BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **227,** 140–146 (1996)
ARTICLE NO. 1480

The Inhibition of Estrogen Receptor-Mediated Responses by Chloro-*S*-triazine-Derived Compounds Is Dependent on Estradiol Concentration in Yeast

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Received August 19, 1996

The chloro-*S*-triazine derived compounds atrazine, atrazine desisopropyl, cyanazine, and simazine are commonly used herbicides. These compounds do not have estrogenic activity in yeast expressing human estrogen receptor (hER) and an estrogen-sensitive reporter. In the presence of a concentration of estradiol (20 nM) that induced maximal reporter activity in yeast, the triazines did not inhibit reporter activity. However, the triazines decreased reporter activity in a dose-dependent manner in the presence of a submaximal concentration of estradiol (0.5 nM). The estradiol-dependent activity of a mutant hER lacking the amino terminus was not inhibited by the triazines in yeast. Competition binding assays demonstrated that the triazines displaced radiolabeled estradiol from recombinant hER. These results suggest that the ability of the triazines to inhibit estrogen receptor-mediated responses in yeast occur through their interaction with hER and is dependent on the concentration of estradiol.

The chloro-*S*-triazine-derived compounds atrazine, atrazine desisopropyl, cyanazine and simazine are herbicides that have been detected in water from the Mississippi River, probably as a result of nonpoint source contamination (1). Sprague-Dawley rats, but not Fischer 344 rats, fed atrazine or simazine had an earlier onset and higher incidence of mammary tumors than control animals (2). These results were further supported by reports showing that atrazine produced irregularities in estrus in Sprague-Dawley rats, suggesting that these chemicals had estrogenic activity. However, only weak interaction of the triazines with ER from rodents in a competition binding assay was measured (3). Furthermore, these chemicals did not increase uterine weight in rats or increase estrogen-specific responses through the hER in yeast or MCF-7 cells, a human mammary carcinoma cell line (4,5).

It was then suggested that triazines may function as anti-estrogens since these compounds inhibited the maximal estradiol-specific increase in uterine DNA synthesis and progesterone receptor induction in rats (4). This inhibition required very high concentrations of triazines. Furthermore, the triazines did not decrease maximal estradiol-induced transcriptional activity of hER in yeast or MCF-7 cells (5).

Consistent with previous reports, herein we show that the triazines have no estrogenic activity in yeast expressing hER. The triazines did function as effective inhibitors of estrogenic activity but only at a submaximal, not a maximal, concentration of estradiol. Competition binding assays indicated that the triazines reduced the binding of radiolabeled estradiol to recombinant hER.

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Abbreviations: DMSO, dimethylsulfoxide; EtOH, ethanol; hER, human estrogen receptor; MeOH, methanol; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

0006-291X/96 $18.00

MATERIALS AND METHODS

Materials. Estradiol-17β and the amino acids for culturing yeast were purchased from Sigma Chemical Co. (St. Louis, MO.). 17β-3,4,6,7[^{3}H](N) estradiol (99 Ci/mmol) was purchased from DuPont/NEN (Wilmington, DE). Atrazine, atrazine desisopropyl, cyanazine and simazine at 99.0% purity were purchased from AccuStandard Inc. (New Haven, CT.). Technical grade cyanazine (97% purity) was generously provided by DuPont (Wilmington, DE). Yeast strain DY150 and the yeast expression vectors containing the ER(wt) and ER179C were also generously provided by Bert O'Malley and Z. Nawaz (Baylor College of Medicine).

Yeast transformation. Yeast strain DY150 (MAT a, ura 3-1, leu 2-3, 112 trp 1-1, his 3-11, 15 ade 2-1, can 1-100) was transformed with ER(wt) or ER179C (6) and a reporter plasmid containing 2 estrogen response elements linked to the *lacZ* gene (7) as described (8). Transformants were selected by tryptophan and uracil auxotrophy.

