase activity in a human adrenocortical carcinoma cell line (20). This response was observed at concentrations in the submicromolar range. In the present study we have continued to examine the effects of triazine herbicides and several of their common

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metabolites on aromatase activity in several human cell lines—the H295R adrenocortical, JEG-3 placental, and MCF-7 breast cancer cell line. The rationale for choosing JEG-3 cells was to examine the inducibility of aromatase in a system where the enzyme is known to be expressed at relatively high levels compared to the H295R cells; we chose MCF-7 cells to test whether the triazines could induce aromatase activity in a system where the enzyme is normally expressed at very low levels. In addition, we have examined the effects of the triazines and their metabolites on estrogen receptor-mediated vitellogenin expression in cultured primary hepatocytes of male carp (21). Increased synthesis of vitellogenin, a yolk-precursor protein in fish and birds, is a response highly sensitive to estrogens and also occurs after exposure to other compounds that are agonists for the estrogen receptor.

Materials and Methods

Cell culture conditions. We obtained H295R, JEG-3, and MCF-7 cells from the American Type Culture Collection (ATCC No. CRL-2128, HTB-36, and HTB-22, respectively). H295R cells were grown in 1:1 (v/v) Dulbecco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F12; GibcoBRL, Breda, The Netherlands) containing 365 mg/mL L-glutamine and 15 mM HEPES (GibcoBRL). The mix was further supplemented with 10 mg/L insulin, 6.7 µg/L sodium selenite, and 5.5 mg/L transferrin (ITS-G; GibcoBRL), 1.25 mg/L bovine serum albumin (Sigma, St. Louis, MO, USA), 100 U/L penicillin/100 µg/L streptomycin (GibcoBRL) and 2% steroid-free replacement serum Ultrosor SF (Soprachem, France). JEG-3 cells and MCF-7 cells were cultured in DMEM containing 4,500 mg/L D-glucose

and 110 mg/L sodium pyruvate (GibcoBRL), 10% heat-inactivated fetal calf serum (ICN, Costa Mesa, CA, USA), and 100 U/L penicillin/100 µg/L streptomycin (GibcoBRL). MCF-7 cells were cultured in DMEM supplemented with L-glutamine, 4,500 mg/L D-glucose, and sodium pyruvate (GibcoBRL). For the aromatase experiments, cells were treated as described previously (20). In brief, cells (about $1-2 \times 10^5$ cells/well) in 24-well culture plates containing 1 mL medium per well were exposed to various concentrations (0, 0.3, 1.0, 3.0, 10.0, and 30 µM) of the triazine herbicides or their metabolites (Riedel-deHaen, Seelze, Germany) (see structures in Figure 1) dissolved in 1 µL of dimethyl sulfoxide (DMSO; Sigma). Negative control cells received 1 µL of DMSO. Positive control cells were exposed to 100 µM of 8-bromo-cyclic adenosine monophosphate (8Br-cAMP) dissolved in medium containing 0.1% DMSO. We included unexposed cells as further controls, and we tested all treatments in quadruplicate.

For the reverse-transcriptase polymerase chain reaction (RT-PCR) experiments, we exposed cells in 12-well plates to 2 µL DMSO or the test chemicals in DMSO; a positive control (100 µM 8Br-cAMP) was included on each plate. We tested each treatment in triplicate and reproduced each experiment three times. DMSO at 0.1% had no effect on CYP19 expression or catalytic activity relative to unexposed cells. The test chemicals did not cause cytotoxicity at concentrations of 30 µM and below, based on visual inspection of the cells, cell attachment, protein content of the wells, and the inability of the triazines to decrease the mitochondrial activity of succinate dehydrogenase determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

test (22). We determined protein concentrations by the fluorometric method of Udenfriend et al. (23), using bovine serum albumin (Sigma) as standard. We added triazines to the cell culture medium at concentrations below their aqueous solubility limit [e.g., 300 µM for atrazine; 50 µM for simazine (24)]. All exposures were for 24 hr.

