Pergamon

0960-0760(94)E0032-G

J. Steroid Biochem. Molec. Biol. Vol. 49, No. 2/3, pp. 139–152, 1994

0960-0760/94 $7.00 + 0.00

Bivalent Ligands as Probes of Estrogen Receptor Action

Kathryn E. Bergmann,[1] Cynthia H. Wooge,[2] Kathryn E. Carlson,[1] Benita S. Katzenellenbogen[2] and John A. Katzenellenbogen[1]*

[1]*Department of Chemistry, University of Illinois, 600 South Mathews Avenue and* [2]*Department of Physiology and Biophysics, University of Illinois, 524 Burrill Hall, 407 South Goodwin Avenue, Urbana, IL 61801, U.S.A.*

The estrogen receptor (ER) is a hormone-regulated transcription factor which is thought to bind to specific DNA sequences as a homodimer. In order to better understand structural requirements for dimerization and its functional role in ER action, we synthesized a series of bivalent ligands based on the non-steroidal estrogen hexestrol. These molecular probes join two hexestrol molecules of the erythro (E, active) configuration with either 4 or 8 carbon linkers (designated E-4-E and E-8-E series, respectively), or with longer linkers comprised of ethylene glycol units (E-eg-E series). Several other bi- and monovalent control compounds were prepared. The bivalent ligands bind to ER with a relative affinity 1–7% that of estradiol. While most of the ligands demonstrated normal monophasic displacement curves in competitive binding assays with [^{3}H]estradiol, uncharacteristic biphasic competitive binding curves were seen for some of the ligands, indicating possible structure-specific, negative site–site interaction. In ER-deficient Chinese hamster ovary (CHO) cells transfected with an expression vector encoding ER, one series of bivalent ligands (E-4-E) had little stimulatory activity and inhibited transcription stimulated by hexestrol, as determined by a transient transfection assay using an estrogen-responsive reporter gene construct [(ERE)$_2$-TATA-CAT, containing two estrogen response elements linked to a TATA promoter and the chloramphenicol acetyl transferase reporter gene]. Monovalent or control bivalent ligands failed to antagonize hexestrol-stimulated activity and were as fully active as hexestrol itself. Studies performed in MCF-7 human breast cancer cells, which contain endogenous ER, yielded similar bioactivity profiles for the E-4-E bivalent inhibitory ligands, showing them to be effective estrogen antagonists, when using either induction of progesterone receptor or (ERE)$_2$-TATA-CAT transcriptional activation as the endpoint. The E-8-E ligand, however, acted as a partial agonist/antagonist of ERE-reporter gene transactivation and a full agonist of progesterone receptor induction in MCF-7 cells, thus showing cell- and response-specific differences in the effects of this bivalent ligand. These bivalent ligands for ER do not show enhanced potency or receptor binding affinity; however, some of them display binding properties that suggest the possibility of structure-specific negative site–site interaction, and some of them function as quite effective estrogen antagonists.

J. Steroid Biochem. Molec. Biol., Vol. 49, No. 2/3, pp. 139–152, 1994

INTRODUCTION

Steroid receptors are ligand-activated regulators of gene transcription. In general, they act by binding to specific DNA sequences, termed response elements, that are found in the promoter and 5′-flanking regions of regulated genes [1, 2]. The consensus response elements for steroid receptors are typically inverted repeats of a specific sequence of five nucleotides, separated by three nucleotides of variable sequence. The steroid receptors interact with these sites in the form of dimers, with the two receptor subunits presumed to bind one another in a head-to-head fashion, matching the C_2-symmetry of the palindromic response element [1]. Other members of the nuclear receptor superfamily, most notably receptors for thyroid hormone, retinoic acid and vitamin D, appear capable of forming either head-to-head homodimers or head-to-tail heterodimers with the RXR receptor [2].

The presence to two equivalents of receptor, bound as a dimer at the response elements, suggests that

*Correspondence to J. A. Katzenellenbogen.
Received 25 Oct. 1993; accepted 4 Feb. 1994.

ligands for these receptors, connected together in a bivalent fashion, might be intriguing chemical probes for steroid receptor structure and function. Bivalent ligands of the general structure *Ligand* 1-(*connector chain*)-*Ligand* 2, have been used to study receptor interactions in a number of systems [3, 4]. Particularly intriguing are the situations where such bivalent ligands appear capable of bridging between the binding sites of the two components of a dimeric complex. Here, increased ligand binding affinity, increased dimer stabilization and unusual bioactivity are anticipated [5, 6]. The possibility of achieving such double occupancy by bivalent ligands depends on a number of geometric factors in the ligand and the receptor dimer—the binding affinity must not be compromised by the positioning of the ligand-linking chain structure, and the chain must be sufficiently long to bridge the separation between the ligand binding sites in the receptor dimer.

While the structure of the DNA binding domain (domain C) of two steroid receptors, the estrogen receptor (ER) and glucocorticoid receptor, is known from X-ray crystallographic [7] and NMR studies [8, 9, 10], the structure of the crucial hormone binding domain (domain E) has not yet been determined. It is known, however, from site-specific mutational studies, that dimer interaction occurs both in the DNA and hormone binding domains [1]. One clue regarding the relative orientation and possible distance separating the ligand binding sites in the dimer comes from one of these studies, where it was found that if mutations are made within a sequence of 25–30 amino acids in the mouse ER, first ligand binding and then receptor dimerization were affected [11]. Thus, these two activities map to a small region in the linear sequence of the receptor and thus might also be reasonably close spatially.

Since such factors as the relative orientation of ligands in these two sites and the direction of ligand entry cannot be elucidated by mutational studies, we undertook the preparation of a series of bivalent ligands for the estrogen receptor to see whether we could probe for site–site interaction and investigate its consequence in terms of the biological activity of the receptor. Thus, in this report, we present a description of the synthesis of several series of bivalent ligands for the ER, based on the non-steroidal receptor ligand hexestrol (**2a**), providing a separation of the ligand centroids by distances from ca. 10 to 60 Å. None of these bivalent ligands showed enhanced binding affinity, although some demonstrated unusual negative site–site interactions in competitive binding assays. In addition, certain of the bivalent ligands also demonstrated antagonist activity in a series of cell-based bioassays.

EXPERIMENTAL

Chemical Synthesis

Complete details on the synthesis of all of the bi- and monovalent hexestrol-based ER ligands is presented elsewhere [12]. The synthesis of the hexestrol acid, thiol and iodide has been published previously [13, 14]. All products were characterized completely and display spectroscopic properties (^{1}H NMR, mass spec.) fully consistent with their proposed structure.

Biological Methods

Materials and general methods

The following compounds were obtained from the sources indicated: tritium-labeled estradiol [[6,7-^{3}H]estra-1,3,5(10)-trien-3,17β-diol], 49–51 Ci/mmol, from Amersham Corp.; [dichloroacetyl-1,2-^{14}C]chloramphenicol (50–60 mCi/mmol) was from New England Nuclear; ethylenedinitrilotetraacetic acid tetrasodium salt (EDTA), and dextran C from Eastman Kodak Co.; sodium azide and 1,4-bis(5-phenyloxazol-2-yl)-benzene (POPOP) from Aldrich Chemical Co.; *N*,*N*-dimethylformamide (DMF) from Fisher Scientific; unlabeled estradiol, activated charcoal, Trizma base, from Sigma Chemical Co.; 2,5-diphenyloxazole (PPO) from Research Products International Corp.; and Triton X-114 from Chem Central-Indianapolis. Rat and lamb uterine cytosols were prepared and stored as described previously [15, 16]. All *in vitro* experiments were done in TEA buffer (0.01 M Tris–HCl, 0.0015 M EDTA, 0.02% sodium azide, pH 7.4 at 25°C). The charcoal-dextran slurry used to remove unbound ligand was prepared as reported previously [15] and was used at 1 part to 10 parts of the cytosol solution. Tritium radioactivity was measured in a Nuclear Chicago Isocap 300 liquid scintillation counter, using a xylene based scintillation cocktail, described previously [17].

