The development and maintenance of reproductive tissues is, to a large extent, controlled by steroid hormones. The effects of hormones are mediated through binding intracellular receptors and the interaction of hormone-receptor complexes with DNA. Recently, it has become apparent that hormonal responses can be generated in cell culture and in animals by environmental chemicals functioning as hormones or antihormones (1). In 1968 Bitman et al. (2) demonstrated that the pesticide o,p'-DDT produced characteristic estrogen responses in the reproductive tracts of rats and birds. Subsequent studies have reported that DDT can induce feminization of male sea gull embryos (3). A more recent study correlated a decrease in the population of alligators in Lake Apopka, Florida with a DDT and dioxin spill in the lake (4). Investigations into the mechanism(s) responsible for the effects of DDT have shown that its effects appear to be primarily mediated by its interaction with the estrogen receptor (5). Thus, environmental chemicals such as DDT, which interact with the estrogen receptor and display estrogenic activity have been classified as environmental estrogens. Numerous other environmental estrogens have been identified, e.g., bisphenol A (6), a byproduct of autoclaving polycarbonate; the phthalates di-n-butylphthalate and bis(2-ethylhexyl)phthalate (7), also in plastic; and the detergents octylphenol and nonylphenol (8,9). Several polychlorinated biphenyls (PCBs), industrialized chemicals associated with adhesives, fire retardants, and waxes, have also been shown to be estrogenic, inducing the development of ovaries in turtles that would have otherwise hatched as males (10).

The identification of a large number of environmental estrogens and their effects on various wildlife species has focused attention on the association of environmental estrogens with human health. The concentration of the DDT metabolite p,p'-DDE in the sera of women has been associated with an increased risk for breast cancer (11,12). A separate study reported that the sera concentration of p,p'-DDE was a risk factor for breast cancer in Caucasian and African American women but not in Asian women (13). Other studies, however, have reported no correlation between levels of environmental chemicals and incidence of breast cancer (14). In addition to the potential impact on women's health, environmental estrogens have been suggested to account for decreased semen quality and increased testicular cancer in men (15). Nonetheless, the findings correlating environmental estrogens with adverse human health are still the focus of scientific debate and investigation.

The well-documented effects of environmental estrogens in animals and their potential for adverse effects in humans have led to the development of assays for identifying chemicals with estrogenic activity. In 1993, McLachlan (16) proposed a screening approach to determine the functional characteristics of environmental chemicals. Soto et al. (17) have utilized the E-SCREENE assay, which measures the proliferation of estrogen responsive MCF-7 cells as a marker of the estrogenicity of chemicals (17). The drawback to the manner in which the E-SCREEN was used is that chemicals identified as estrogenic were not tested for interaction with the estrogen receptor by determining the proliferation of MCF-7 cells in the presence of the estrogen receptor antagonists tamoxifen or ICI 164,384. It has been suggested that the hormone activity of environmental chemicals can be measured by determining their interaction with hormone receptors and the production of functional responses in reporter gene assays (16). We have developed YES by expressing human estrogen receptor (hER) and two estrogen response elements (EREs) linked to the lacZ gene in yeast (18). Yeast do not contain steroid or nuclear receptors, but they do possess proteins homologous to mammalian cells necessary for activated transcription, allowing for the identification of chemicals that induce hER transcriptional activity. To further examine the activities of environmental chemicals in human cells, an estrogen-responsive reporter assay in MCF-7 cells transiently transfected with an ERE-luciferase plasmid was developed.

In this report we demonstrate that o,p'-DDD and p,p'-DDD, which have been previously identified as estrogenic in animal studies, display estrogenic activity. Furthermore, using this combination of assays, alachlor and cis-nonachlor and trans-nonachlor were identified as having weak estrogenic activity.