Isolation and amplification of RNA. We isolated RNA using the RNA Insta-Pure System (Eurogentec, Liège, Belgium) according to the enclosed instructions and stored it at -70°C . We performed RT-PCRs using the Access RT-PCR System (Promega, Madison, WI, USA) with various modifications reported previously (20). We verified the purity of the RNA preparations by denaturing agarose gel electrophoresis. We obtained suitable primer pairs by entering the human CYP19 cDNA sequence obtained from the European Molecular Biology Laboratories database (Heidelberg, Germany) into the software program Geneworks (version 2.4; IntelliGenetics, Mountain View, CA, USA). The primer pair used for CYP19 mRNA amplification was 5'-TTA-TGA-GAG-CAT-GCG-GTA-CC-3' and 5'-CTT-GCA-ATG-TCT-TCA-CGT-GG-3', producing an amplification product of 314 base pairs. As reference, RT-PCR was performed on β -actin mRNA using the primer pair 5'-AAA-CTA-CCT-TCA-ACT-CCA-TC-3' and 5'-ATG-ATC-TTG-ATC-TTC-ATT-GT-3', according to the instructions of the Access RT-PCR kit, except using 1 mM MgSO_4 , an annealing temperature of 54°C , and 25 cycles. We found β -actin mRNA unaffected by any of the treatments (DMSO, triazines, metabolites or 8Br-cAMP) and could be used reliably as a reference amplification response. Detailed information on PCR conditions and reproducibility and ability of the method to be used (semi)quantitatively was published previously (20). We detected amplification products using agarose gel electrophoresis and ethidium bromide staining. We quantified intensity of the ethidium bromide stains using a FluorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Aromatase assay. We determined the catalytic activity of aromatase using the method of Lephart and Simpson (25) with minor modifications. Cells were exposed to 54 nM 1β -3H-androstenedione (New England Nuclear Research Products, Boston, MA, USA) dissolved in serum-free (Ultrosor SF-free) culture medium and incubated for 1.5 hr at 37°C in an atmosphere of 5% CO_2 and 95% air. All further steps proceeded as reported previously (20,26). Aromatase activity was expressed in picomoles of androstenedione converted per hour per milligram cellular protein. We verified the

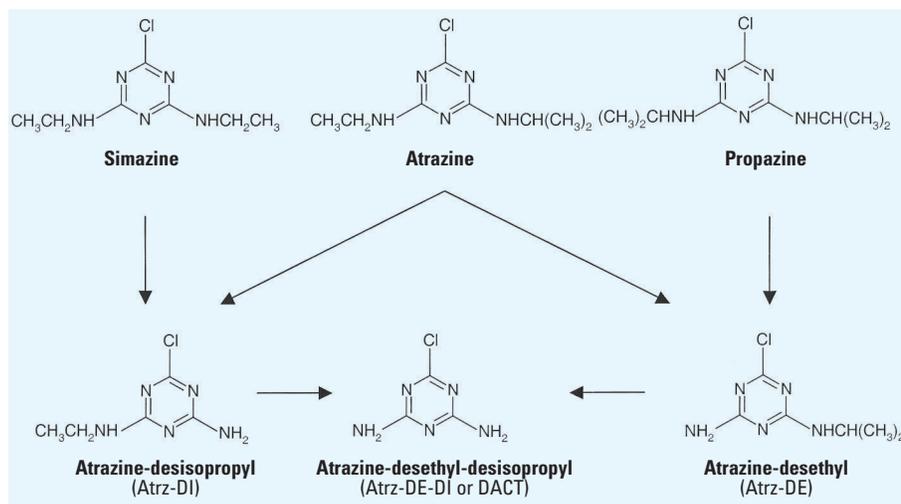


Figure 1. The various routes of metabolism of the 2-chloro-s-triazines herbicides atrazine, simazine, and propazine to several common dealkylated metabolites. Each of the three dealkylated metabolites can be further dechlorinated via 2-hydroxylation.

specificity of the aromatase assay based on the release of tritiated water by measuring the production of estrone (the aromatization product of androstenedione), using a ^{125}I -labeled double-antibody radioimmunoassay kit (ICN), and by using 4-hydroxyandrostenedione, an irreversible inhibitor of the catalytic activity of aromatase, to block the formation of tritiated water (27).