Receptor binding affinity

Binding assays and calculation of the relative binding affinity (RBA) values were performed as reported previously [15], using rat or lamb uterine cytosol diluted to $\sim$1.5 nM of receptor. Several concentrations of unlabeled competitor (prepared in 1:1 DMF–TEA to ensure solubility), together with 10 nM [^{3}H]estradiol were incubated at 0 or 25°C for 18–24 h. Three assay conditions were examined: untreated rat or lamb uterine cytosol, Na molybdate (20 nM) added to lamb uterine cytosol, and KCl (0.4 M) added to lamb uterine cytosol. Free ligand was removed by adsorption to dextran-coated charcoal. In all cases, the RBA values given in Tables 3–6 are the average of at least two determinations; interassay reproducibility has a coefficient of variance that is $<30\%$.

Progesterone receptor induction (PgR)

MCF-7 cells were grown in phenol red-free and insulin-free medium containing 10% charcoal-dextran treated serum for 1 week before treatment for 3 days with compound (10^{-6} M), or control vehicle (0.1% EtOH) in the presence or absence of hexestrol (10^{-10} M). Dose–response studies with hexestrol

showed this (10^{-10} M) concentration to be slightly above the minimal concentration of hexestrol giving maximal stimulation of PgR. Fresh medium and compound were added daily during the 3-day period. Cells were harvested and PgRs were measured by using a whole cell PgR assay as described previously [18]. In brief, aliquots of cell suspension (2–5 × 10^6 cells/ml of tissue culture medium) were incubated with 10 nM [^{3}H]R5020 in the absence and presence of 1000 nM unlabeled R5020 for 0.5 h at 37°C. Cells were then rinsed 3 times with 2 ml of iced phosphate-buffered saline (PBS) with 1% Tween-80 and transferred with 200 μl of PBS to scintillation vials. 1 ml of EtOH was added, and samples were counted in xylene-based scintillation fluid containing 25% Triton X-114.

Transient transfection (CAT) assays

Cell culture conditions and transfections. Chinese hamster ovary (CHO) cells, an ER-deficient cell line, were passaged in phenol red-free Dulbecco's modified Eagle's–Ham's F-12 tissue culture media (DME/F12) supplemented with 10% charcoal-dextran-treated fetal calf serum (Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml; Gibco). CHO cells were plated in 60-mm dishes in DME/F12 and 5% charcoal-treated calf serum for gene transfer studies at a density of 1.2 × 10^5 cells/plate in a humidified 5% CO_2 atmosphere. MCF-7 cells were grown and plated exactly as described previously [19]. At 36–40 h after cell plating, the media were changed, and the cells were transfected 5–6 h later. Cells were transfected by the calcium phosphate technique, with modifications as described previously [20, 21].

DNA constructs. All cloning was performed by standard procedures. The human ER expression vector, pRER, was constructed by us as described previously [21]. The estrogen-responsive plasmid $(ERE)_2$-TATA-CAT, used for transcriptional activation studies, was provided by Dr David Shapiro and is described elsewhere [22]. The β-galactosidase plasmid pCH110 (Pharmacia) was used as an internal control for transfection efficiencies in all gene transfer experiments.

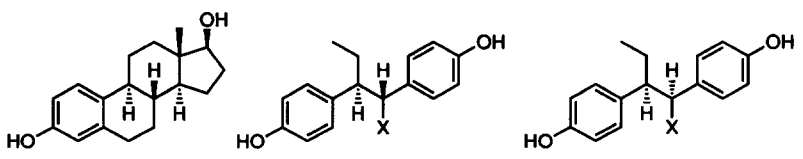
CAT assay. Forty-eight hours after the addition of hormone, the plates were washed with cold PBS and collected in 1 ml TNE [40 mM Tris–HCl (pH 7.5), 140 mM NaCl, and 1.5 mM EDTA], and the pellet was resuspended in 300–400 μl cold 250 mM Tris–HCl, pH 7.5. Cells were lysed by three cycles of freeze–thawing. Aliquots of 30–50 μl were used in a β-galactosidase assay, as described previously [19, 20]. Extracts containing equal amounts of β-galactosidase activity were used for the CAT assay. The reaction products were separated in a solvent of $CHCl_3$–MeOH (95:5, v/v). After autoradiography, radioactive spots were excised for quantification by scintillation counting. CAT activity was expressed as the percent conversion of chloramphenicol to its acetylated forms.

RESULTS

Design of bivalent hexestrol ligands

While estradiol (**1**), the steroidal ligand for ER, has been shown to tolerate sizable substituents at the 7α [23], 11β [24], 16α [25], and 17α [26] positions, in preparing bivalent ligands for the ER, we selected the non-steroidal ligand hexestrol (**2a**). This ligand affords several advantages: (1) long appendages off the hexane backbone chain of hexestrol are known to be tolerated reasonably well by ER, (2) hexestrol and its derivatives are relatively simple to synthesize, and (3) while *meso*-hexestrol **2a** (and its corresponding erythro hexestrol derivatives, E, **2bc**) with the *R**,*S** relative stereochemistry, have high affinity for ER, *dl*-hexestrol **3a** (and its corresponding threo hexestrol derivatives T, **3bc**) with the *R**,*R** relative configuration, have low affinity [13, 27], and therefore can serve as convenient control compounds.

The ligands we have prepared for ER are shown in Tables 1 (bivalent ligands) and 2 (monovalent ligands). The bivalent ligands we prepared (Table 1) contain two hexestrol moieties linked via either an ester or a thioether function to a spacer group. The spacer chain distances range from the short methylenes units (2, 3, 4, and 8 carbons) to the longer ethylene glycol units (2, 3, 4, 5, 10, 14, and 18 units). These bivalent ligands are of two types: (1) "*true*" *bivalent ligands*, in which two high affinity erythro hexestrols are connected together (*E–E series*); in this system, both ends have the possibility of binding to a receptor molecule, and (2) "*control*" *bivalent ligands*, in which one high affinity erythro hexestrol is connected to one low affinity threo



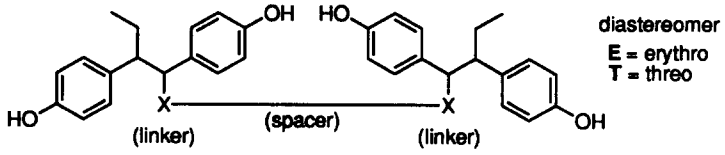
1 17β-Estradiol **2a** X = -Et (*meso*-Hexestrol) **3a** X = -Et (*dl*-Hexestrol)

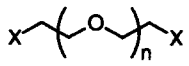
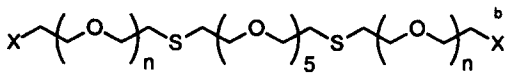
2b X = $-CO_2R$ [erythro or E Hexestrol derivatives] **3b** X = $-CO_2R$ [threo or T Hexestrol derivatives]

2c X = $-CH_2SR$ [erythro or E Hexestrol derivatives] **3c** X = $-CH_2SR$ [threo or T Hexestrol derivatives]

Structures 1–3

Table 1. Hexestrol bivalent compounds and shorthand symbols [a]



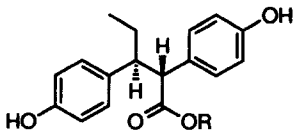
spacer	Shorthand Symbol (linker type X) —C(=O)—O— Ester	-CH₂S— Thioether (s)
X—$(CH_2)_n$—X	n = 2 **E-2-E**	**E-2(s)-E**
	3 **E-3-E**	**E-3(s)-E**
	4 **E-4-E (E-4-T)**	**E-4(s)-E (E-4(s)-T)**
	8 **E-8-E (E-8-T)**	**E-8(s)-E (E-8(s)-T)**
	n = 1 **E-2*eg*-E (E-2*eg*-T)** 2 **E-3*eg*-E (E-3*eg*-T)** 3 **E-4*eg*-E (E-4*eg*-T)** 4 **E-5*eg*-E (E-5*eg*-T)**	—
	—	n = 1 **E-10*eg*(s)-E** 3 **E-14*eg*(s)-E** 5 **E-18*eg*(s)-E**

[a]See text for an explanation for the shorthand symbols for these structures.
[b]In this series, ***eg*** is represented in *italics* to indicate that some *thio*ethylene glycol units are present, as shown.

hexestrol (*E–T system*); though closely related to the E–E system, only one end of this E–T bivalent ligand can bind to ER, so its behavior acts as a control for the E–E series. Since the ester-linked bivalent ligands are potentially subject to hydrolysis, we have also prepared some monovalent ligands to serve as control systems for such hydrolysis. These monovalent ligands (Table 2) contain one erythro hexestrol moiety, attached via an ester function to a spacer system with a terminal methyl ($-CH_3$) or hydroxyl (-OH) group.

