Chloro-*s*-triazine Herbicides and Certain Metabolites Induce Aromatase (CYP19) Activity in H295R Human Adrenocortical Carcinoma Cells

J.Thomas Sanderson, Marjoke Heneweer, Willem Seinen, John P. Giesy* and Martin van den Berg

Research Institute for Toxicology, Utrecht University, PO Box 80176, 3508 TD Utrecht, The Netherlands
*Department of Zoology, National Food Safety and Toxicology Center, Institute of Environmental Toxicology, Michigan State University, East Lansing, MI 48824, USA.

Introduction

There is growing concern that certain environmental contaminants have the potential to cause endocrine disruption, which may lead to reproductive problems and other toxicities related to sexual differentiation, growth and development. Current research has focused on potential interactions with the sex hormone receptors, particularly the estrogen receptor. However, other mechanisms of potential interference with endocrine functions exist, including effects on steroidogenic enzyme systems.

We have evaluated the H295R human adrenocortical carcinoma cell line [1,2] as an *in vitro* tool for examining potential interferences of chemicals with levels of mRNA and/or catalytic activities of several steroidogenic cytochrome P450 (CYP) enzymes [3]. These cells are known to express numerous steroidogenic cytochrome P450s (CYPs) including aromatase (CYP19) [4,5], which is the rate-limiting enzyme in the conversion of androgens to estrogens. To elucidate an underlying mechanism of action for the reported estrogen-related endocrine disrupting properties of the 2-chloro-*s*-triazine herbicides [6], we examined the effects of atrazine, simazine, propazine, and several common metabolites on mRNA expression and catalytic activity of aromatase (CYP19) in H295R cells.

Materials and Methods

Cell culture conditions. H295R cells were obtained from the American Type Culture Collection (ATCC # CRL-2128) and grown under culture conditions published previously [2,5]. Cells, in 24-well plates containing 1 ml medium per well, were exposed to the test compounds (0-30 μM) (Riedel deHaen, Germany), administered as 1000-fold stock solutions in 1 μl dimethyl sulfoxide (DMSO). Negative control cells were exposed to DMSO. Positive control cells received 100 μM of 8-bromo-cyclic adenosine monophosphate (8Br-cAMP) dissolved in medium containing 0.1% DMSO. After a 24 h exposure, cells were prepared for RNA analysis or aromatase assay.

RNA isolation and amplification. RNA was isolated using the RNA Insta-Pure System (Eurogentec, Belgium) and stored at -70°C. Reverse-transcriptase polymerase chain reactions (RT-PCRs) were performed using the Access RT-PCR System (Promega, USA). The purity of the RNA preparations was verified by denaturing agarose gel electrophoresis. The primer pair used for CYP19 mRNA amplification was: 5'-TTA-TGA-GAG-CAT-GCG-GTA-CC-3'; 5'-CTT-GCA-ATG-TCT-TCA-CGT-GG-3', resulting in a single amplification product of 314 base pairs, when using 100 ng total RNA per reaction, an annealing temperature of 57°C and $MgSO_4$

concentration of 0.75 mM. As reference, RT-PCR was performed on β-actin mRNA using a commercially available primer pair (Promega, USA). Serial dilutions of total RNA concentrations were amplified using the primer pairs to determine the 'linear' range of the PCR reaction, in order to make semi-quantitative inferences. Amplification products were detected using agarose gel electrophoresis and ethidium bromide staining and quantified using a FluorImager (Molecular Dymanics, USA).

Aromatase assay. The catalytic activity of aromatase was determined by the ^3H_2O-release assay [7] with modifications. Cells were exposed to 54 nM 1β-^{3}H-androstenedione (New England Nuclear Research Products, USA) dissolved in serum-free culture medium for 1.5 h. The specificity of ^3H_2O-release was verified by measuring the aromatization product estrone, using a ^{125}I-labeled double-antibody radioimmunoassay kit (DSL-8700; Diagnostic Systems Inc, USA), and by using 4-hydroxyandrostenedione, an irreversible inhibitor of the catalytic activity of aromatase.

Results

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), simazine (2-chloro-4,6-bis(ethylamino)-*s*-triazine) and propazine (2-chloro-4,6-bis(isopropylamino)-*s*-triazine) induced aromatase activity in H295R cells after a 24 h exposure (Fig. 1). The induction was concentration- and time-dependent (not shown), with an apparent maximum of about 2.5 fold at 30 µM. 4-HA was able to block completely basal and atrazine- or 8Br-cAMP-induced activity of aromatase (Fig. 1).

