Certain Styrene Oligomers Have Proliferative Activity on MCF-7 Human Breast Tumor Cells and Binding Affinity for Human Estrogen Receptor α

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To examine the estrogenic activities of styrene oligomers, we carried out cell proliferation assays with estrogen-sensitive MCF-7 cells and competitive binding assays to human estrogen receptor α (hERα). The styrene oligomers tested were 1,3-diphenyl propane (SD-1), 2,4-diphenyl-1-butene (SD-2), *cis*-1,2-diphenyl cyclobutane (SD-3), *trans*-1,2-diphenyl cyclobutane (SD-4), 2,4,6-triphenyl-1-hexene (ST-1), 1a-phenyl-4a-(1´-phenylethyl)tetralin (ST-2), 1a-phenyl-4e-(1´-phenylethyl)tetralin (ST-3), 1e-phenyl-4a-(1´-phenylethyl)tetralin (ST-4), 1e-phenyl-4e-(1´-phenylethyl)tetralin (ST-5), 1e,3e,5a-triphenylcyclohexane (ST-6), and 1e,3e,5e-triphenylcyclohexane (ST-7). In the MCF-7 cell proliferation assay, styrene trimers (ST-1, ST-3, ST-4, and ST-5) had the highest proliferative activities of the compounds tested. The relative potency of these chemicals was 0.0002–0.0015%, which was comparable with that of bisphenol A (0.0001–0.0025%), and their relative proliferative effect was 51–104%. Styrene dimers (SD-3 and SD-4) also significantly increased the cell yields. However, SD-1, SD-2, ST-2, ST-6, and ST-7 had insignificant proliferative activities. The competitive binding assay revealed the binding affinity of some styrene oligomers for hERα. The order of their binding potency for hERα was as follows: ST-4 > ST-2 > ST-3 > ST-5 > ST-1 > SD-3 > SD-4 > SD-2 > SD-1. ST-6 and ST-7 did not appear to bind to hERα. The present studies indicate that styrene dimers SD-3 and SD-4 and styrene trimers ST-1, ST-3, ST-4, and ST-5 have estrogenic activity on MCF-7 cells and binding affinity for hERα. These compounds might be endocrine disrupters. *Key words*: binding affinities, cell proliferative activities, estrogenic activities, human estrogen receptor α, MCF-7 cells, styrene oligomers.

Environ Health Perspect 109:699–703 (2001). [Online 29 June 2001]
http://ehpnet1.niehs.nih.gov/docs/2001/109p699-703ohyama/abstract.html

Polystyrene used for food containers, such as takeout food containers, coffee cups, meat trays, soup bowls, and salad boxes (*1*), contains high levels of styrene dimers (90–1,030 μg/g) and styrene trimers (650–20,770 μg/g) as impurities (*2,3*). These styrene oligomers migrate from the polystyrene containers into the containers' contents (*4–6*). When polystyrene containers containing vegetable oil were treated by heating in a microwave oven or were incubated for 24 hr at 20°C, a styrene dimer, 2,4-diphenyl-1-butene (SD-2; 0–1.6 ng/cm^2) and styrene trimers 2,4,6-triphenyl-1-hexene (ST-1; 1.3–69.7 ng/cm^2), 1a-phenyl-4a-(1´-phenylethyl)tetralin (ST-2; 9.2–156 ng/cm^2), 1a-phenyl-4e-(1´-phenylethyl)tetralin (ST-3; 18.1–501 ng/cm^2), 1e-phenyl-4a-(1´-phenylethyl)tetralin (ST-4; 13.9–294 ng/cm^2), and 1e-phenyl-4e-(1´-phenylethyl)tetralin (ST-5; 18.7–306 ng/cm^2) migrated into the vegetable oil (*4*). When instant foods such as Chinese noodles, Japanese noodles, buckwheat noodles, chow mein, spaghetti, and rice were packed in polystyrene containers, styrene trimers ST-1 (0–8.1 μg/cup), ST-2 + ST-3 (0–13.8 μg/cup), ST-4 (0–5.2 μg/cup), and ST-5 (0-8.4 μg/cup) migrated (0–33.8 μg total styrene oligomers detected in a cup) from the containers into the foods after cooking in the cups, but the dimers did not (*5*). The maximum quantity of styrene trimers that migrated from containers to foods was higher than that of bisphenol A leached from the lacquer coating of vegetable cans (4–23 μg/can) (*7*).