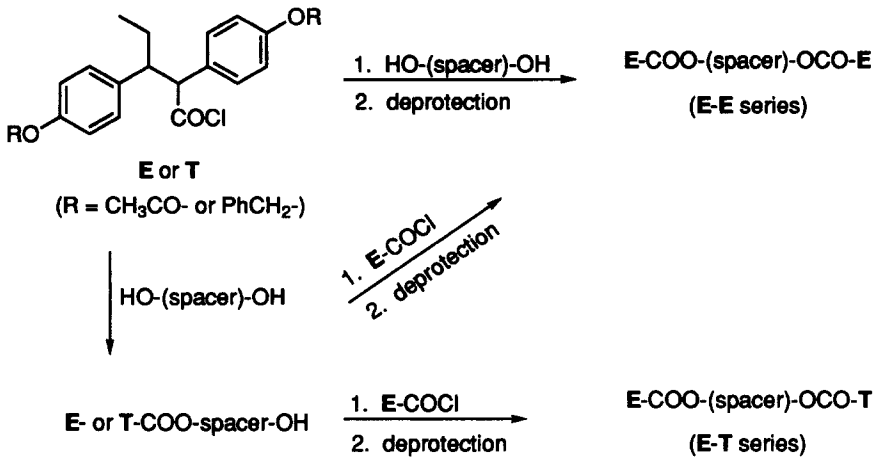
To facilitate discussion of these ligands, we have given them shorthand symbols that provide a simple key to their structure; the symbols represent the *ligands* that are connected through *linkers* to a *spacer*. The

Table 2. Hexestrol monovalent compounds and shorthand symbols [a]



spacer (R)		Shorthand Symbols	
$-(CH_2)_n$-X	n = 2	**E-2-H**	—
	4	**E-4-H**	**E-4-OH**
	8	**E-8-H**	**E-8-OH**
$-(CH_2CH_2O)_n-CH_2X$	n = 1	**E-1*eg*-CH_3**	**E-1*eg*-CH_2OH**
	2	**E-2*eg*-CH_3**	**E-2*eg*-CH_2OH**

[a]The shorthand symbols for the monovalent ligands are a simple extension of those for the bivalent ones (see text): a single erythro hexestrol (E) is linked through an ester group to a carbon (#) or ethylene glycol (#*eg*), which is terminated with the group X as shown.



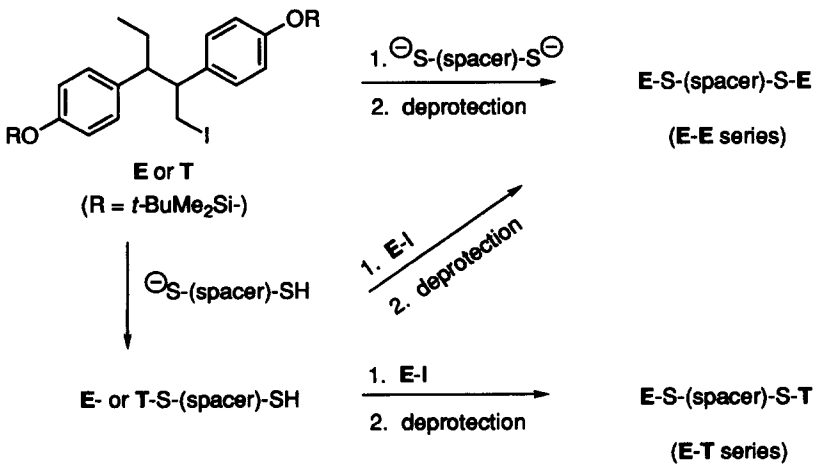
Scheme 1

ligand is either **E** (for erythro hexestrol) or **T** (for threo hexestrol); the *spacer* is either a polymethylene carbon chain (designated by a *number* that corresponds to the number of carbon atoms) or a series of ethylene glycol units (designated by an "**eg**", preceded by a number corresponding to the number of eg units); the *linker* is either an ester (given no further designation) or a thioether [designated by an (**s**) that follows the spacer]. Thus, **E-8-E** represents two erythro hexestrols connected through ester groups to an octamethylene spacer, **E-4eg-T** represents an erythro and a threo hexestrol linked through ester groups to a tetraethylene glycol spacer; **E-10*eg*(s)-E** are two erythro hexestrols linked via thioether functions to a long ethylene glycol chain (cf. Table 1). The shorthand for the monovalent ligands is related (cf. footnote to Table 2). The structures and names in Tables 1 and 2 further illustrate this naming system.

Synthesis of hexestrol mono- and bivalent ligands

A complete description of the synthesis of the mono- and bivalent hexestrol ligands for ER is presented elsewhere [12]. The synthesis of the key hexestrol acid and thiol components used in these preparations (**2bc** and **3bc**) has also been published elsewhere [13, 14]. Therefore, only a brief overview of the synthetic schemes will be shown here.

The ester-linked derivatives were prepared by reaction of acid chloride derivatives of either the erythro or threo hexestrol-related acids (**2b** and **3b**, respectively, R = H) with either a 1,ω-diol or an ethylene glycol system, as shown in Scheme 1. For the symmetrical system **E–E** series, this double esterification could be achieved in one step. However, for the unsymmetrical **E–T** series, efficient synthesis required sequential reaction of the threo (**T**) unit with an excess of the diol, followed, after isolation of the monoester, by reaction with the erythro (**E**) unit. This sequential synthesis proved to be more efficient for some of the longer symmetrical systems as well. The synthesis of the monovalent esters proceeded in a similar fashion. Throughout the esterification reactions, the phenolic hydroxyl groups on the hexestrols systems were protected either as acetates (for the monomers and the shorter, more reactive systems) or as benzyl ethers (for the larger, less reactive systems). These



Scheme 2

protecting groups are readily removed at the end of the syntheses by hydrolysis or hydrogenolysis, respectively.

A related strategy was used for the preparation of the thioether-linked bivalent ligands (Scheme 2). Here, the hexestrol unit was converted to the corresponding iodide, which was then subjected to reaction with the spacer system in the form of a 1,ω-dithiolate. This strategy proved to be more efficient than the complementary approach of using the hexestrol unit as the thiolate in reaction with a 1,ω-dihalide. Protection of the phenolic hydroxyl groups as the tert-butyldimethylsilyl ethers was convenient, as this group is stable to non-aqueous alkali, but can be cleaved readily with toluenesulfonic acid or tetrabutylammonium fluoride at the end of the sequence.

Separation of two E-4-E diastereomers

We have distinguished two series of bivalent ligands, one based on the same hexestrol diastereomer (**E–E**), and the other utilizing two different hexestrol diastereomers (**E–T**). Because each hexestrol unit is itself chiral, the bivalent ligands within each series are, in fact, mixtures of diastereomers. For example, the **E-4-E** dimer could be constructed either from two (+) or two (−) units, or from one (+) and one (−) unit, and in principle the (+ , + / − , −) pair of enantiomers would be separable from (+ , −) or meso form.

All of the bivalent ligands were purified by HPLC before binding and biological experiments were performed, and while the corresponding members of the **E–E** and **E–T** series were well separated, only in the case of the **E-4-E** bivalent ligand were the internal (+ , + / − , −) and (+ , −) diastereomers separable. These were isolated separately and are designated as **E-4-E(1)** and **E-4-E(2)**, respectively. Because we had available from previous work a sample of the (−)-hexestrol-derived acid [13], we were able to prepare a sample of the (− , −) diastereomer and to demonstrate upon normal phase silica gel HPLC, that the first-eluting diastereomer (40 min) is the (+ , + / − , −) diastereomer, and therefore, the last one (47 min), was the (+ , −) diastereomer.

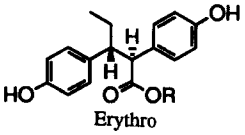
Receptor binding affinity

The RBA of the hexestrol derivatives for the ER was determined by a competitive radiometric binding assay using [^{3}H]estradiol as the tracer [14]. The values reported are relative to estradiol, which is assigned a value of 100%. RBA values are given for the hexestrol monomers (Table 3) and for the dimeric ligands (Tables 4–6).