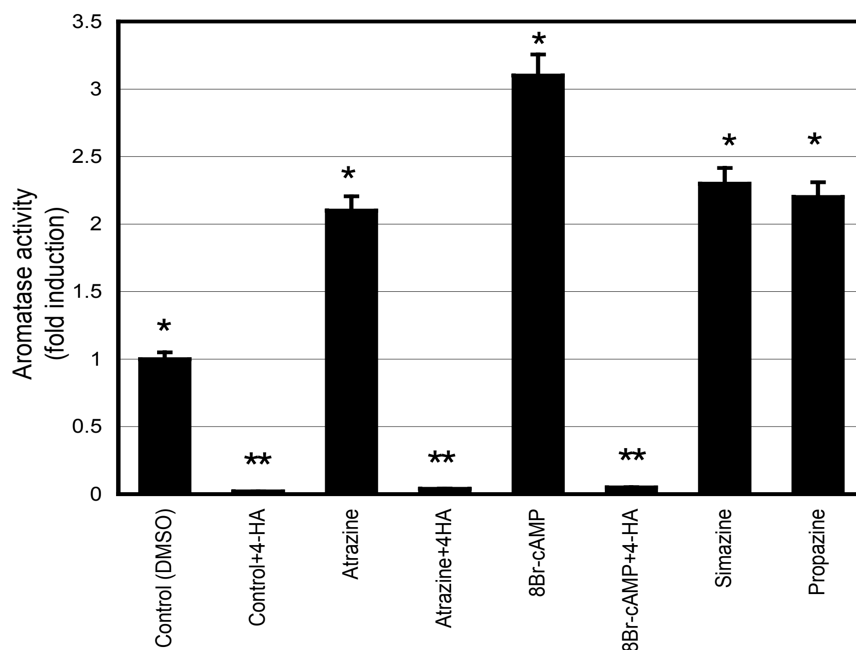

Figure 1. *Effect of 30 µM atrazine, simazine or propazine, or 100 µM 8Br-cAMP on aromatase activity in H295R cells. The aromatase inhibitor 4-HA (100 µM) was able to block completely basal and atrazine- or 8Br-cAMP-induced activity of aromatase. Basal aromatase activity was*

*0.36 ±0.03 pmole/h/mg protein. Bars represent means with standard deviations (n=4). *) significantly greater or **) significantly less than control (p<0.05).*

Similarly, two metabolites common to these three herbicides, deethylatrazine (2-chloro-4-amino-6-isopropylamino-*s*-triazine) and deisopropylatrazine (2-chloro-4-ethylamino-6-amino-*s*-triazine), were capable of inducing aromatase activity, while the fully dealkylated metabolite (2-chloro-4,6-amino-*s*-triazine) and the three 2-hydroxylated (and thus dechlorinated) metabolites were not active (Fig. 2). The induction of aromatase activity by the triazines and their metabolites was verified by the measurement of ^3H_2O-release and estrone production in the same cellular incubations. Estrone production and ^3H_2O-release increased in atrazine- and 8Br-cAMP-treated cells, in the expected one-to-one ratio (data not shown).

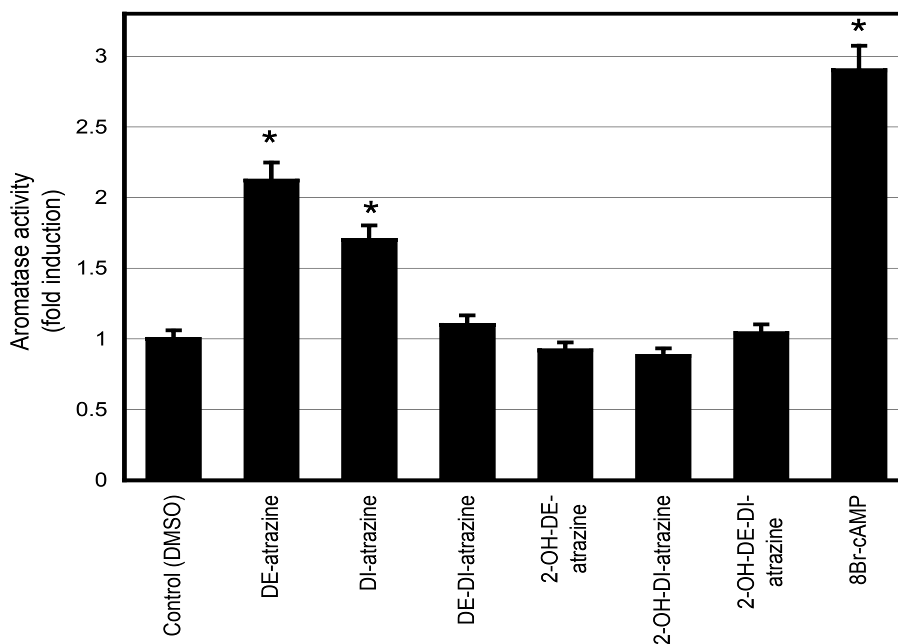

Figure 2. *Effect of 30 µM of several triazine metabolites or 100 µM 8Br-cAMP (n=4) on aromatase activity in H295R cells. *) significantly greater than control (p<0.05). DE = deethyl; DI = deisopropyl.*

RT-PCR analysis of RNA isolated from cells exposed to the triazines, their metabolites or 8Br-cAMP demonstrated that CYP19 mRNA was consistently increased relative to control cells (Fig. 3) by the compounds that were able to induce the catalytic activity of aromatase. The amplification response to β-actin mRNA, used as a reference, was not affected by the treatments (data not shown).