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AccuStandard phenyl)ethane purchased was from Amersham Corporation (Arlington Heights, IL) and o,p'-DDT [1-(1,1-trichloro-2-(p-chlorophenyl)-2-(o-chlorophenyl)ethane] was purchased from Sigma Chemical Co. (St. Louis, MO), o,p'-DDD [1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane] and p,p'-DDD [2,2-bis(4-chlorophenyl)-1,1-dichloroethane] were purchased from Aldrich Chemical Co. (Milwaukee, WI). Alachlor [2-chloro-N-(2,6-diethylphenyl)-N-(mehoxy-methyl)acetamide], benomyl [1,1-(butyl-lamino)carbonyl]-1H-benzimidazol-2-yl]carbamic acid methyl ester, and cis- and trans-nonachlor were purchased from AccuStandard (New Haven, CT).

The yeast strain BJ2407 was transformed with the p5C2W231-hER expression plasmid and the YRpE2 reporter plasmid that contains 2 EREs linked to the lacZ gene to create yeast strain hER-ERE as previously described (18). A single yeast colony was grown in SD-ura, trp medium overnight at 30°C. The next day, 50 μl of the overnight culture was diluted into 950 μl fresh medium and grown overnight in the presence of the test chemicals. All chemicals were prepared in DMSO and added to the medium so the concentration of DMSO did not exceed 2%. For the β-galactosidase assay, yeast cells were collected by centrifugation and resuspended in 700 μl Z-buffer (60 mM Na2HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, and 35 mM β-mercaptoethanol). The cells were permeabilized by the addition of 6 μl CHCl₃ and 4 μl 0.1% SDS followed by vortexing for 25 sec. The reactions were equilibrated at 30°C for 10 min, then 160 μl o-nitrophenyl-β-D-galactopyranoside (ONPG; 4 mg/ml Z-buffer) was added and the reactions returned to 30°C. The reactions were terminated by the addition of 400 μl 1M NaCO₃, the cell debris removed by centrifugation at 15,000 × g for 5 min, and the A₄₂₀ of the samples measured. Miller units were determined using the following formula: [A₄₂₀(A₅₀₀ of 1/10 dilution of cells × volume of culture × length of incubation)] × 1000. The Miller units produced by the chemicals divided by the Miller units produced by vehicle was used to calculate fold-induction. The data are representative of two independent experiments of three replicates.

Recombinant hER was produced in Sf9 insect cells by using the baculovirus expression system and prepared as ammonium sulfate precipitates. For competition binding assays, recombinant hER at a concentration of approximately 0.4 nM was incubated in the binding buffer [10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA (ethyleneglycol-bis(β-aminopropyl ether)N,N',N″,N‴-tetraacetic acid), 1 mM NaVO₄, 10% glycerol, 10 mg/ml γ-globulin, 0.5 mM PMSE (phenylmethyl sulfonyl fluoride), and 0.2 mM leupeptin] for 1 hr at 25°C with 2.5 nM [3H]17β-estradiol in the presence or absence of radioligand environmental chemicals or 17β-estradiol. The specific binding of [3H]17β-estradiol was assessed by adding a 300-fold molar excess of radioligand 17β-estradiol. For Scatchard analysis, recombinant hER was dissolved in the binding buffer with 0.5, 1, 2.5, 5, or 10 nM [3H]17β-estradiol and a 300-fold molar excess of 17β-estradiol, 10 mM p,p'-DDD, or 50 μM alachlor. [3H]17β-estradiol and test chemicals were dissolved in DMSO or ethanol and added to the reaction so the concentration of solvent did not exceed 2.5%. Free [3H]17β-estradiol was removed by incubation with charcoal (5% activated charcoal/0.5% dextran) for 10 min at 4°C and centrifugation for 3 min at 15,000 × g. Bound [3H]17β-estradiol was measured by scintillation counting. The data shown are representative of two independent experiments with three replicates.

MCF-7 cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS (fetal bovine serum; Gibco-BRL, Gaithersburg, MD), BME amino acids, MEM nonessential amino acids, L-glutamine, sodium pyruvate, penicillin-streptomycin (BME and MEM amino acids, L-glutamine, sodium pyruvate, and penicillin-streptomycin were diluted in the medium to a 1X concentration from either 100X or 50X stocks), and porcine insulin (10⁻⁸ M) (Sigma). Stocks were maintained in 75-cm² culture flasks in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