Carp hepatocyte/vitellogenin production assay. Male carp (*Cyprinus carpio*) hepatocytes were freshly perfused by a two-step retrograde technique, isolated and cultured as described previously in 96-well plates (21). Culture conditions included the use of

phenol red-free DMEM/F12 medium (Sigma) supplemented with 14.3 mM NaHCO_3 , 20 mM HEPES, 50 $\mu\text{g/L}$ gentamycin, 1 μM insulin, 10 μM hydrocortisone, 2% Ultrosor SF and 2 mg/L of the protease inhibitor aprotinin (Fluka, Buchs, Switzerland). Cells were seeded in 96-well plates at a density of 1×10^6 cells/mL (180 $\mu\text{L/well}$). For the estrogenicity studies, we exposed cells to various concentrations of 17β -estradiol (0.06–6 μM) or the triazines and their metabolites (0.3–30 μM), from DMSO stocks. For the antiestrogenicity studies, we used the same triazine concentrations but added them in culture medium

containing 100 nM 17β -estradiol (approximate EC_{50}). The final concentration of DMSO did not exceed 0.2% (v/v). As positive controls we included on every plate either a 100 nM 17β -estradiol (for estrogenicity studies) or 0.1, 1.0, and 10 μM tamoxifen, a known estrogen receptor antagonist (for antiestrogenicity studies). All treatments were in sextuplet; each concentration–response experiment was reproduced three times. Exposures were for 6 days. We quantified vitellogenin production by an indirect competitive ELISA, and we determined cell viability as described in detail previously (21).

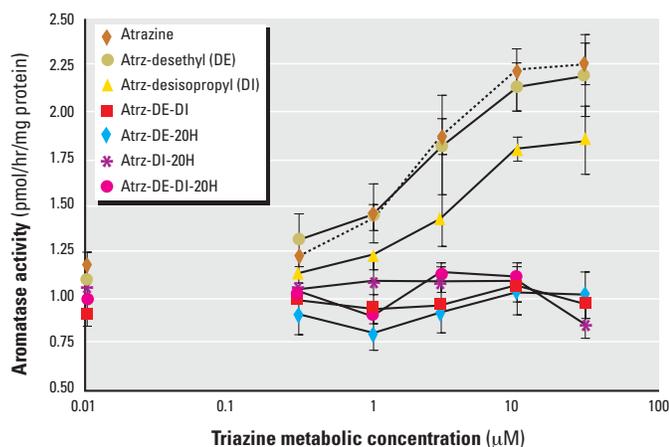


Figure 2. Concentration–response curves for induction of aromatase activity in H295R human adrenocortical carcinoma cells after 24-hr exposure to atrazine and six of its metabolites. Each concentration was tested in quadruplicate.

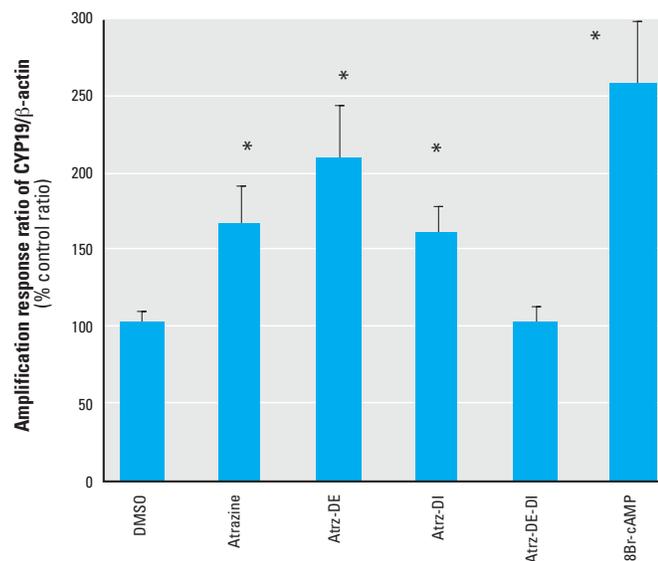


Figure 4. Levels of CYP19 mRNA in H295R human adrenocortical carcinoma cells exposed for 24 hr to DMSO vehicle, 30 μM atrazine, atrazine-desethyl (Atrz-DE), atrazine-desisopropyl (Atrz-DI), or atrazine-desethyl-desisopropyl (Atrz-DE-DI or DACT), or 100 μM 8-bromo-cAMP. Each treatment was tested in triplicate.

*Significantly different from control (DMSO) (two-tailed Student *t*-test, $p < 0.05$).