Yeast assays. Yeast strains ER(wt) and ER179C were grown overnight at 30 °C in synthetic medium-tryptophan, uracil. The next day, 25 μl of the overnight culture was diluted into 975 μl of fresh medium and grown overnight with MeOH, DMSO or estradiol-17β in the presence or absence of atrazine, atrazine-desisopropyl, cyanazine or simazine. Atrazine, atrazine-desisopropyl, cyanazine and simazine were purchased in MeOH at 414 μM and added to the medium so that the concentration of MeOH did not exceed 0.5%. MeOH concentrations greater than 0.5% significantly reduced the reproducibility of the experiments. Cyanazine from DuPont was prepared in DMSO at 10 mM and added so that the concentration of DMSO in the medium was 0.1%. Estradiol-17β was prepared in DMSO and the concentration of DMSO in the assay was 0.1%. The same volume of solvent was used for each concentration of triazine and estradiol tested. None of the chemicals inhibited the growth of the yeast at the concentrations tested.

β-galactosidase assays. The yeast cells were collected by centrifugation, resuspended in 700 μl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM β-mercaptoethanol) and permeabilized by the addition of 6 μl of $CHCl_3$ and 4 μl 0.1% SDS followed by vortexing for 25 s. The reactions were equilibrated at 30 °C for 10 min, then 160 μl of ONPG (4 mg/ml in Z-buffer) was added and the reactions returned to 30 °C for between 5 and 60 min. The reactions were terminated by the addition of 400 μl 1M $NaCO_3$, the cell debris removed by centrifugation and the absorbance at 420 nm measured. The growth of the yeast strains was monitored by measuring the absorbance at 600 nm. Miller units were determined using the following formula: [A_{420}/(A_{600} of 1/10 dilution of cells $\times$ volume of culture $\times$ length of incubation)] $\times$ 1000. The data are representative of three experiments with three replicates.

Binding assays. Recombinant hER was produced in Sf9 insect cells using the baculovirus expression system and prepared as ammonium sulfate precipitates (9). The hER produced in Sf9 insect cells has approximately the same affinity for [^{3}H]estradiol-17β as the hER produced in yeast cells (unpublished data). Also, the Sf9 extract containing hER had less proteolytic activity than an extract from strain ER(wt). Recombinant hER at a concentration of 0.4 nM was dissolved in the 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_4$, 10% glycerol, γ-globulin (10

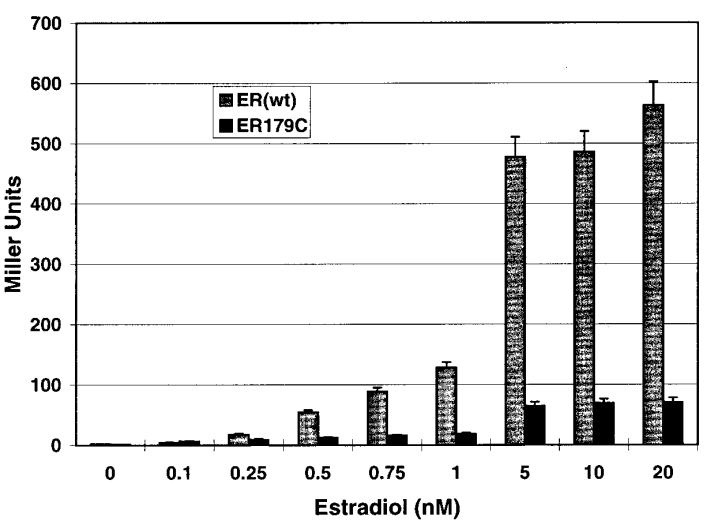


FIG. 1. Dose-response curves of estradiol-dependent β-galactosidase activity in yeast strains ER(wt) and ER179C. The yeast strains ER(wt) and ER179C were cultured in the presence of increasing concentrations of estradiol for 12 h and then β-galactosidase activity measured. β-Galactosidase activity is expressed as Miller units as described under Materials and Methods.