The RBA values for the monomeric ligands are presented in two categories—those with methylene spacer chains and those with ethylene glycol spacer chains. Each of these sets includes monomers with terminal methyl groups and with terminal hydroxyl groups all in the erythro (**E**) series. In one case, we prepared the threo isomer (**T-2-H**), and it had a 7-fold lower affinity for the ER than the corresponding erythro isomer (**T-2-H** = 13%; **E-2-H** = 85%). No other monomers in the threo series were prepared, as these invariably would have lower affinities. The monomers with the higher RBA values are those with a short chain that have a terminal methyl group (**E-2-H** = 85%, **E-4-H** = 71% and **E-1eg-CH$_3$** = 20%). All other monomers have relatively low receptor binding affinities (< 10%).

The dimeric ligands were also divided into two categories—those with methylene spacer chains (Table 4) and those with ethylene glycol spacer chains (Table 5). Some of the **E–E** dimers have a corresponding **E–T** dimer. Two of the methylene dimeric ligands, **E-4-**

Table 3. RBA and partition coefficients (Log P) of the hexestrol monomers

	ROH	RBA[a] 0 °C (threo)[b]	RBA[a] 25 °C	Log P[c]
 Erythro	**H**	0.05	–	
	E-2-H	85 (13)	–	
	E-4-H	71	30	4.23
	E-8-H	2	2	6.14
	E-4-OH	8	–	3.22
	E-8-OH	3	–	4.68
	E-1eg-CH$_3$	20	–	3.31
	E-2eg-CH$_3$	1	–	3.47
	E-1eg-CH$_2$OH	1	–	
	E-2eg-CH$_2$OH	0.09	–	

[a]RBA were determined by a competitive binding assay. Estradiol = 100% [15].
[b]Threo isomer = (2*R**,3*R**).
[c]Octanol–water partition coefficients (Log P) were determined by an HPLC method [28]. Log P (Estradiol) = 3.50.

Table 4. RBA and partition coefficients (Log P) of the E–E and E–T bivalent ligands with methylene spacers

E—E isomer				E—T isomer		
(linkage)	RBA (inflections)[a]				RBA (inflections)[a]	
compound	0 °C	25 °C	log P[b]	compound	0 °C	log P[b]
(Ester:)						
E-2-E	0.79 (1)	–	4.93	–	–	–
E-3-E	2.34 (1)	–	5.49	–	–	–
E-4-E (1) (--,++)[c]	1.44 (1)	1.26 (1)	5.68	**E-4-T**	1.27 (1)	5.71
	1.58[d] (1)	–				
E-4-E (2) (-+,+-)	0.40; 0.010 (2)	3.16; 0.010 (2)	5.69	–	–	–
E-8-E	3.24; 0.024 (2)	6.92; 0.018 (2)	6.95	**E-8-T**	0.324 (1)	7.11
(Thioether:)						
E-2(s)-E	2.95 (1)	–	5.96	–	–	–
E-3(s)-E	3.8 (1)	–	6.26	**E-3(s)-T**	0.73 (1)	–
E-4(s)-E	1.2 (1)	–	6.54	**E-4(s)-T**	0.41 (1)	6.76
E-8(s)-E	0.75; 0.020 (2)	–	8.38	–	–	–

[a]RBA was measured by a competitive binding assay at 0 or 25°C [15], where estradiol is defined as 100%. The number in parentheses corresponds to a monophasic (1) or biphasic (2) binding curve. When the second component in the biphasic displacement is sufficiently large, an RBA value for each case can be determined; in such cases, two values are given (e.g. 3.24; 0.23).

[b]Octanol–water partition coefficients (Log P) were determined by the HPLC method [15]. Log P (E_2) = 3.50.

[c]The two diastereomers of **E-4-E** were separated by HPLC, and the RBA data are given for each diastereomer separately; the number in parentheses refers to the isomers that eluted first (1) or second (2) off the HPLC, and the pair of enantiomers to which each peak corresponds is denoted (see text).

[d]RBA of the (– , –) enantiomer of **E-4-E** obtained by synthesis from the (–)-erythro acid (see text).

E(1) and **E-8-E** were also examined at 25°C. The RBA values for the **E–E** dimeric ligands (1–7%) were higher for the methylene spacer chain isomers, especially for the shorter chains, than for the ethylene glycol spacer chains (<1%). The affinities for the ethylene glycol dimers were lowest for the systems with the largest spacers. While lipophilicity increases with spacer length in all three systems (see increasing Log P values [28] in Tables 3–5), this effect is moderated in the ethylene glycol spacer systems. Here, the most widely spaced dimers (10*eg*, 14*eg* and 18*eg*) have affinities comparable to or only slightly less than those of the shorter ethylene glycol spacer dimers. In most cases, as expected, the **E–T** isomers had lower affinities for the ER than the corresponding **E–E** isomers.

An interesting phenomenon was observed with four of the (**E–E**) dimeric ligands; the competitive binding curve, normally sigmoidal [as seen with E_2 and the **E-8-T** dimer; Fig. 1(B)], showed a biphasic or double inflection profile, suggestive of some negative site–site interaction [e.g. Fig. 1 (A), **E-8-E**]. The other compounds which showed this profile, though not as pronounced as the **E-8-E** dimer, were **E-8(s)-E**, **E-4-E(2)** and **E-2eg-E**. All other **E–E** dimeric compounds, the monomers, and the **E–T** dimers, showed normal, single inflection, competitive binding curves.

Since the **E-8-E** dimer showed the most pronounced biphasic binding curve, it was chosen for further study of the effect of several different assay conditions on its binding affinity for the ER: namely increased temperature to 25°C (believed to encourage receptor dimer formation), incubation in the presence of sodium molybdate (believed to keep the receptor in an 8S aggregated form), and incubation in the presence of 0.4 M KCl (believed to dissociate the receptor into the 4S monomer form). The results of the binding assays under these conditions are given in Table 6. The two values, RBA(a) and RBA(b), are the RBA values at the two inflection points of the biphasic curve; the ratio a : b is the relative amount of the high vs low affinity components of the binding competition curve [cf. Fig. 1(A)]. In each case, the biphasic curve is more pronounced at 0 than at 25°C (i.e. the a/b values are closer to 1). Also, at each temperature, the biphasic character is increased by the addition of molybdate or KCl. The origin of the biphasic character of these displacement curves is obviously complex and is addressed further in the Discussion.

Table 5. RBA and partition coefficients of the bivalent ligands with polyethylene glycol spacers [a]

(linkage)	E—E isomer			E—T isomer		
compound	RBA (inflections)		Log P	compound	RBA (inflections)	Log P
(Esters:)						
E-2eg-E	1.122; 0.020	(2)	5.30	**E-2eg-T**	0.81 (1)	5.04
E-3eg-E	0.56	(1)	5.40	**E-3eg-T**	0.35 (1)	5.15
E-4eg-E	0.44	(1)	5.54	**E-4eg-T**	0.27 (1)	5.34
E-5eg-E	0.093	(1)	5.63	**E-5eg-T**	0.085 (1)	5.38
(Thioether:)						
E-10eg(s)-E	0.48	(1)	7.72	–	–	–
E-14eg(s)-E	0.25	(1)	7.91	–	–	–
E-18eg(s)-E	0.12	(1)	–	–	–	–

[a]See explanation footnotes for Table 4.

Effects of dimeric ligands on ER function

The effectiveness of the bivalent ER ligands as estrogen agonists of antagonists was determined by two cell-based assays, namely induction of PgR in ER-positive MCF-7 human breast cancer cells [18] and stimulation of estrogen-responsive reporter gene transactivation in MCF-7 cells and in ER-deficient CHO cells transfected with an expression plasmid encoding wild type ER [20, 21]. The estrogen responsive reporter plasmid used, $(ERE)_2$-TATA-CAT, contains two consensus EREs linked to a minimal TATA promoter and CAT reporter gene. Our previous and current studies show that its transcription is markedly stimulated by estradiol- or hexestrol-liganded ERs [19, 21]. The results from the PgR and reporter gene transactivation assays are presented in Figs 2–4.