Colborn et al. (*8*) designated styrene dimers and trimers as endocrine disrupters in the Wingspread statement, and the Environmental Agency, Government of Japan, cited styrene dimers and trimers as compounds suspected of having endocrine-disruptive effects in its Strategic Programs on Environmental Endocrine Disrupters (*9*). However, styrene oligomers were reported to have no endocrine disruptive effect both in a MCF-7 cell proliferation assay (*10*) and in a radioisotope (RI) receptor competitive-binding assay using rat estrogen receptor (*10,11*). Therefore, we tested 11 styrene oligomers including those found in food (*4,5*) in a proliferation assay at an optimal initial cell concentration using human breast tumor, highly estrogen-sensitive MCF-7 cells. We also examined the binding potency of these styrene oligomers to human estrogen receptor α (hERα) in a non-RI receptor competitive-binding assay.

Materials and Methods

Chemicals. The styrene dimers 1,3-diphenyl propane (SD-1), SD-2, *cis*-1,2-diphenyl cyclobutane (SD-3), and *trans*-1,2-diphenyl cyclobutane (SD-4) and styrene trimers ST-1, ST-2, ST-3, ST-4, ST-5, 1e,3e,5a-triphenylcyclohexane (ST-6), and 1e,3e,5e-triphenylcyclohexane (ST-7) were purchased from Hayashi Pure Chemical Industry. (Osaka, Japan). The positive control, 17β-estradiol (E_2), was obtained from Calbiochem (Richmond, CA, USA). The chemical structures of these compounds are shown in Figure 1, and the purity of the compounds is summarized in Table 1.

Solvent for styrene oligomers. Styrene oligomers and E_2 were dissolved in ethanol for the MCF-7 cell proliferative assay. Styrene oligomers were dissolved in ethanol at the concentration of 10^{-2} M, except for ST-2 and ST-6, which were dissolved at 10^{-3} M due to the lower solubility of these compounds. The cell proliferation assay was performed at $\leq 10^{-5}$ M styrene oligomers.

For a competitive binding assay, styrene oligomers and E_2 were dissolved in dimethyl sulfoxide (DMSO). The assay was performed at $\leq 5 \times 10^{-3}$ M styrene oligomers. Glass Pasteur capillary pipettes were used in handling the chemical solutions.

Culture medium. Dulbecco's modification of Eagle's Medium (DME) containing phenol red and fetal bovine serum (FBS) were purchased from Nissui (Tokyo, Japan) and Hyclone (Logan, UT, USA), respectively. Phenol red-free DME (Cat. no. 23800-022) was obtained from Gibco BRL (Grand Island, NY, USA)

Removal of sex steroids by charcoal-dextran treatment of serum. We removed sex steroids from FBS by charcoal-dextran stripping (CDFBS) (*12*). Charcoal and dextran T70 were purchased from Sigma (St. Louis, MO, USA) and Amersham Pharmacia Biotech (Uppsala, Sweden), respectively.

Cell line and cell culture conditions. Estrogen-sensitive human breast tumor MCF-7 cells were provided by Ana M. Soto (Tufts University School of Medicine, Boston, MA, USA). For routine maintenance,

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We thank A. Hamaoka and T. Yamamoto for technical assistance.

Received 6 December 2000; accepted 5 February 2001.

cells were grown in 5% FBS medium (DME with phenol red supplemented with 80 mg/L kanamycin, 50 mg/L gentamycin, 4 mM L-glutamine, 2.24 g/L sodium hydrogen carbonate, and 5% FBS) in an atmosphere of 5% CO_2/95% air with saturating humidity at 37°C. Cells were subcultured every 2 weeks. The cells detached by 0.05% trypsin were plated at an initial concentration of 12,500 cells/mL. The 5% FBS medium in the cell cultures was replaced with fresh medium twice a week.