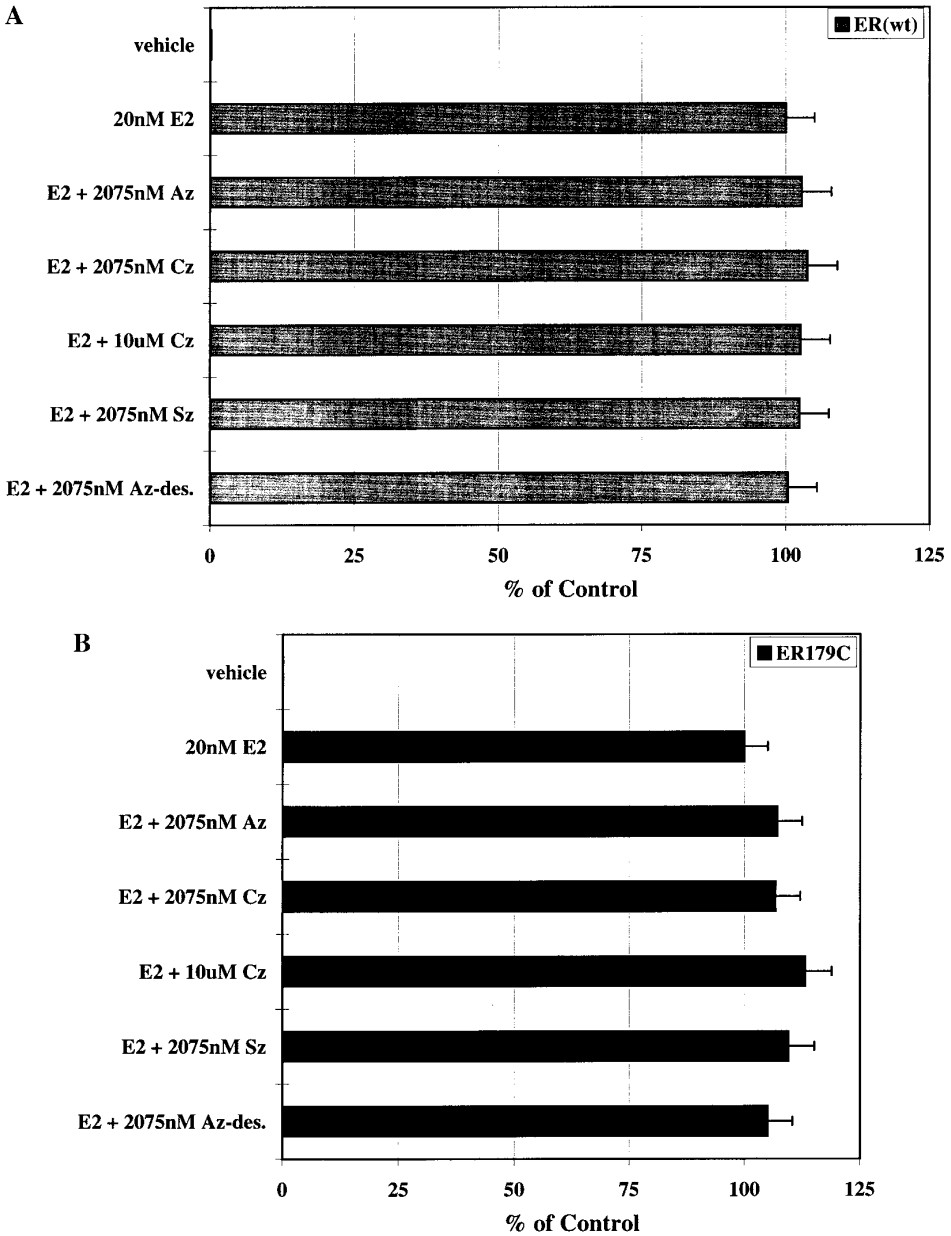
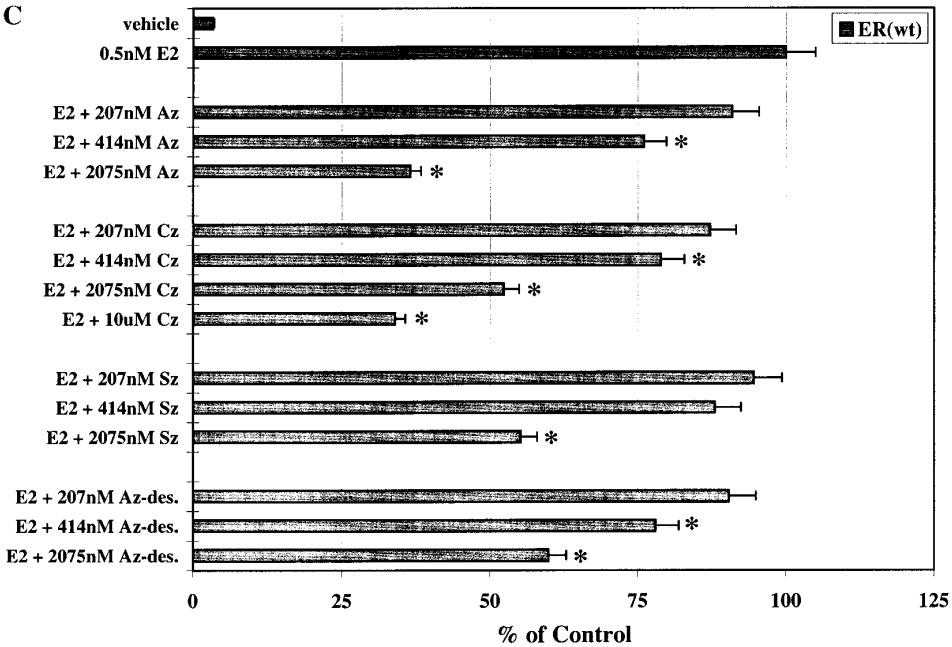