Because of the complexity of these assays, only 11 compounds were tested; these were selected to be the highest affinity and most interesting of the bivalent ligands and their corresponding control compounds (**E–T** series and monovalent analogs). The compounds investigated in the PgR assay (Fig. 2) include **E-4-E** (1 and 2) and **E-8-E**, their monomer and hydrolysis products, their **E–T** counterparts, and one of the ethylene glycol dimers, **E-2eg-E** and its monomer. Most of these compounds appear to be simple estrogen agonists; this includes all of the members of the **E-8** series and in the **E-4** series, the control bivalent ligands **E-4-T**, and the two monomers **E-4-H** and **E-4-OH**. By contrast, the two diastereomeric **E-4-E** bivalent ligands appear to be rather effective antagonists of PgR induction, having very limited activity themselves, and causing substantial depression of hexestrol-induced PgR levels. Marginal, but far less complete antagonism may be demonstrated by two compounds in the ethylene glycol series, **E-2eg-E** and **E-2eg-CH_3**; the same may be true for **E-8-T**.

Some of these compounds were investigated in the reporter gene transactivation assay, in both MCF-7 cells which contain endogenous ER and in CHO cells transfected with an expression plasmid for ER so as to

Table 6. RBA of the E-8-E bivalent ligand for ER under various assay conditions [a]

Assay conditions	Temp	RBA(a)[b]	RBA(b)[b]	a:b[c]
untreated	0 °C	3.24	0.024	1:1
untreated	25 °C	6.92	0.018	2.3:1
20 mM Na molybdate	0 °C	3.98	0.05	2:1
20 mM Na molybdate	25 °C	5.78	0.032	4.9:1
0.4 M KCl	0 °C	2.46	0.032	1.5:1
0.4 M KCl	25 °C	10.0	0.051	6.7:1

[a]Experiments utilize lamb uterus as source of receptor.

[b]RBA(a) and RBA(b) are relative binding affinities determined at the inflection points of the high and low affinity components of the biphasic displacement curve, respectively [cf. Fig. 1(A)].

[c]a : b represents ratio of quantity of high and low affinity components, respectively [cf. Fig. 1(A)].

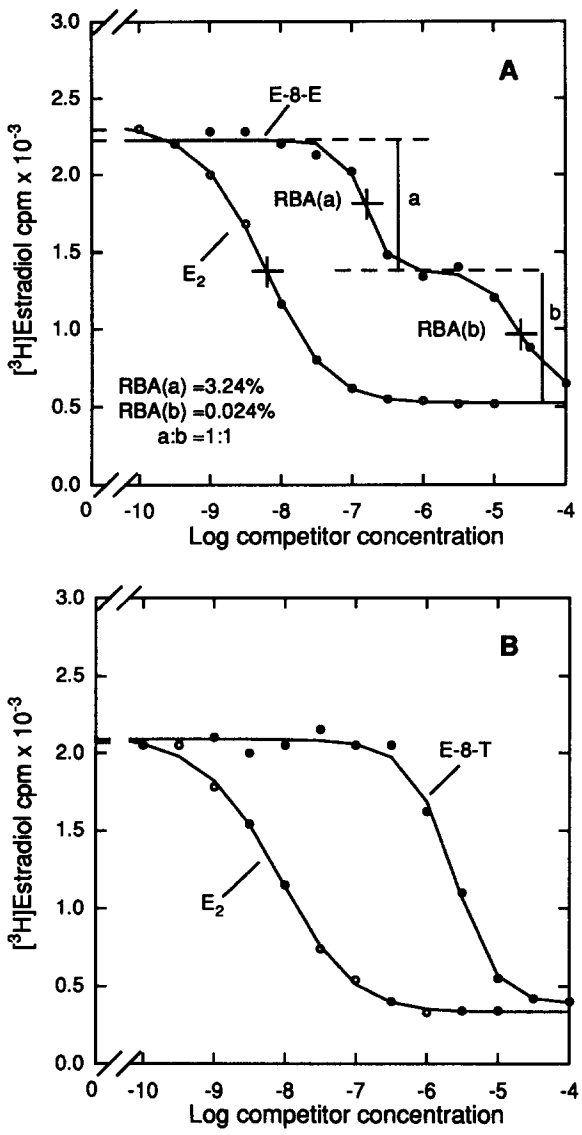


Fig. 1. Competitive binding assay to determine relative affinity of bivalent ligands (E-8-E and E-8-T) for lamb uterine ER. Receptor preparations were incubated for 18 h at 0°C with 10 nM [^{3}H]estradiol (E_2) and the indicated concentrations of E_2 or bivalent ligands. After incubation, free tracer was removed by charcoal-dextran treatment. Numbers indicate the RBA values for RBA(a) and RBA(b) components, with the RBA of E_2 being set at 100%.

introduce ER into these ER-deficient cells (Fig. 3). With one exception, the results of the reporter gene transactivation assays were similar to the PgR assay. Again, the two diastereomeric **E-4-E** bivalent ligands were effective antagonists in both MCF-7 and CHO cell systems. By contrast, the **E-8-E** bivalent ligand, that was a simple agonist in the PgR assay system, proved to be a rather effective antagonist in the reporter gene transactivation assay, and more so in CHO cells than in MCF-7 cells (Fig. 3).

The potency of these compounds as antiestrogens was investigated further in the CHO cell transactivation CAT assay in which CHO cells were transfected with the ER expression plasmid and the reporter plasmid $(ERE)_2$-TATA-CAT (Fig. 4). Although the antagonism by the three bivalent ligands is nearly complete, their potency is relatively modest, with half inhibition of CAT stimulation by hexestrol requiring 1–3 × 10^3-fold excess of the bivalent ligand. This is perhaps a 10-fold greater ratio than might be expected based on the ratio of RBA values for hexestrol (300) vs these three bivalent ligands (1.5–3.2). This difference might be accounted for by the larger size and increased lipophilicity of the bivalent ligands relative to hexestrol, factors that may further reduce their relative potency in a cell-based assay (CAT or PgR assay), as opposed to a cell-free one (RBA measurement in cell extracts containing ER).

DISCUSSION

In this study, a series of mono- and bivalent ligands of varying lengths were prepared for ER, and were tested for their affinity for the receptor and for their agonist or antagonist activity in several cell-based assays. By examining the binding characteristics and bioactivity of these bivalent ligands, we hoped to be able to demonstrate the adjacency of the ligand binding sites in the ER dimer. If, indeed, the binding sites of the ER complex are within bridging distance, then a bivalent ligand of sufficient length should form a very stable receptor–receptor complex. Bivalent ligand systems have been prepared for other receptor systems and have been shown to exhibit increased potencies and affinities for their respective receptors that were more than double those of the corresponding monovalent series, suggesting simultaneous occupation of vicinal binding sites [4, and references cited therein].

Our preparation of bivalent ligands for ER was stimulated by the known dimeric nature of the ER and the recent report by Parker [11] demonstrating, by site-specific mutagenesis, that adjacent regions in the linear sequence of the ER were involved in receptor dimerization and ligand binding; this suggested that the ligand binding sites might be close to the dimer interface and, thereby, close to one another. The experiences of laboratories involved in the design of affinity matrices for the purification of ER has been somewhat different [29]. Here, adequate binding affinity was achieved only with relatively long spacers between ligand and matrix, leading Green [30] to propose that the ER binding site is a deep cleft in the receptor, so that a spacer chain of at least 14 atoms long per receptor would be needed to allow binding of a second valence of a bivalent ligand. In this situation short chain bivalent ligands would bind only univalently to the receptor, as the second valence would be sterically incapable of binding. The entropic freedom of the very long spacer chains needed to span the distance between the binding sites in the ER dimer would reduce the efficiency of their bivalent binding.