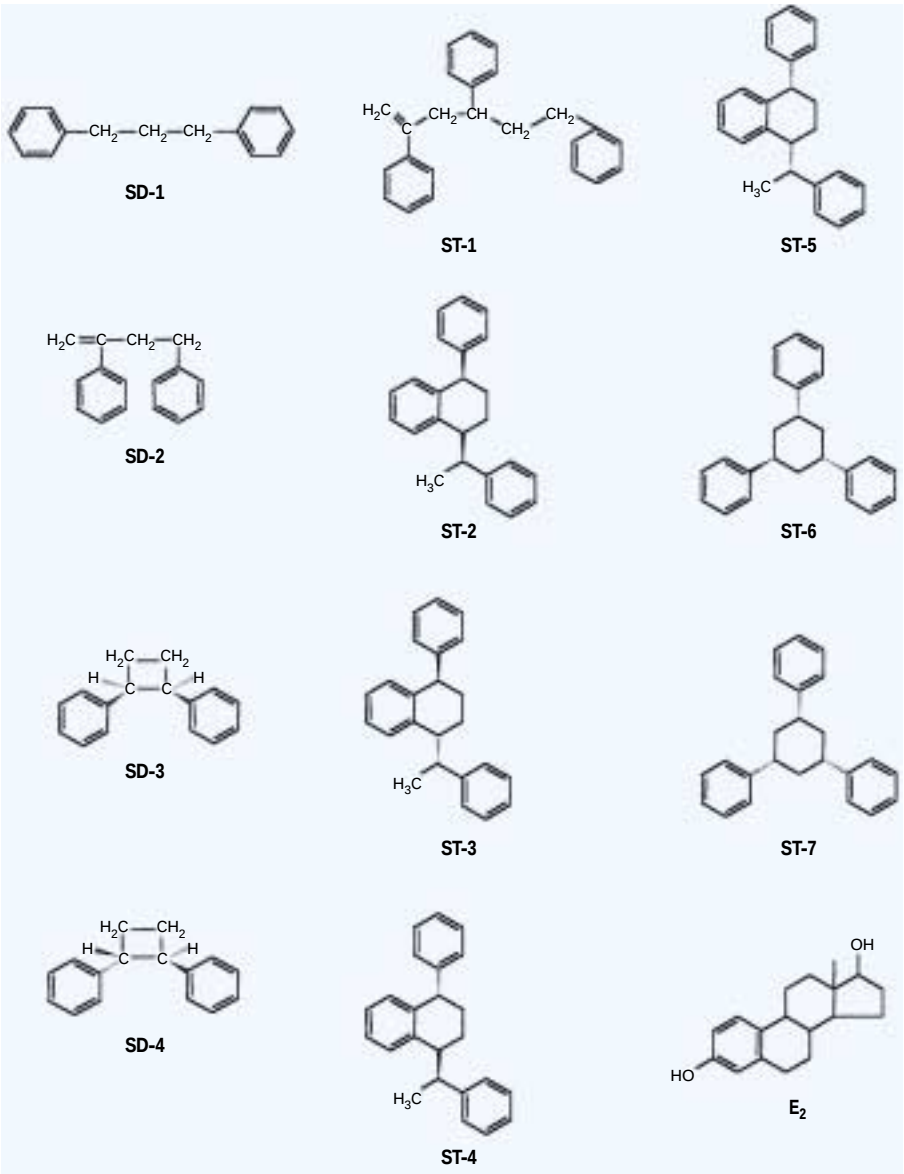

Figure 1. Chemical substances tested.

Table 1. Data on tested chemicals

Compound	Original concentration (mM) Ethanol solution[a]	DMSO solution[b]	Supplier (code no.)	Purity (%)[c]
E_2	0.001	0.1	Calbiochem (3301)	> 99.5
SD-1	10	100	Hayashi PC (990-52331)	97.9
SD-2	10	100	Hayashi PC (990-52334)	99.0
SD-3	10	100	Hayashi PC (990-52332)	99.9
SD-4	10	100	Hayashi PC (990-52333)	99.4
ST-1	10	10	Hayashi PC (990-52335)	98.2
ST-2	1	1	Hayashi PC (990-52336)	98.2
ST-3	10	10	Hayashi PC (990-52337)	99.9
ST-4	10	10	Hayashi PC (990-52338)	99.5
ST-5	10	10	Hayashi PC (990-52339)	99.2
ST-6	10	3.6	Hayashi PC (990-52387)	99.6
ST-7	1	10	Hayashi PC (990-52388)	99.5

[a]Ethanol solution was used for MCF-7 cell proliferation assay. [b] DMSO solution was used for competitive binding assay. [c]Purity of the chemicals as reported by the suppliers.