Discussion and Conclusions

This is the first consistent demonstration that several 2-chloro-*s*-triazine herbicides induce the human aromatase enzyme *in vitro*. The relatively weak induction of just over 2 fold may be of physiological relevance locally in tissues, as aromatase is the rate-limiting enzyme in the conversion of androgens to estrogens. Increased local production of estrogens via this mechanism

may have the potential to cause or contribute to estrogen-mediated pathologies, such as the tumor promotion observed experimentally in atrazine-exposed rats [6,8,9]. The H295R cell system deployed in the present study has proven useful in identifying a potential key target for the endocrine-disrupting effects of triazine herbicides. Future studies *in vivo* are needed to support our hypothesis linking aromatase induction in specific target tissues to the endocrine toxicities of the triazines. If our hypothesis is supported this may have implications for the regulation of this class of herbicides as potential endocrine disrupters and tumor promoters.

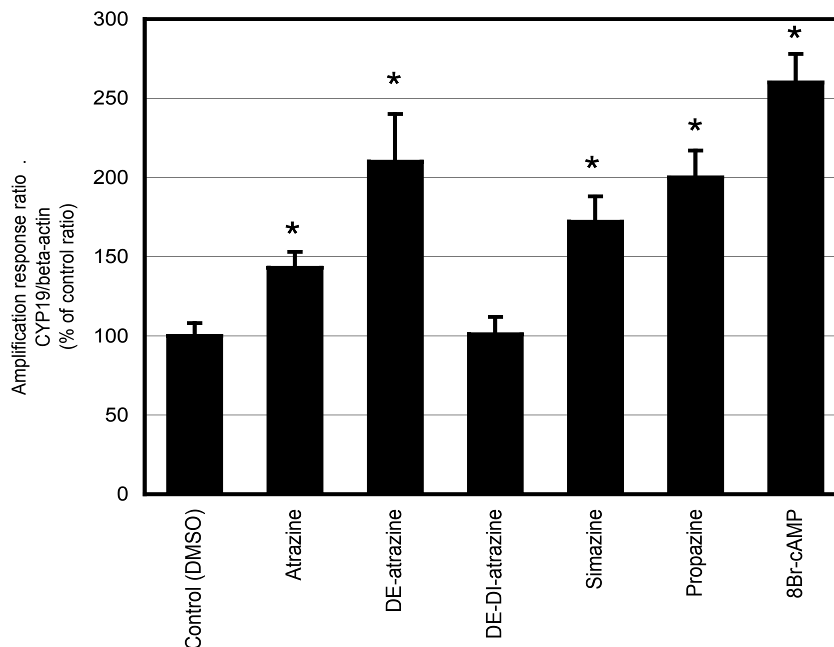

Figure 3. *Effect of several triazines and metabolites (30 µM) or 8Br-cAMP (100 µM) on the level of CYP19 mRNA in H295R cells (n=3). *) significantly greater than control (p<0.05).*

References

1. Gazdar, A.F., H.K. Oie, C.H. Shackleton, T.R. Chen, T.J. Triche, C.E. Myers et al. **1990**. *Cancer Res.* 50:5488-5496.
2. Rainey, W.E., I.M. Bird and J.I. Mason. **1994**. *Mol. Cell. Endocrinol.* 100:45-50.
3. Sanderson, J.T. and M. Van den Berg. **1998**. *Organohalogen Compounds* 37:77-80.
4. Staels, B., D.W. Hum and W.L. Miller. **1993**. *Mol. Endocrinol.* 7:423-433.
5. Rainey, W.E., I.M. Bird, C. Sawetawan, N.A. Hanley, J.L. McCarthy, E.A. McGee et al. **1993**. *J. Clin. Endocrinol. Metab.* 77:731-737.
6. Stevens, J.T., C.B. Breckenridge, L.T. Wetzel, J.H. Gillis and L.G. Luempert III. **1994**. *J. Toxicol. Environ. Health* 43:139-153.
7. Lephart, E.D. and E.R. Simpson. **1991**. *Methods Enzymol.* 206:477-483.
8. Wetzel, L.T., L.G. Luempert III, C.B. Breckenridge, M.O. Tisdel and J.T. Stevens. **1994**. *J. Toxicol. Environ. Health* 43:169-182.
9. Pinter, A., G. Torok, M. Borzsonyi, A. Surjan, M. Calk, Z. Kelecsenvi et al. **1990**. *Neoplasma* 37:533-544.