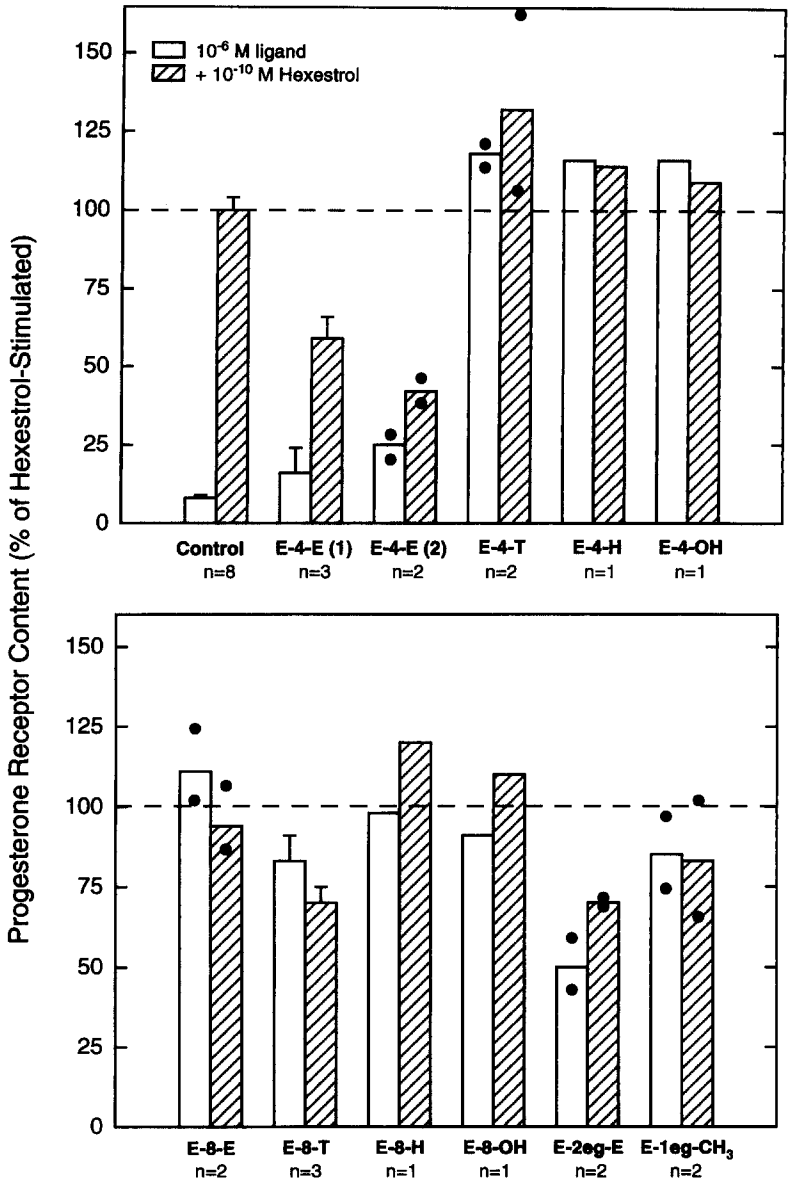


Fig. 2. PgR levels in MCF-7 cells treated with control vehicle (control) or the indicated ligands at 10^{-6} M concentration in the presence or absence of 10^{-10} M hexestrol. The cellular PgR content in the presence of 10^{-10} M hexestrol, which was found to be 80 ± 3 fmol/10^6 cells, is set at 100%. PgR levels were determined after 3 days in the presence of the indicated compounds. Fresh medium and compounds were added daily during the 3-day period. The cells were then harvested and assayed for PgR as described in Experimental. Each value represents data obtained from duplicate flasks of cells. Values represent the mean + SEM with the number of separate experiments indicated. In the case of duplicate experiments ($n = 2$), the mean and range (individual values as dots) are shown.

The bivalent hexestrol ligands we prepared for ER had spacer chains that range from short polymethylene units (2–8 atoms), through medium length ethylene glycol spacers (2–5 glycol units; 6–15 atoms), to very long spacer chains (10, 14, and 18 mixed ethylene glycol/thioethylene glycol units; 30–54 atoms). None of the bivalent ligands—even those with very long spacers—demonstrated unusually high binding affinity, certainly not in comparison with the affinity of hexestrol itself, nor with the monomeric ligands prepared as controls for possible hydrolysis of the ester-linked bivalent systems. Even the more critical comparison between the E–E type true bivalent ligand and the E–T type control bivalent ligands, failed to demonstrate any preferential binding of the bivalent species. Thus, it appears that the two ligand binding sites in the ER dimer are not both accessible by the two ends of even an amply spaced bivalent ligand. Alternatively, as noted above, once the spacing ligands become too great, the affinity advantage of a bivalent system may become compromised by entropic factors. Further studies of the stability of ER dimer complexes with mono- vs bivalent ligands might permit differentiation between these two alternatives.

Despite the apparent lack of the dual occupancy of the ligand binding sites in the ER dimer by bivalent ligands, there were two unusual observations we made about these compounds: some of them demonstrate biphasic competitive displacement curves, suggestive of some type of negative site–site interaction, and some appear to be rather complete antagonists, albeit of only moderate potency. A biphasic competitive binding curve is normally considered to indicate the presence of two sites with differing affinities. In fact, such biphasic competition is often the first evidence for receptor subtypes, with selective ligand showing a differential in

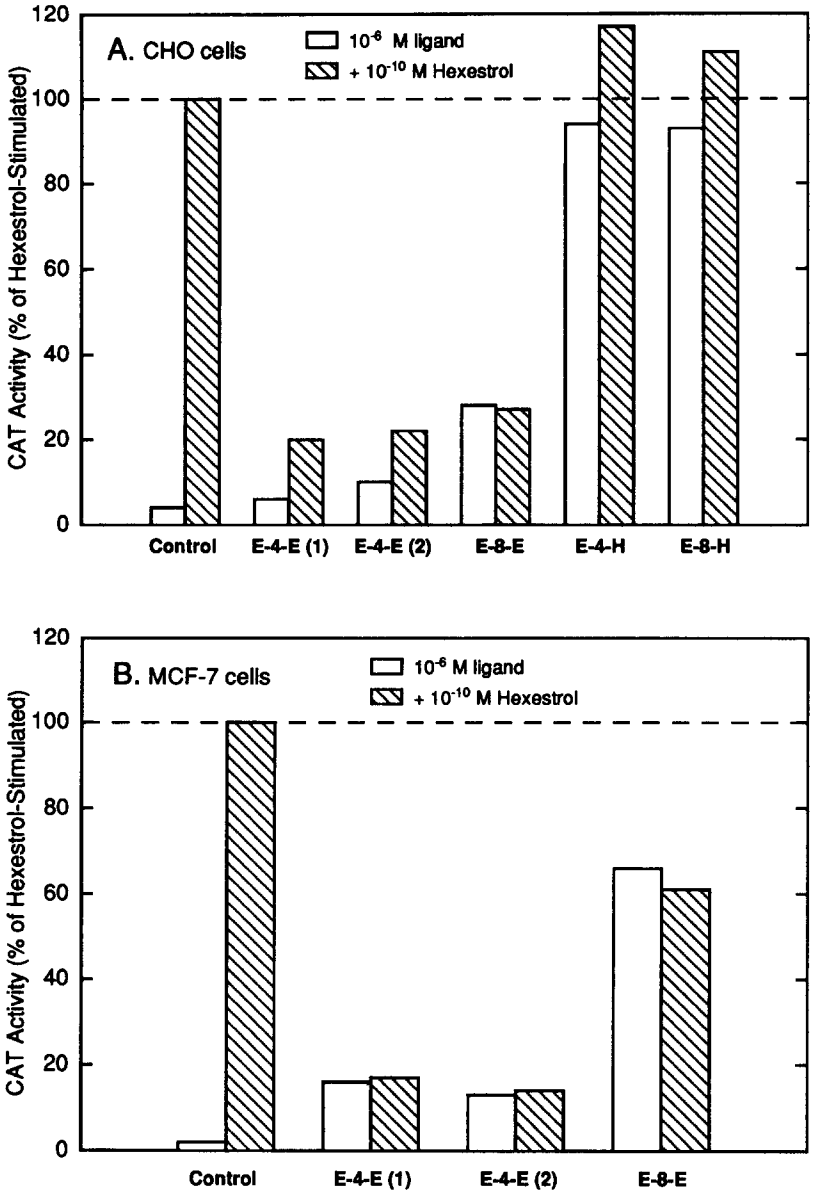


Fig. 3. Effect of bivalent ligands and hexestrol on estrogen-responsive reporter gene transactivation: studies in CHO cells and MCF-7 cells. (A) CHO cells were cotransfected with an expression vector encoding wild type human ER, the estrogen-responsive reporter gene [$(ERE)_2$-TATA-CAT], and carrier DNA (pTZ19), using the calcium phosphate-DNA coprecipitate method. An expression vector coding for β-galactosidase was also included in the coprecipitate as an internal control for transfection efficiency. Cells were treated with 10^{-6} M ligand in the presence and absence of 10^{-10} M hexestrol for 48 h. This concentration of hexestrol was slightly above the minimal hexestrol concentration found to evoke maximal stimulation of CAT activity. The amount of CAT activity obtained was normalized with reference to the internal control β-galactosidase activity. Data are presented as the percent of hexestrol (10^{-10} M) stimulated reporter gene activity which is set at 100%. Values are the mean of two closely corresponding, duplicate determinations. (B) MCF-7 human breast cancer cells were transfected with the [$(ERE)_2$-TATA-CAT] reporter plasmid and an internal control plasmid that expresses β-galactosidase. Cells were treated with 10^{-6} M ligand in the presence or absence of 10^{-10} M hexestrol for 48 h. Cell extracts were prepared and assayed for β-galactosidase activity to normalize for transfection efficiency. CAT activity is reported as the percent of hexestrol-stimulated activity where 10^{-10} M hexestrol activity is set at 100%. Values are the mean of two closely corresponding, duplicate determinations.