FIG. 2. Coincubation of yeast strains ER(wt) and ER179C with estradiol and various triazines. The yeast strains ER(wt) (A) and ER179C (B) were incubated with vehicle, 20 nM estradiol in the presence or absence of various triazines (Az, atrazine; Az-des, atrazine-desisopropyl; Cz, cyanazine; E2, estradiol; Sz, simazine) at the indicated concentrations. The strain ER(wt) (C) or strain ER179C (D) was incubated with 0.5 nM estradiol in the presence of absence of various triazines at the indicated concentrations. The 10 μM cyanazine was obtained from DuPont. The % of control is defined as a percentage of β-galactosidase activity of estradiol in the absence of competitor.

mg/ml), 0.5 mM phenylmethylsulfonyl fluoride and 0.2 mM leupeptin for 1 h at 25 °C with 2 nM [^{3}H]estradiol-17β in the presence or absence of increasing concentration of radioinert environmental chemicals or estradiol-17β. The [^{3}H]estradiol-17β was dissolved in 10% EtOH and the triazines in MeOH as described earlier. The concentration of EtOH and MeOH was 0.25% and 2.5%, respectively, in the binding reaction. Cyanazine from DuPont was prepared



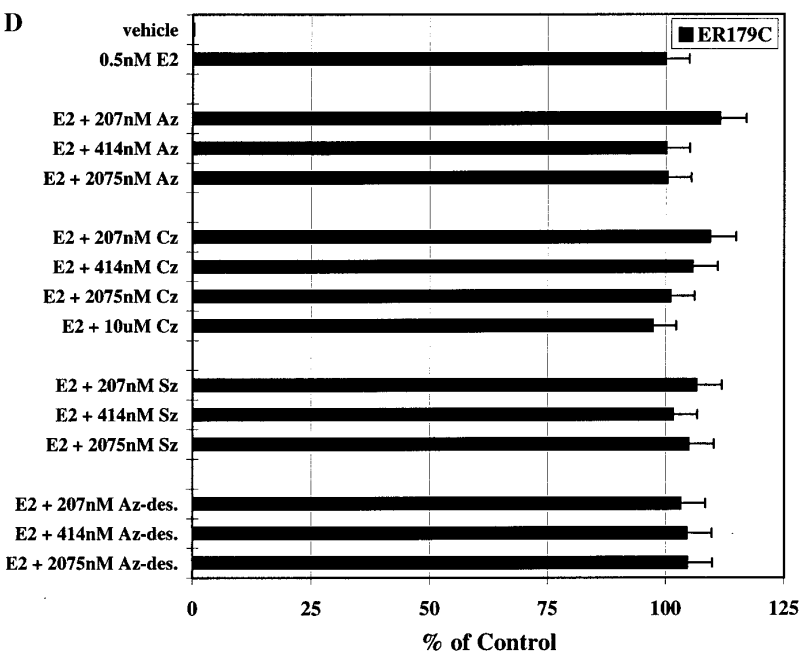


FIG. 2.—*Continued*

in DMSO at 10 mM and added so that the concentration of DMSO in the reaction was 1.0%. The same volume of solvent was used for each concentration of triazine tested. Non-specific binding of [^{3}H]estradiol-17β was assessed by the addition of 300-fold molar excess of radioinert estradiol-17β. Free [^{3}H]estradiol-17β was removed by incubation with Chardex (contains 5% activated charcoal and 0.5% dextran dissolved in phosphate-buffered saline) for 10 min at 4 °C and centrifugation for 3 min at 15,000 $\times$ g. The bound [^{3}H]estradiol-17β was measured using liquid scintillation counting. The data are representative of three experiments with three replicates.

RESULTS AND DISCUSSION

We have expressed full-length hER (ER(wt)) or a mutant ER lacking the amino-terminus from amino acids 1 to 179 (ER179C) and a reporter containing 2 estrogen response elements linked to β-galactosidase in yeast. Incubation of yeast strain ER (wt) with estradiol showed a dose-dependent increase in β-galactosidase activity with 20 nM estradiol producing a maximal response (Fig. 1). The yeast strain ER179C was maximally activated between 5 and 20 nM estradiol but had approximately 10-fold less activity than ER(wt) probably due to the removal of activation function-1 located in the amino-terminus (6). Coincubation of yeast strains ER (wt) or