MCF-7 cells were transfected to phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) for 48 hr prior to plating. Cells were plated at a density of 5 x 10⁵ cells/35-mm plate and maintained at 37°C for an additional 24 hr in phenol red-free DMEM with 5% DCC-FBS. Cells were transfected in serum-free, phenol red-free DMEM using 12 μg of Lipofectamine (Gibco BRL) with 2 μg of vector CRE2uc, containing two copies of the vitellogenin ERE linked to the luciferase gene, and 1 μg of pCMVβ-galactosidase plasmid for 5 hr. After transfection, the medium was replaced with phenol red-free DMEM with 5% DCC-FBS and vehicle, 17β-estradiol, or environmental chemicals for 18 hr. All chemicals were prepared in DMSO or ethanol and added to the medium so the final concentration of solvent did not exceed 1%. Cells were harvested by incubation in lysis buffer (Analytical Luminescence Laboratory, Ann Arbor, MI) for 15 min at 25°C and cell debris was removed by centrifugation for 3 min at 15,000 × g. Protein concentrations were measured using the BioRad protein assay (BioRad Laboratories, Hercules, CA). β-galactosidase activity was determined by the addition of 40 μg protein to 500 μl Z-buffer and 100 μl ONPG and incubated at 37°C. Reactions were terminated by the addition of 500 μl 1 M NaCO₃, and the A₄₅₄ for each sample was measured. The volume of sample measured in the luciferase assay was normalized for β-galactosidase activity and protein concentration. Luciferase activity was determined in a Monolight 2010 luminometer (Analytical Luminescence Laboratory) using manufacturer's reagents and instructions. Luciferase activity of samples treated with chemicals divided by the luciferase activity of those treated with vehicle was used to determine fold induction. The data shown are representative of at least two independent experiments with three replicates.

For the 96-well plate transfections, MCF-7 cells were plated at a density of 1.5 x 10³ cells/well in phenol red-free DMEM containing 5% DCC-FBS and allowed to attach overnight. The next day, cells were transfected in serum-free, phenol red-free DMEM using 75 μg of Lipofectamine with 12.5 μg pERE2uc/96-well plate for 5 hr. After transfection, the medium was aspirated from the wells and replaced with phenol red-free DMEM containing 5% DCC-FBS and vehicle, 17β-estradiol, o,p'-DDT, or p,p'-DDD for 18 hr. For luciferase assays, the medium was removed and the cells were incubated in lysis buffer for 15 min at 25°C. Luciferase activity was determined in a Monolight 9600 luminometer (Analytical Luminescence Laboratory) using manufacturer's reagents and instructions. Luciferase activity of samples treated with chemicals divided by the luciferase activity of those treated with vehicle was used to determine fold induction. The data shown are representative of at least two independent experiments with three replicates.

### Results

**Identification of Environmental Chemicals with Estrogenic Activity in the Yeast Estrogen Screen**

We have created the YES by expressing hER and two EREs linked to the lacZ gene in the yeast strain hER-ERE as previously described by Arnold et al. (18). Yeast strain hER-ERE was grown overnight in the pres-
ence or absence of 10 nM 17β-estradiol or increasing concentrations of various environmental chemicals. Ten nanomolar estradiol produced a 100-fold induction of β-galactosidase activity (Table 1). At 10 μM, α,p'-DDT increased β-galactosidase activity by 95-fold, to a similar extent as 10 nM 17β-estradiol. p,p’-DDD at 10 μM induced β-galactosidase activity to a level similar to that of α,p'-DDT. At 10 μM, alachlor, cis-nonachlor, and trans-nonachlor all induced β-galactosidase activity, but to a lesser extent than α,p'-DDT. Benomyl did not increase β-galactosidase activity in this assay.