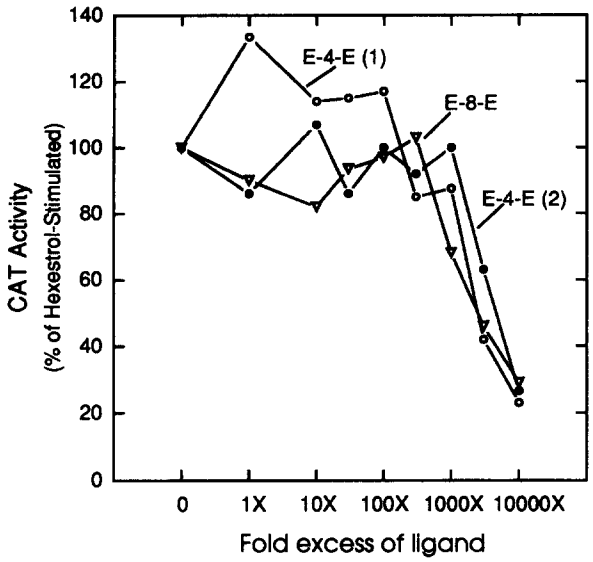
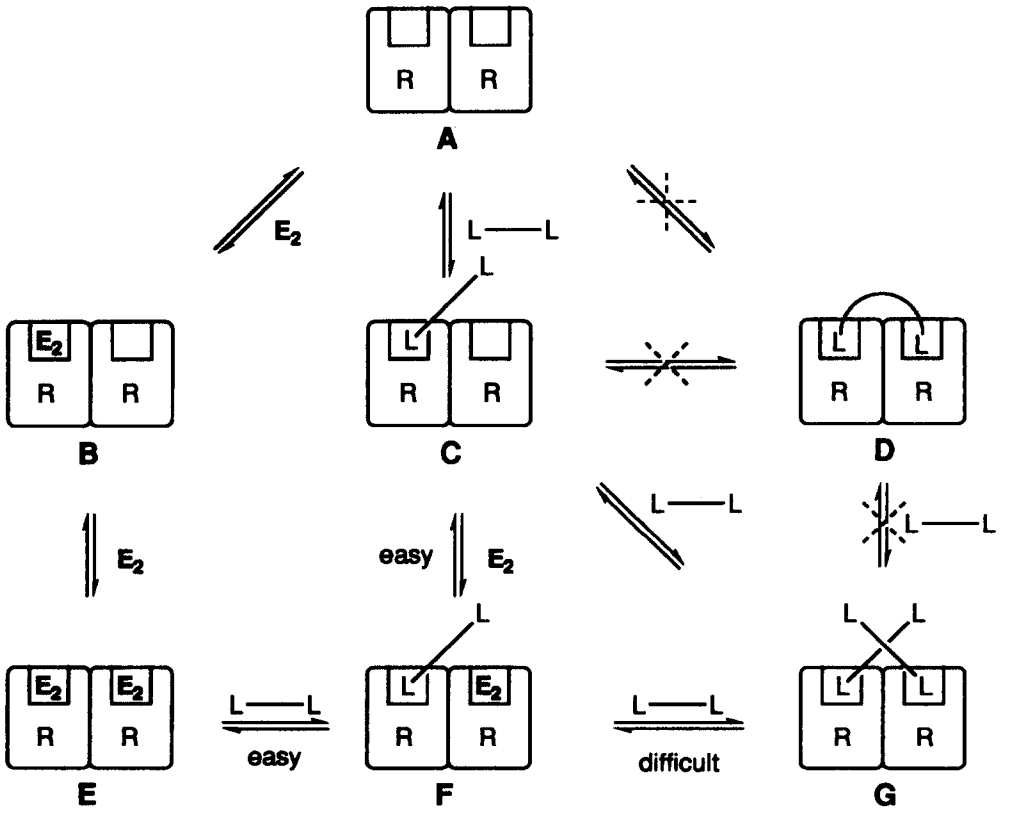


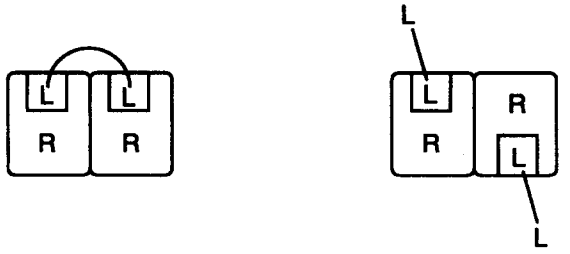
Fig. 4. Effect of bivalent ligands on hexestrol-stimulated CAT activity. CHO cells were transfected, as described in Fig. 3(A), with an ER expression plasmid, the estrogen responsive reporter gene [$(ERE)_2$-TATA-CAT], and an internal control plasmid that expresses β-galactosidase. The ability of increasing concentrations of bivalent ligand to suppress CAT activity induced by 10^{-10} M hexestrol was determined after a 48 h incubation with hexestrol and the indicated fold excesses of bivalent ligand. Results (mean of two separate experiments) are expressed as a percent of CAT activity observed in the presence of 10^{-10} M hexestrol alone.

potency in the displacement of a non-selective tracer from the receptor subtypes.

It would be unexpected to find receptor subtypes for ER in the preparations we use for binding assays; so, we favor an explanation suggested by Scheme 3. If ER is a dimer (**A**), then, as illustrated in Scheme 3, the displacement characteristics of a large (bivalent) ligand could be different from those of a small ligand, even if it does not bind directly in a simple bivalent fashion. According to this scheme, both ligand binding sites in the dimer have equivalent affinities for the small tracer estradiol (**A** ⇄ **B** ⇄ **E**). However, when the larger (bivalent) ligand binds to one site (**C**), it has an allosteric effect such that the access of another large (bivalent) ligand to the second site is hindered (**C** ⇄ **G**), whereas the binding of the smaller tracer ligand estradiol is not affected (**C** ⇄ **F**). The result is that displacement of the second equivalent of tracer estradiol from the dimer (**F** ⇄ **G**) requires a much greater concentration of the large (bivalent) ligand than the first (**E** ⇄ **F**), which causes the displacement curve to appear to be biphasic. While such a biphasic displacement from pure ER dimer would give equal fractions of the first and second component, various mixtures of ER monomer and dimer could result in diminished fractions of the second component, since in the monomer state, all ligands should show simple, monophasic displacement curves.



Scheme 3



Scheme 4

It may seem curious to have a situation where a large (bivalent) ligand blocks its own binding to the second site in a receptor dimer, without blocking the binding of a small ligand. This may be understood better by considering that there is a continuum of possibilities for the relative dispositions of the two ligand binding sites in a receptor dimer. Illustrated in Scheme 4 are the limits of this continuum, designated as being "accessible" for bivalent binding vs "inaccessible" for bivalent binding. The situation outlined in Scheme 3 might operate if the ER dimer existed in some state that was intermediate between these two extremes, with the two sites being inaccessible yet mutually interacting.

While such schemes can provide an explanation for the observed biphasic displacement, it is curious that this phenomenon is observed only in four cases of the many we have studied—most clearly in the cases of **E-8-E** and **E-4-E** (2, (+, −)) [but not for **E-4-E** (1, (−, − / +, +)), and only weakly for **E-8(s)-E** and **E-2eg-E**]. It is not clear why the biphasic displacement behavior is not observed for some closely related structures. Also, while we were able to perturb the fraction of the displacement accounted for by the second, low affinity component, we could not achieve the behavior predicted for increased ER dimer levels at 25°C nor for increased monomer levels in 0.4 M KCl-containing ER preparations.