ER179C with 2075 nM atrazine, atrazine desisopropyl, cyanazine or simazine did not inhibit the maximal β-galactosidase activity produced by 20 nM estradiol in either strain (Fig. 2A and B). The concentration of the triazines tested in the yeast strains were limited by the concentration of the compounds that was commercially available and the adverse effects of solvent concentration. To determine whether a concentration of a triazine greater than the compounds tested had inhibitory activity, technical grade cyanazine provided by DuPont was used. At 10 μM, technical grade cyanazine did not decrease the maximal estradiol-dependent reporter activity in either strain (Fig. 2A and B).

The cotreatment of yeast strain ER(wt) with estradiol and the various triazines was repeated using a concentration of 0.5 nM estradiol which induced 12% of the maximum β-galactosidase activity. At a concentration of 0.5 nM estradiol in strain ER(wt), all triazines tested showed a dose-dependent inhibition of β-galactosidase activity (Fig. 2C). Atrazine was the most effective triazine at decreasing estradiol-dependent reporter activity. Atrazine, at a concentration of 2075 nM, decreased the activity of 0.5 nM estradiol in strain ER(wt) to 55% of control. The next most effective inhibitor was cyanazine, followed by simazine and atrazine-desisopropyl. Techinal grade cyanazine was able to inhibit the estradiol-dependent β-galactosidase activity to 35% of control at a concentration of 10 μM.

Coincubation of strain ER179C with 5 nM estradiol, which had the same activity as 0.5 nM in strain ER(wt), and the various triazines had no effect (data not shown). Furthermore, the triazines did not reduce the activity of 0.5 nM estradiol in strain ER179C (Fig. 2D). This suggests that the amino terminus of the ER may mediate the inhibitory effect of these compounds.

To determine if the triazines directly interacted with the hER in yeast, competition binding assays using [^{3}H]estradiol-17β in the presence or absence of radioinert triazines were performed. The binding of [^{3}H]estradiol-17β was reduced to 11% of control in the presence of radioinert estradiol (Fig. 3). All the triazines tested in the yeast strains showed a dose-dependent inhibition of [^{3}H]estradiol-17β binding. Technical grade cyanazine, at a concentration of 100 μM, reduced [^{3}H]estradiol-17β binding to 48% of control. The binding assays suggests that the inhibitory effect of the triazines is mediated by the interaction of these compounds with the hER.