MCF-7 cell proliferation assay. Phenol red-free DME 8.3 g/L was supplemented with 1 g/L glucose, 110 mg/L sodium pyruvate, 80 mg/L kanamycin, 50 mg/L gentamycin, and 12 mM HEPES (plain DME). The 5% CDFBS medium for proliferation assay consisted of plain DME, 4 mM L-glutamine, 2.24 g/L sodium hydrogen carbonate, and 5% CDFBS. The E-SCREEN assay to evaluate MCF-7 cell proliferation was performed according to a technique modified from that originally described by Soto et al. (*13*). Briefly, MCF-7 cells cultured for 11 days were trypsinized and plated in 24-well plates (Falcon, Franklin Lakes, NJ, USA) at an initial concentration of 40,000 cells/mL of 5% FBS medium/well. After the cells were allowed to attach for 24 hr, 0.9 mL of 5% CDFBS medium was substituted for the seeding medium. The solution of chemicals in ethanol was diluted with plain DME to various concentrations, and 0.1 mL of that was added in wells. The ethanol concentration in culture medium did not exceed 0.1%. The cells were cultured for 6 days in an atmosphere of 5% CO_2/95% air with saturating humidity at 37°C. The medium was not changed at all over the course of the experiment. The assay was terminated by removing the medium from wells. We calculated the number of cells by measuring the amount of protein stained with sulforhodamine-B (SRB; Wako PC, Osaka, Japan) as described by Brotons et al. (*7*) and Villalobos et al. (*14*). In this assay, the cell yield in 10^{-10} M E_2 was 3.6-fold (SD = 0.825) higher than the solvent control. Differences between the values obtained in the presence of the test chemicals and those obtained in the solvent controls were assessed using the Newman-Keuls test. A p-value of < 0.01 was regarded as significant.

Competitive binding assay. The binding potency of test chemicals to hERα was measured by non-RI receptor binding assay using the Estrogen-R(α) Competitor Screening Kit (Wako PC) according to the manufacturer's instructions. Briefly, the test chemical dissolved in DMSO and other reagents including fluorescence-labeled E_2 were mixed and competitively bound to the hERα coated on the microplate wells (*15*). DMSO was not effective in this assay. The fluorescence intensity was measured at excitation (485 nm) and emission (535 nm) with a fluorescence microplate reader apparatus, Spectra Fluo (Tecan, Austria). We calculated the binding levels of the chemicals to hERα from the decrease of fluorescence intensity.

Results

MCF-7 cell proliferation assay. We compared the increase of cell yield obtained at different concentrations of test chemicals with that obtained in 10^{-10} M E_2 (Figure 2). The increase of cell yield with 10^{-10} M E_2 (= the cell yield in 10^{-10} M E_2 – the cell yield in the solvent control) was expressed as 100%. Data were expressed as the means ± SDs of three independent assays performed in triplicate. This cell proliferation assay was performed at ≤ 10^{-5} M styrene oligomers because of low solubility in culture media. EC_{50} is the concentration of test compound that produces 50% of the cell yield by 10^{-10} M E_2. The values of relative potency (RP), defined as the ratio of the EC_{50} of E_2 to that of the test compound, and the values of relative proliferative effect (RPE), defined as the ratio of the highest cell yield obtained with the test compound to that with 10^{-10} M E_2, are shown in Table 2. Results are summarized below:

- SD-1 and SD-2: No effect was observed at 10^{-8}, 10^{-7}, and 10^{-6} M; however, a slight increase of cell yield was found in 10^{-5} M.
- SD-3: Significant cell proliferation ($p < 0.01$) was induced by this compound at ≥ 10^{-6} M, and the highest cell yields were obtained at 10^{-5} M. RPE was 31%.
- SD-4: A slight increase in cell yield appeared at 10^{-6} M, and significant cell proliferation ($p < 0.01$) was induced by this chemical at 10^{-5} M; RPE was 29%.
- ST-1: Significant cell proliferation ($p < 0.01$) was induced at ≥ 10^{-6} M. The highest cell yields were obtained at 10^{-5} M; therefore, RP and RPE were 0.0015% and 81%, respectively.
- ST-2: The cell yield decreased at 10^{-8} and 10^{-7} M compared to that in the solvent control. A slight increase in cell yields was found at 10^{-6} M; at > 10^{-5} M, the effect on proliferation could not be examined due to the insolubility of this chemical.
- ST-3: Significant cell proliferation ($p < 0.01$) was induced by this chemical at ≥ 10^{-6} M, and the highest cell proliferation was observed at 10^{-5} M. RP and RPE were 0.0005% and 86%, respectively.
- ST-4: Significant cell proliferation ($p < 0.01$) was induced at 10^{-6} M, and the highest cell proliferation was observed at 10^{-5} M. RP and RPE were 0.0006% and 104%, respectively, the highest of the tested styrene oligomers.
- ST-5: An increase in cell yields was seen from 10^{-6} M, and significant cell proliferation ($p < 0.01$) was caused by this compound at 10^{-5} M. RP and RPE were 0.0002% and 51%, respectively.
- ST-6 and ST-7: These chemicals decreased cell yields.

Binding of styrene oligomers to hERα. The inhibition of the binding of fluorescence-

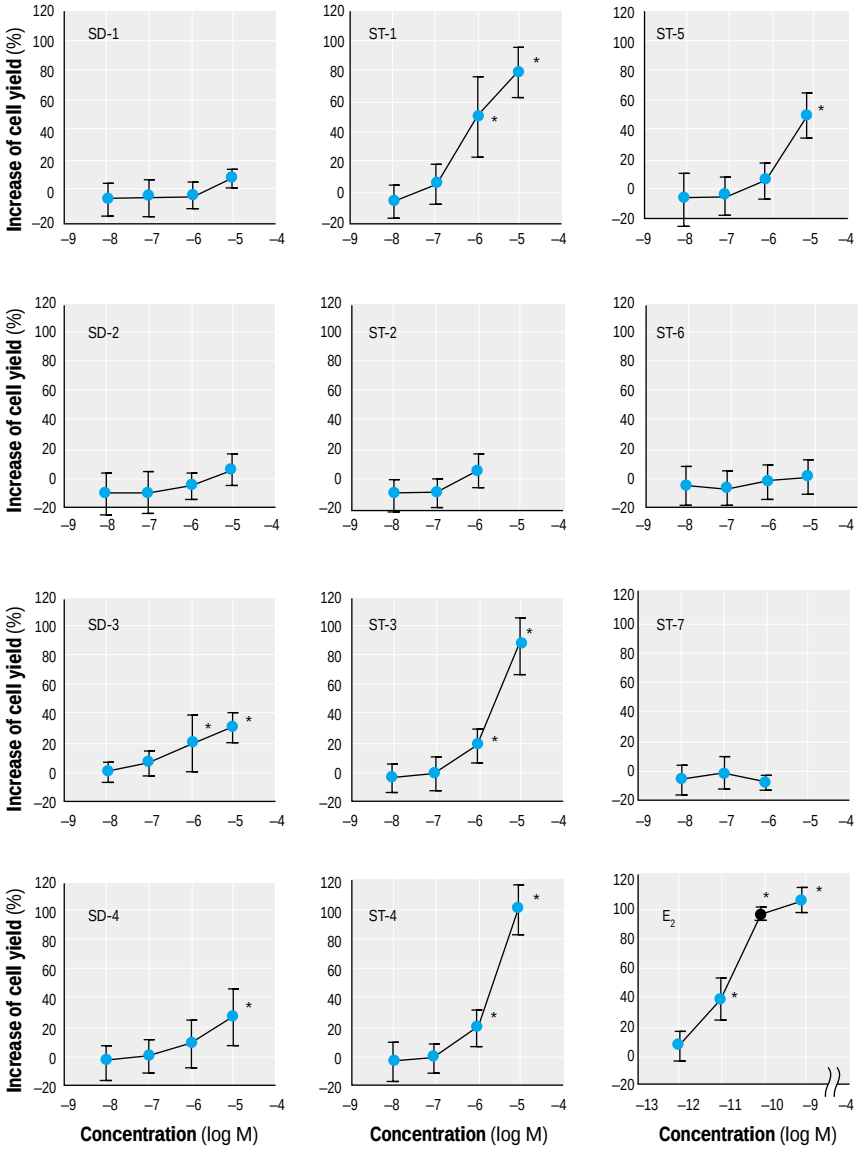

Figure 2. Proliferative effects of styrene oligomers on MCF-7 cells. Increase of cell yield was calculated as [(the cell yield in various concentrations of test chemicals) – (cell yield in the solvent control)] ÷ [(the cell yield in 10^{-10} M E_2) – (cell yield in the solvent control)] × 100. Each point is the mean ± SD of three independent assays in triplicate.
*Significantly different from hormone-free control ($p < 0.01$).