**Inhibition of [3H]17β-estradiol Binding by Environmental Chemicals**

The chemicals tested in YES were measured for their ability to inhibit the binding of [3H]17β-estradiol to hER in competition binding assays. Recombinant hER was incubated with [3H]17β-estradiol in the presence or absence of varying concentrations of radioinert chemicals. Unbound [3H]17β-estradiol was removed by incubation with chardex, and bound [3H]17β-estradiol was measured by scintillation counting. Not surprisingly, 17β-estradiol was the most effective chemical tested at inhibiting the binding of [3H]17β-estradiol to hER with an IC50 (concentration of chemical necessary to inhibit the binding of [3H]17β-estradiol by 50%) of 1 nM (Fig. 1). α,p'-DDT was the most effective environmental chemical at reducing [3H]17β-estradiol binding with an IC50 of 1 μM or 1000-fold greater than the IC50 for estradiol. This result is consistent with previous studies which showed that α,p'-DDT has a 1000-fold lower binding affinity for ER in rat cytosol than estradiol. p,p’-DDD had an IC50 of 11 μM, which was approximately 10,000-fold greater than the IC50 for 17β-estradiol. The chemicals α,p’-DDD, alachlor, cis-nonachlor, and trans-nonachlor only weakly reduced the binding of [3H]17β-estradiol, and none of the chemicals reduced binding by 50% at the concentrations tested. The inability of the chemicals to significantly reduce the binding of [3H]17β-estradiol may have been due to the insolubility of these chemicals at concentrations greater than 50 μM (unpublished observations).

A Scatchard analysis was performed with [3H]17β-estradiol in the presence or absence of p,p’-DDD or alachlor to determine their mechanism of binding. Both p,p’-DDD and alachlor competitively inhibited [3H]17β-estradiol binding to hER as demonstrated by the nonparallel slopes in the Scatchard plots (Fig. 2).

**Activation of an ERE–Luciferase Reporter in MCF-7 cells by Environmental Chemicals**

To examine the ability of the environmental chemicals to facilitate hER-mediated transcriptional activation in mammalian cells, MCF-7 human breast cancer cells were transiently transfected with a plasmid containing two ERs linked to the luciferase gene. The cells were incubated in the presence or absence of increasing concentrations of environmental chemicals for 18 hr, and then cell extracts were assayed for luciferase activity. Consistent with the results of the competition binding assays, 17β-estradiol at 100 pM was the most effective chemical at inducing luciferase activity, with a 46-fold induction above control (Fig. 3). p,p’-DDD at 100 nM induced luciferase activity to a level similar to 100 pM estradiol. α,p’-DDD induced luciferase activity to the same extent as p,p’-DDD, but at a concentration of 1 μM (Fig. 3). This is inconsistent with the competition binding studies which showed that α,p’-DDD was more effective than p,p’-DDD at inhibiting [3H]17β-estradiol bind-

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**Table 1. Fold-induction of β-galactosidase by various environmental chemicals in the YES**

| Chemicals | Concentration (μM) | Fold-induction |
|-----------|-------------------|---------------|
| DMSO      | 0                 | 0             |
| Estradiol | 0.0001            | 50            |
|           | 0.0010            | 80            |
|           | 0.0100            | 100           |
| α,p’-DDT  | 0.100             | 40            |
|           | 1.0               | 85            |
|           | 10.0              | 95            |
| α,p’-DDD  | 0.100             | 0             |
|           | 1.0               | 10            |
|           | 10.0              | 35            |
| p,p’-DDD  | 0.100             | 25            |
|           | 1.0               | 65            |
|           | 10.0              | 85            |
| Alachlor  | 0.100             | 0             |
|           | 1.0               | 10            |
|           | 10.0              | 40            |
| Benomyl   | 0.100             | 0             |
|           | 1.0               | 0             |
|           | 10.0              | 0             |
| cis-Nonachlor | 0.100        | 0             |
|           | 1.0               | 0             |
|           | 10.0              | 0             |
| trans-Nonachlor | 0.100        | 0             |
|           | 1.0               | 0             |
|           | 10.0              | 12            |

Fold induction represents the β-galactosidase activity induced by the chemical compared to the β-galactosidase activity in the presence of DMSO. Each value represents the mean ± SE of two independent experiments with three replicates. The SE was less than 10% for the experiments performed.

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**Figure 1.** Inhibition of specific [3H]17β-estradiol binding to human estrogen receptor (hER) by environmental chemicals. Recombinant hER was incubated with 2.5 nM [3H]17β-estradiol in the presence or absence of increasing concentrations of radioinert 17β-estradiol, α,p’-DDT, α,p’-DDD, p,p’-DDD, benomyl, alachlor, cis-nonachlor, or trans-nonachlor. Data are representative of at least three independent experiments, and each data point is presented as mean ± SE.