The concentrations of the triazines required to displace [^{3}H]estradiol-17β from the hER in the binding assay were greater than those necessary for the inhibition of estradiol-dependent β-galactosidase activity. This may be the result of variation between the two different assays. We have identified other compounds which demonstrated greater activity in yeast than in the hER binding assay (10). Alternatively, the results from the binding assay may indicate that the triazines inhibit multiple targets (e.g., protein kinases) in yeast which could affect hER activity. Nonetheless, the binding of the triazines to the hER was greater than the binding of the triazines to the rat ER described in a previous report (3). This may be due to a different preparation of the ER for the binding assays or that the hER is more sensitive to the effects of the triazines than the rat ER. The use of a tritium-labeled triazine compound, which is not currently commercially available, will be necessary to directly determine if these compounds interact with the hER and rodent ER.

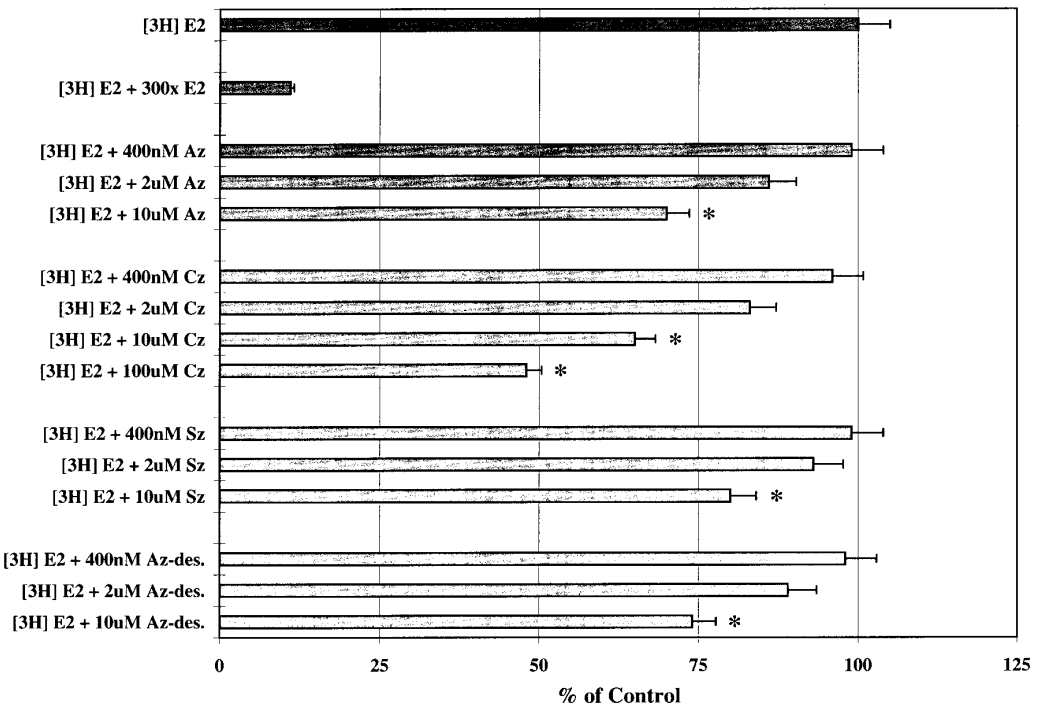


FIG. 3. Competition binding assay with recombinant hER and the various triazines. Recombinant hER was incubated with 2 nM [^{3}H]estradiol-17β in the presence or absence of radioinert estradiol (600 nM) or increasing concentrations of the various triazines. The 100 μM cyanazine was obtained from DuPont. The bound [^{3}H]estradiol-17β was measured by liquid scintillation counting and expressed as percentage of [^{3}H]estradiol-17β in the absence of competitor (5100 dpms).

In conclusion, the concentration of estradiol regulates the ability of the triazines to inhibit ER-mediated transcriptional activity in yeast. The amino terminus of the hER is required for the inhibitory effect of the triazines in yeast. The inhibitory activity of the triazines appears to occur through the interaction of these compounds with hER. These observations suggest that the ER has developed a regulatory mechanism for controlling the availability of binding sites for some anti-estrogens based on the concentration of estradiol.

ACKNOWLEDGMENTS

We thank Jon Cook, John O'Connor, and John Obourn of Haskell Laboratory for providing cyanazine and comments on the manuscript. We also thank Diane Klotz for reading the manuscript.

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