Table 2. Estrogenic effects of styrene oligomers.

Compound	MCF-7 cell proliferation assay			Competitive binding assay	
	EC_{50} (M)[a]	RP (%)[b]	RPE (%)[c]	IC_{20} (M)[d]	RBA (%)[e]
E_2	1.4×10^{-11}	100	100	6.0×10^{-11}	100
SD-1	NE	–	–	1.2×10^{-4}	0.005
SD-2	NE	–	–	7.6×10^{-5}	0.008
SD-3	R < 50	–	31	2.4×10^{-5}	0.025
SD-4	R < 50	–	29	5.6×10^{-5}	0.011
ST-1	9.5×10^{-7}	0.0015	81	1.2×10^{-5}	0.05
ST-2	NE	–	–	6.2×10^{-6}	0.097
ST-3	2.9×10^{-6}	0.0005	86	9.7×10^{-6}	0.062
ST-4	2.3×10^{-6}	0.0006	104	2.6×10^{-6}	0.228
ST-5	9.5×10^{-6}	0.0002	51	1.0×10^{-5}	0.058
ST-6	NE	–	–	NE	–
ST-7	NE	–	–	NE	–

[a]EC_{50}, the concentration of test compound producing 50% of the cell yield by 10^{-10} M E_2; NE, value could not be estimated from the response curve; $R < 50$, maximal response observed for the test chemical at the concentrations tested was below 50%. [b]RP = [(EC_{50} of E_2) ÷ (EC_{50} of the test compound)] × 100. [c]RPE = [(the highest cell yield obtained with the test compound) ÷ (the cell yield obtained with the solvent control –1)] ÷ [(the cell yield obtained with 10^{-10} M E_2) ÷ (the cell yield obtained with the solvent control) –1] × 100. [d]IC_{20} = the concentration of test chemicals for 20% inhibition of binding of fluorescence-labeled E_2 to ERα. [e]RBA = (IC_{20} of E_2) ÷ (IC_{20} of the test compound) × 100.

labeled E_2 to hERα by various concentrations of tested compounds is shown in Figure 3. The inhibition by styrene dimers (SD-1, SD-2, SD-3, and SD-4) was detected at ≥ 5 × 10^{-5} M, and was concentration dependent. The maximum inhibition was 51–76% by each compound at 5 × 10^{-4} M. The inhibition by styrene trimers (ST-1, ST-2, ST-3, ST-4, and ST-5) was detected at ≥ 5 × 10^{-6} M. This concentration (5 × 10^{-6} M) was lower by one order of magnitude than the concentrations of styrene dimers that caused comparable inhibition. However, complete inhibition could not be obtained. The maximum inhibition was 28–44% at 5 × 10^{-5} M. Inhibition by E_2 as a positive control was detected starting at the lower concentration of 5 × 10^{-9} M and was concentration dependent. E_2 at 5 × 10^{-7} M caused 86% inhibition. A slight inhibition by ST-6 was seen at 1.8 × 10^{-5} M. ST-7 could not cause inhibition at any concentration. Styrene trimers were insoluble at ≥ 5 × 10^{-4} M in the reaction solution containing fluorescence-labeled E_2. The concentration for 20% inhibition of the binding (IC_{20}) and the ratio of IC_{20} of E_2 to that of each styrene oligomer (relative binding affinity; RBA) are shown in Table 2. The RBAs of styrene dimers SD-1, SD-2, SD-3, and SD-4 were 0.005, 0.008, 0.025, and 0.011%, respectively, and those of styrene trimers ST-1, ST-2, ST-3, ST-4, and ST-5 were 0.05, 0.097, 0.062, 0.228, and 0.058%, respectively. The styrene trimers, except for ST-6 and ST-7, had relatively high affinity for hERα.