**Figure 2.** Scatchard analysis of human estrogen receptor (hER) with [3H]17β-estradiol in the presence or absence of p,p’-DDD and alachlor. Recombinant hER was incubated with increasing concentrations of [3H]17β-estradiol and 10 μM p,p’-DDD (A) or 50 μM alachlor (B). The data were analyzed by the method of Scatchard (28) and are representative of at least two independent experiments.
ing to hER. In this assay, o,p'-DDT and p,p'-DDD appear to be full agonists of hER-mediated transactivation because they increased luciferase activity to the same extent as estradiol, albeit at higher concentrations.

None of the other environmental chemicals, at the concentrations tested, induced luciferase activity to the same extent as estradiol (Fig. 3). At 1 μM alachlor, luciferase activity was increased 23-fold or approximately 50% of the maximal activity induced by estradiol. 10 μM alachlor did not further increase luciferase activity, indicating that alachlor is only a partial agonist of hER. o,p'-DDD increased luciferase activity 15-fold above control at a concentration of 1 μM. Higher concentrations of o,p'-DDD were not tested for induction of luciferase activity due to their toxic effects on MCF-7 cells. cis-Nonachlor and trans-nonachlor displayed minimal estrogenic activity in this assay. At the maximum concentration tested, trans-nonachlor (4.5 μM) induced luciferase activity 14-fold above control levels and cis-nonachlor (20 μM) induced luciferase activity seven-fold above control levels. Benomyl was not able to induce luciferase activity even at 20 μM (Fig. 3).

To demonstrate that the chemicals interacted with the hER in MCF-7 cells, MCF-7 cells were incubated in the presence of environmental chemicals alone or environmental chemicals and a 100-fold molar excess of 4-OH-tamoxifen, an ER antagonist. The luciferase activity induced by all of the chemicals tested was eliminated in the presence of 4-OH-tamoxifen (Fig. 4), demonstrating that the chemicals interact with the hER in MCF-7 cells.

Next, we examined the feasibility of testing environmental chemicals for estrogenic activity using MCF-7 cells in a 96-well plate. MCF-7 cells in 96-well plates were transfected with the ERE–luciferase reporter plasmid, treated with various concentrations of 17β-estradiol, o,p'-DDT, p,p'-DDD, or vehicle for 18 hr, and then assayed for luciferase activity. Luciferase activity was induced by environmental chemicals in MCF-7 cells plated in 96-well plates (Fig. 5). The induction of luciferase activity by the chemicals was reduced compared to the induction seen in the 35-mm wells, as shown in Figure 3. The maximum induction by the chemicals was 46-fold in 35-mm wells, whereas the maximum induction in the 96-well plates was fourfold.

Discussion

We have used a combination of three assays to study the estrogenic activity of several environmental chemicals. This
herein show that \( p,p' \)-DDD activated estrogen-dependent \( \beta \)-galactosidase activity in yeast, inhibited the binding of \( \text{[H]} \)-estradiol to hER, and activated an estrogen-responsive reporter gene in MCF-7 cells. In fact, \( p,p' \)-DDD appears to be a full agonist of hER because it was able to activate an estrogen-responsive gene in MCF-7 cells to the same level as \( 17\beta \)-estradiol.

The reasons for differences between our studies and previous reports in whole animals may be several-fold. \( p,p' \)-DDD may still function as an estrogen in the rat; however, due to its low affinity for ER, the time course of response may be delayed compared to estradiol. Studies have shown that, in the rat, chlordecone (Kepone), also a chlorinated insecticide, elicits the same degree of estrogenic response as estradiol when measured by redistribution of ER to the nucleus, uterine weight gain, and synthesis of progesterone receptor (24). The uterine responses to chlordecone occurred over a matter of days, whereas they occurred within several hours in response to estradiol. Species differences may also play a role in the differential ability of environmental chemicals to act as estrogens in separate systems. In different animals or tissues, the dose of environmental chemical necessary to elicit a response may vary, as may the time of treatment necessary to detect the response. The multitoxin approach presented here, however, provides investigators with insight into the mechanism of action of these environmental compounds with respect to whether ER is mediating the estrogenic response of the chemical.