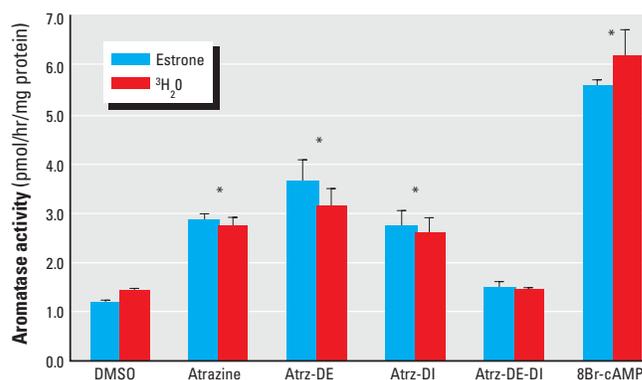


Figure 3. Comparison of aromatase activity based on estrone production of tritiated water release in H295R human adrenocortical carcinoma cells treated for 24 hr with 30 μM atrazine, its metabolites atrazine-desethyl (Atrz-DE), atrazine-desisopropyl (Atrz-DI), and atrazine-desethyl-desisopropyl (Atrz-DE-DI or DACT), or 100 μM 8-bromo-cAMP. Each concentration was tested in quadruplicate.

*Significantly different from control (DMSO) (two-tailed Student *t*-test, $p < 0.05$).

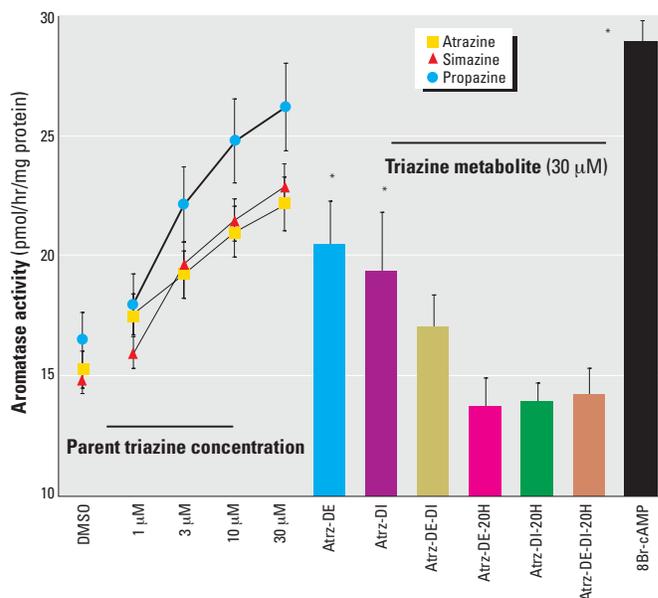


Figure 5. Concentration–response curves for induction of aromatase activity in JEG-3 human placental choriocarcinoma cells after 24 hr exposure to atrazine, simazine, or propazine, and the effect of 24-hr exposure of JEG-3 cells to a single concentration of 30 μM of six triazine metabolites, or 100 μM 8-bromo-cAMP. Each concentration was tested in quadruplicate.

*Significantly different from control (DMSO) (two-tailed Student *t*-test, $p < 0.05$).

Results

Aromatase induction in H295R cells. Atrazine, atrazine-desethyl (Atrz-DE), and atrazine-desisopropyl (Atrz-DI) were able to induce the catalytic activity of aromatase concentration-dependently to an apparent maximum of just over 2-fold (Figure 2). Greater concentrations of these compounds demonstrated slight cytotoxicity (about 20% decrease in MTT reduction at 100 μ M). The fully dealkylated metabolite of atrazine—atrazine-desethyl-desisopropyl (Atrz-DE-DI or DACT)—and the metabolites that were hydroxylated at the 2 position of the triazine ring [and thus dechlorinated (Figure 1)] had no effect on aromatase activity (Figure 2). We verified further the differential effects of the triazine compounds on aromatase activity by measuring the ability of the cells to convert androstenedione to estrone. Tritiated water release and estrone production were increased in a 1:1 ratio in cells exposed to atrazine or Atrz-DE, Atrz-DI, and 8Br-cAMP, whereas the metabolite Atrz-DE-DI had no effect on either measurement (Figure 3). The mechanism of induction of aromatase activity appeared to involve the induction of CYP19 mRNA, because atrazine, Atrz-DE, Atrz-DI, and 8Br-cAMP were able to increase mRNA levels for CYP19 relative to control, whereas Atrz-DE-DI had no effect (Figure 4). None of the triazine metabolites could inhibit or enhance the activity of aromatase when added directly to the medium used for the aromatase assay (data not shown). The same was true for the parent triazines (20).