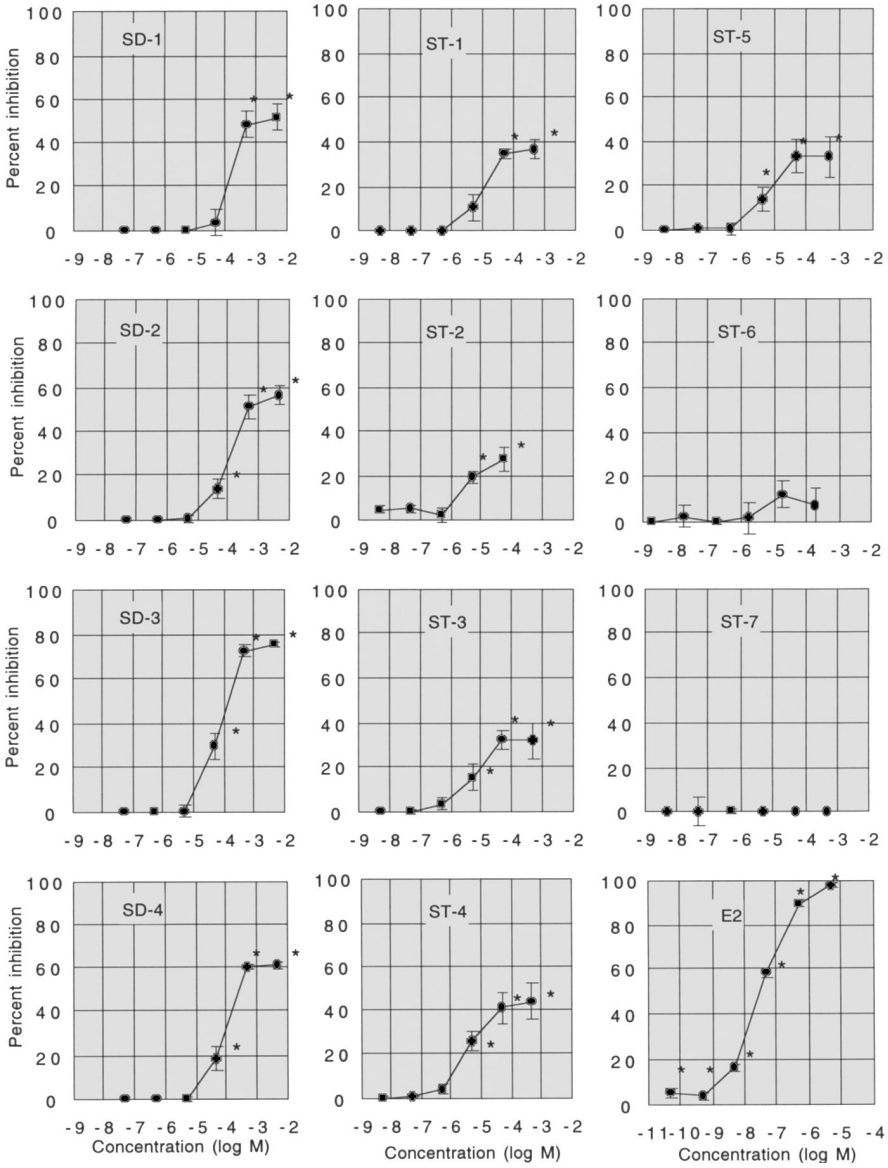

Figure 3. The inhibition of fluorescence-labeled E_2 binding to hERα by various concentrations of styrene oligomers. Percent of inhibition was calculated as [1 – (optical density in the presence of competitor) ÷ (optical density in the absence of competitor)] × 100. Each point is the mean ± SD of two independent assays performed in duplicate.
*Significantly different from hormone-free control ($p < 0.01$).