Whether the concentrations of environmental chemicals necessary to induce estrogenic activity are too high to be physiologically relevant is an important consideration. In competition binding studies, the IC\(_{50}\) values for \( o,p' \)-DDD and \( p,p' \)-DDD were 1 \( \mu \)M and 11 \( \mu \)M, respectively. Luciferase assays, however, demonstrated that nanomolar concentrations of some environmental chemicals such as \( o,p' \)-DDD (100 nM–1 \( \mu \)M) and \( p,p' \)-DDD (100 nM), are sufficient for full agonistic activity. These differences indicate that competition binding assays do not necessarily reflect the effective concentration of a chemical and that luciferase assays may be more sensitive at determining the concentration at which an environmental chemical exerts estrogenic activity. Competition binding assays are also limited in that they can not distinguish between an ER agonist and an ER antagonist, whereas functional assays, such as the luciferase assays, are able to do so.

Discrepancies between competition binding data and luciferase data for \( o,p' \)-DDT and \( p,p' \)-DDD may be based on the fact that, in luciferase assays, the environmental chemicals bind to unliganded ER as opposed to competition binding assays in which the environmental chemicals must compete with \( 17\beta \)-estradiol for binding to ER. In competition binding assays, the structure of \( o,p' \)-DDD, compared to that of \( p,p' \)-DDD, may make it better able to compete with \( 17\beta \)-estradiol for binding to hER. In contrast, \( p,p' \)-DDD may, upon binding to hER, create a more potent transcriptionally active complex than does the binding of \( o,p' \)-DDD to hER, allowing greater transcription of the luciferase reporter gene.

In terms of comparable levels of environmental estrogens that may occur in the environment, one investigation has determined the concentrations of some environmental chemicals, including \( \text{trans} \)-nonachlor, to be as high as 200 nM in alligator eggs from Lake Apopka, Florida (25), demonstrating that it is possible for local concentrations of environmental chemicals to reach the concentrations that could elicit a biological response.

Finally, we were interested in determining whether the ability of environmental chemicals to induce the expression of the luciferase reporter gene could be determined in a microassay. By using the microassay, a large number of chemicals can be evaluated. However, the decrease induction of luciferase activity observed using the 96-well plate procedure compared to the induction observed with the 35-mm well transfection protocol limits this assay to identifying only a positive or negative response with respect to the ability of an environmental chemical to induce estrogen-responsive reporter gene activity. At the present time, in order to determine whether a chemical acts as a partial or full agonist, a more sensitive assay such as the 35-mm well transfection procedure will be necessary.

Proliferation assays were not included in these studies. The ability to bind to hER and regulate the transcription of estrogen-regulated genes may have effects in addition to the proliferation of the breast cancer cell. For example, the induction of estrogen-regulated gene transcription may result in the production of growth factors or other proteins that act to regulate the growth of cells surrounding the breast cancer cell. These surrounding cells may ultimately produce other factors that affect the breast cancer cell. Recent studies have also shown that different MCF-7 stocks respond uniquely to both estradiol and environmental estrogens in proliferation assays (26,27), possibly adding to discrep-
The in vitro assays used here were not selected to definitively determine whether an environmental chemical is an estrogen (as the definition of a true estrogen is still the focus of much debate) or to replace the testing of environmental chemicals for estrogenic activity in animals. Rather, this approach was designed as a multifaceted procedure to examine several different aspects of estrogenic activity, namely, the ability to bind to ER and the capacity to activate estrogen-responsive genes through ER. The ability of a chemical to elicit any of these responses may indicate it will be estrogenic in vivo.

This three-tiered approach will aid in the characterization of large numbers of chemicals for estrogenic activity and, more importantly, provide insight into the mechanisms involved in the mediation of the estrogenic activity of environmental chemicals. We suggest, therefore, that these in vitro assays are an important addition to other biological assays, including whole animal studies, which examine the estrogenicity of environmental chemicals, as well as an effective method for beginning to dissect the molecular mechanisms involved in the estrogenic responses elicited by many environmental chemicals.

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