Aromatase induction in JEG-3 and MCF-7 cells. Several 2-chloro-*s*-triazine herbicides were able to induce aromatase activity in JEG-3 cells (Figure 5). Atrazine, simazine, and propazine induced aromatase activity concentration-dependently, producing statistically significant increases in activity above control at concentrations above 1 μ M (Student *t*-test, $p < 0.05$). Among the metabolites of atrazine tested at 30 μ M, only Atrz-DE and Atrz-DI significantly increased aromatase activity above control. The most noticeable difference between JEG-3 and H295R cells is the basal activity of aromatase, which was at least an order of magnitude greater in JEG-3 cells (about 15 pmol androstenedione/hr/mg cellular protein) than in H295R cells (about 1–1.5 pmol androstenedione/hr/mg cellular protein); also, basal activity in JEG-3 cells was inducible by only 2-fold after 24-hr exposure to 100 μ M 8Br-cAMP, whereas aromatase activity was inducible by over 5-fold in H295R cells. In MCF-7 cells, basal aromatase activity was undetectable, and neither 8Br-cAMP, atrazine, simazine, nor propazine was able to induce the activity to detectable

levels, under our culture and assay conditions; the same was true for mRNA levels (data not shown).

Vitellogenin production in carp hepatocytes. Vitellogenin concentrations in unexposed or DMSO-exposed male carp hepatocytes were undetectable. A lowest-observed-effect concentration of 17 β -estradiol of about 2 nM produced a detectable amount of vitellogenin of about 100–400 ng/mg cellular protein; the EC₅₀ of 17 β -estradiol induced vitellogenin concentrations to 4,000–6,000 ng/mg protein. Coexposure of hepatocytes to 100 nM 17 β -estradiol and 0.1, 1, or 10 μ M tamoxifen inhibited 17 β -estradiol-induced vitellogenin synthesis by 54%, 89%, and 91%, respectively. The readily aromatizable androgens testosterone and 17 α -methyltestosterone did not induce vitellogenin synthesis at concentration between 0.6 nM and 1 μ M (6-day exposures), indicating that aromatase activity is either very low or not present in male carp hepatocytes in primary culture under our conditions. Exposure of male carp hepatocytes to various concentrations (0–30 μ M) of the triazines or their metabolites did not significantly induce vitellogenin production (Figure 6A). The only exception was a slight, but statistically significant ($p < 0.05$) and concentration-dependent estrogenic response by Atrz-DE-DI (DACT), which increased vitellogenin production from 2% of the response by 100 nM 17 β -estradiol at 1 μ M (not shown) to about 8% of the response by 100 nM 17 β -estradiol at 30 μ M (Figure 6A). None of the compounds could produce a concentration-dependent antiestrogenic response in the presence of 100 nM 17 β -estradiol (Figure 6B).

Discussion

We recently reported that several chloro-*s*-triazine herbicides induce the catalytic activity and mRNA expression of human aromatase

in vitro in H295R adrenocortical carcinoma cells (20). The present study extends these observations by demonstrating that atrazine, simazine, propazine, and two metabolites shared by these Atrz-DE and Atrz-DI—were able to induce aromatase activity in H295R cells, whereas the fully dealkylated metabolite Atrz-DE-DI (DACT) and the three hydroxylated metabolites of atrazine were not active. In addition, the compounds that induced aromatase activity in H295R cells also induced this activity in JEG-3 cells, although with lesser efficacy. A difference between the two cell lines is that JEG-3 cells exhibited a 15-fold greater basal aromatase activity than H295R cells, and inducibility by 8Br-cAMP was lower (< 2-fold) than in H295R cells (over 5-fold). Thus, the relatively high level of basal aromatase gene expression in JEG-3 cells and relatively low inducibility by the cAMP analog partly explains the lesser response to the triazines.

MCF-7 cells did not exhibit aromatase activity in this study, nor did they respond to induction by cAMP analogs or triazine herbicides. The expression of aromatase in MCF-7 cells has been the subject of conflicting reports. Although many studies have not detected aromatase activity in MCF-7 cells (28), some report the presence of low aromatase activity (29–31) and of stimulation of estrogen-receptor-mediated cell proliferation by androgens in this cell line (30). The expression of aromatase in MCF-7 cells is poorly understood, and although at least one study reported stimulation of this enzyme by cAMP (31), we have not been able to stimulate MCF-7 cells to express detectable levels of activity using 8Br-cAMP or forskolin. The above suggests that major qualitative differences exist in characteristics among batches of MCF-7 cells in culture, which may complicate the use of this cell line as an *in vitro* screening tool for effects