Discussion

We demonstrated that proliferation of MCF-7 cells was induced by styrene oligomers such as SD-3, SD-4, ST-1, ST-3, ST-4, and ST-5. The maximal proliferation occurred at a 10^{-5} M concentration of styrene oligomers. ST-1, ST-3, ST-4, or ST-5 produced complete concentration–response curves up to 10^{-5} M in the MCF-7 cell proliferation assay. ST-4 had the highest proliferative activity among the tested styrene oligomers and was a full agonist. ST-1, ST-3, and ST-5 had relatively high activity (RPE = 51–81%). The RP of styrene trimers ST-1, ST-3, ST-4, and ST-5 was 0.0002–0.0015% in the MCF-7 cell proliferation assay; these values were comparable to that of bisphenol A (0.0001–0.0025%) (*16*) and higher than that of 4-*n*-nonylphenol (RP = 0.000008–0.00007%) (*16*). The proliferative activities of styrene dimers were weaker than those of styrene trimers. Nobuhara et al. (*10*) reported that SD-3, SD-4, ST-1, and a mixture of tetralin ring trimers were not able to induce the proliferation of MCF-7 cells. They used MCF-7 cells (American Type Culture Collection; ATCC) purchased from Dainippon P. (Osaka, Japan) at an initial cell concentration of 2 × 10^4 cells/well in 12-well plates. Villalobos et al. (*14*) reported that MCF-7 supplied by A.M. Soto had the highest proliferative response to E_2, and that the ATCC strain responded to E_2 with a much smaller increase in cell yield. They also reported that ATCC MCF-7 cells should not be used in cell proliferation tests such as the E--SCREEN assay (*14*). Our results were obtained using MCF-7 cells provided by A.M. Soto at an initial concentration of 4 × 10^4 cells/well in 24-well plates. We confirmed that the initial concentration of 4 × 10^4 cells/well in 24-well plates was optimal for cell proliferation assays and a concentration < 2 × 10^4 cells/well in 24-well plates tended to increase the minimal concentration of test compound needed for maximal cell yield and the value of EC_{50} (*17*).

Styrene trimers such as ST-1, ST-2, ST-3, ST-4, and ST-5 and styrene dimers such as SD-1, SD-2, SD-3, and SD-4 had binding affinity for hERα. RBAs of ST-1, ST-3, ST-4, and ST-5 were higher than those of SD-1, SD-2, SD-3, and SD-4, although the high affinity for hERα was revealed at 5 × 10^{-4} M styrene dimers. It seems that styrene trimers at ≥ 5 × 10^{-5} M had low solubility in the reaction solution. We found that the binding potency of styrene trimers except for ST-6 and ST-7 were higher than that of styrene dimers. ST-2

had binding affinity for hERα and the RBA was higher than that of ST-1, ST-3, and ST-5, which had strong proliferative activity, although the proliferative activity was not significant. ST-2 may be estrogenic, although the proliferative activity could not be ascertained due to extremely low solubility in the solvent for the MCF-7 cell proliferation assay. We do not think that ST-6 and ST-7 are estrogenic because the values could not be estimated from the response curves in the cell proliferative assay and the competitive binding assay.

Azuma et al. (*11*) and Nobuhara et al. (*10*) reported that SD-1, SD-3, SD-4, ST-1, ST-2, ST-3, and ST-5 had no affinity for ER in an RI competitive binding assay. Although they examined the binding affinity of styrene oligomers at $\leq 10^{-5}$ M for ER of rat uterus, we tested them at the concentrations up to 5×10^{-3} or 5×10^{-4} M for purified human ER α. If they had tested at a concentration $> 5 \times 10^{-5}$ M, the binding activity would have been observed.

Estrogenic activities of styrene trimers differed depending on their chemical structures. Styrene trimers with a linear structure (ST-1) and a tetralin structure (ST-2, ST-3, ST-4, and ST-5) had estrogenic activity, but those with a cyclohexane structure (ST-6 or ST-7) did not.

The value of RPE from the MCF-7 cell proliferation assay correlated with the value of RBA from the competitive binding assay. This result suggested that the cell proliferative effect of these styrene oligomers was caused by their binding to hERα.

Styrene trimers such as ST-1, ST-3, ST-4, and ST-5 tested here moved from containers into foods upon heat treatment, preservation for 24 hr at 20°C, or cooking (*3*,*4*), and they are incorporated into the body with the foods. The present study demonstrated that styrene oligomers, particularly styrene trimers such as ST-1, ST-3, ST-4, and ST-5, had relatively high estrogenic activities in the MCF-7 cell proliferation assay and the competitive binding assay. These compounds might be endocrine disrupters. The effects of styrene trimers on uteri have not been found in *in vivo* studies using 21-day-old rats (*10*). However, fetuses are more vulnerable to estrogenic chemicals than are adults. The hormonal effects of these styrene trimers with regard to reproduction and the nervous system should be investigated using experimental animals, particularly in embryos.

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