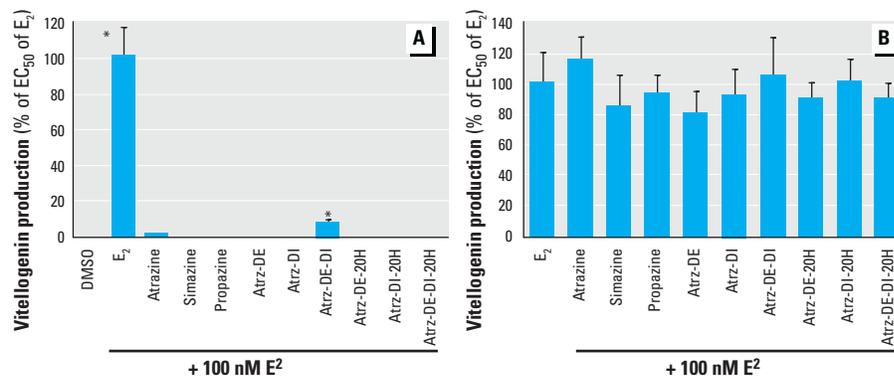


Figure 6. Exposure of male carp hepatocytes to triazines and metabolites. (A) Effect of 100 nM estradiol (E₂) or 30 μ M of the three parent triazines and six metabolites on vitellogenin production in freshly isolated male carp hepatocytes. (B) Effect of a coexposure of freshly isolated male carp hepatocytes to 100 nM E₂ and 30 μ M of each of the three parent triazines or their six metabolites individually, on the induction of vitellogenin synthesis. Each concentration was tested in quadruplicate.

*Significantly different from control (DMSO) (two-tailed Student *t*-test, $p < 0.05$).

of androgens and estrogens, or effects of xenobiotics on steroidogenic and/or steroid metabolizing enzymes. In any case, our findings indicate that, unlike in cell systems in which the expression of aromatase activity is clearly cAMP-dependent (H295R and JEG-3), triazine herbicides do not induce aromatase activity in MCF-7 cells, in which this expression is relatively refractory to cAMP.

Regarding a structure–activity relationship for aromatase induction by the different triazine compounds, it appeared that the relatively lipophilic parent triazines and mono-dealkylated metabolites were active, whereas the more hydrophilic fully dealkylated and 2-hydroxylated (dechlorinated) metabolites were inactive. These results indicate that biokinetic factors such as metabolism may play a considerable role in the biologic activity of triazine herbicides. Whether the structure–activity relationship observed for aromatase induction *in vitro* corresponds with the potential to increase estradiol levels or cause estrogen-mediated toxicities *in vivo* is not certain. Indeed, atrazine, simazine, and propazine appear to have similar effects on mammary tumor incidences *in vivo* (32), but toxicologic information on the fully dealkylated metabolite of the triazines is insufficient to make a judgment. To substantiate the aromatase induction hypothesis, additional experimental evidence is required to determine whether aromatase induction occurs *in vivo* and in which target tissues this induction would take place. Given the recent evidence that plasma estradiol and estrone levels are increased in atrazine-treated male Wistar rats (33), it is apparent that the presence of ovarian aromatase is not essential for the effects of atrazine. The further observation that estrone levels appear to be preferentially increased *in vivo* (33) may indicate a tissue-specific effect on aromatase. If aromatase induction is shown to play a role *in vivo*, it could be hypothesized that the induction would occur in tissues that contain relatively greater levels of androstenedione than testosterone as precursor—tissues such as adrenal cortex and adipose.

The lack of response of male carp hepatocytes to the triazines and their metabolites is consistent with the observed lack of interaction of these compounds with the estrogen receptor and inability to cause estrogen receptor-mediated responses *in vitro* (13–15). In addition, measurable aromatase activity was not found in the hepatocytes because readily aromatizable androgens were not able to elicit vitellogenin induction. However, we do not rule out the presence of low levels of aromatase activity in male carp hepatocytes

in primary culture, and future investigations will be needed to address this question.

In conclusion, the effects of the triazine herbicides and some of their metabolites on aromatase activity may provide a partial explanation for the observed increases in plasma estradiol concentrations in female Sprague-Dawley rats (7) and estradiol and estrone concentrations in male Wistar rats (33) exposed to atrazine, together with the observed estrogen-mediated toxicities *in vivo* (7). Future studies are needed to investigate the inducibility of aromatase by the various triazine herbicides and their metabolites *in vivo*, and compare the developed structure–activity relationship for induction to *in vivo* estrogenic toxicities and to the *in vitro* results of the present study.

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