The antagonist activity of certain of the bivalent ligands is intriguing. Two of the **E-4-E** bivalent systems are rather complete antagonists in the PgR induction system, and the **E-8-E** ligand is a partial antagonist in the reporter gene transfection assay, and more so in CHO than in MCF-7 cells. However, it is not apparent why closely related bivalent ligands do not demonstrate antagonist activity. Clearly, the lack of antagonism by the members of the **E–T** series indicates that the antagonism is not simply a characteristic of a hexestrol derivative bearing a large side chain substituent.

Finally, while some of the hexestrol-based bivalent ligands we have prepared for the ER demonstrate unusual site–site interaction and some have antagonist activity, further studies would be required to establish whether dual binding site occupancy is being achieved by these ligands.

Acknowledgements—We are grateful for support of this research through grants from the National Institutes of Health [PHS 5R37 CA18119 (to B.S.K.) and PHS 5R37 DK15556 (to J.A.K.)]. High resolution mass spectra were obtained on instruments supported by the National Institutes of Health (GM 27029); ^{1}H NMR were obtained on a Varian QE 300 MHz instrument supported by the National Institutes of Health (PHS 1S10 RR 02299).

REFERENCES

1. *Nuclear Hormone Receptors, Molecular Mechanisms, Cellular Functions, Clinical Abnormalities* (Edited by M. G. Parker). Academic Press, London (1991).
2. Parker M. G.: Steroid and related receptors. *Curr. Op. Cell Biol.* 5 (1993) 499–504.
3. Kizuka H. and Hanson R. N.: β-Adrenoceptor antagonist activity of bivalent ligands. 1. diiamide analogues of practolol. *J. Med. Chem.* 30 (1987) 722–726.
4. Portoghese P. S.: The role of concepts in structure–activity relationship studies of opioid ligands. *J. Med. Chem.* 35 (1992) 1927–1937.
5. Portoghese P. S., Larson D. L., Sayre L. M., Yim C. B., Ronisisvalle G., Tam S. W. and Takemori A. E.: Opioid agonist and antagonist bivalent ligands. The relationship between spacer length and selectivity at multiple opioid receptors. *J. Med. Chem.* 29 (1986) 1855–1861.
6. Shimohigashi Y., Costa T., Matsuura S., Chen H.-C. and Rodbard D.: Dimeric enkephalins display enhanced affinity and selectivity for the delta opiate receptor. *Molec. Pharmac.* 21 (1982) 558–563.
7. Luisi B. F., Xu W. X., Otwinowski Z., Freedman L. P., Yamamoto K. R. and Sigler P. B.: Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* 352 (1991) 497–505.
8. Härd T., Kellenbach E., Boelens R., Maler B., Dahlman K., Freedman L. P., Carlstedt-Duke J., Yamamoto K. R., Gustafsson J.-A. and Kaptein R.: Solution structure of the glucocorticoid receptor DNA-binding domain. *Science* 248 (1990) 157–160.
9. Schwabe J. W. R., Neuhaus D. and Rhodes D.: Solution structure of the DNA-binding domain of the estrogen receptor. *Nature (London)* 348 (1990) 458–461.
10. Lee M. S., Kliewer S. A., Provencal J., Wright P. E. and Evans R. M.: Structure of the retinoid x-receptor α-DNA-binding domain. *Science* 269 (1993) 1117–1121.
11. Fawell S. E., Lees J. A., White R. and Parker M. G.: Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. *Cell* 60 (1990) 953–962.
12. Bergmann K. E.: Design, synthesis, and biochemical evaluation of three types of hexestrol-based probes for estrogen receptor: Fluorine-18 radiopharmaceuticals, bivalent ligands and diazirine photoaffinity reagents. Ph.D. Thesis submitted to the University of Illinois (1993).
13. Landvatter S. W. and Katzenellenbogen J. A.: Stereochemical considerations in the binding of non-steroidal estrogens to the estrogen receptor. *Molec. Pharmac.* 20 (1981) 43–51.
14. Zablocki J. A., Katzenellenbogen J. A., Carlson K. E., Norman M. J. and Katzenellenbogen B. S.: Estrogenic affinity labels: Synthesis, irreversible receptor binding, and bioactivity of aziridine-substituted hexestrol derivatives. *J. Med. Chem.* 30 (1987) 829–838.
15. Katzenellenbogen J. A., Johnson H. J., Jr and Myers H. N.: Photoaffinity labels for estrogen binding proteins of rat uterus. *Biochemistry* 12 (1973) 4085–4092.
16. Carlson K. E., Sun L.-H. K. and Katzenellenbogen J. A.: Characterization of trypsin-treated forms of the estrogen receptor from rat and lamb uterus. *Biochemistry* 16 (1977) 4288–4296.
17. Katzenellenbogen J. A., Johnson H. J., Carlson K. E. and Myers H. N.: The photoreactivity of some light sensitive estrogen derivatives. The use of as exchange assay to determine their photoinactivation with rat uterine estrogen binding protein. *Biochemistry* 13 (1974) 2986–2994.

18. Katzenellenbogen B. S. and Norman M. J.: Multihormonal regulation of the progesterone receptor in MCF-7 human breast cancer cells: Interrelationships among insulin/IGF-I, serum and estrogen. *Endocrinology* **126** (1990) 891–898.
19. Cho H. and Katzenellenbogen B. S.: Synergistic activation of estrogen receptor-mediated transcription by estradiol and protein kinase activators. *Molec. Endocr.* **7** (1993) 441–452.
20. Reese J. C., Wooge C. H. and Katzenellenbogen B. S.: Identification of two cysteines closely positioned in the ligand binding pocket of the human estrogen receptor: roles in ligand binding and transcriptional activation. *Molec. Endocr.* **6** (1992) 2160–2166.
21. Reese J. C. and Katzenellenbogen B. S.: mutagenesis of cysteines in the hormone binding domain of the human estrogen receptor: Alterations in binding and transcriptional activation by covalently and reversibly attaching ligands. *J. Biol. Chem.* **266** (1991) 10880–10887.
22. Chang T., Nardulli A., Lew D. and Shapiro D.: The role of estrogen response elements in expression of the *Xenopus laevis* Vitellogenin B1 gene. *Molec. Endocr.* **6** (1992) 346–354.
23. French A. N., Wilson S. R., Welch M. J. and Katzenellenbogen J. A.: A synthesis of 7α-substituted estradiols: Synthesis and biological evaluation of a 7α-pentyl-substituted Bodipy^{tm} fluorescent conjugate and a fluorine-18 substituted 7α-pentyl estradiol analog. *Steroids* **58** (1993) 157–169 and references cited therein.
24. Van Brocklin H. F., Pomper M. G., Carlson K. E., Welch M. J. and Katzenellenbogen J. A.: Preparation and evaluation of 17-ethynyl-substituted 16α-[^{18}F]fluoroestradiols: selective receptor-based PET imaging agents. *Nucl. Med. Biol.* **19** (1992) 363–374, and references cited therein.
25. Fevig T. L., Mao M. K. and Katzenellenbogen J. A.: Estrogen receptor binding tolerance of 16α-substituted estradiol derivatives. *Steroids* **51** (1988) 471–497.
26. Pomper M. G., Van Brocklin H., Thieme A. M., Thomas R. D., Kiesewetter D. O., Carlson K. E., Mathias C. J., Welch M. J. and Katzenellenbogen J. A.: 11β-methoxy-, 11β-ethyl-, and 17α-ethynyl-substituted 16α-fluoroestradiols: receptor-based imaging agents with enhanced uptake efficiency and selectivity. *J. Med. Chem.* **33** (1990) 3143–3155 and references cited therein.
27. Kilbourn M. R., Arduengo A. J., Park J. T. and Katzenellenbogen J. A.: Conformational analysis of non-steroidal estrogens: The effect of conformer populations on the binding affinity of *meso*- and *dl*-hexestrol to the estrogen receptor. *Molec. Pharmac.* **19** (1981) 388–398.
28. Minick D. J., Frenz J. H., Patrick M. A. and Brent D. A.: A comprehensive method for determining hydrophobicity constants by reversed-phase high-performance liquid chromatography. *J. Med Chem.* **31** (1988) 1923–1933.
29. Redeuilh G., Secco C., Baulieu E-E.: The use of the biotinyl estradiol-avidin system for the purification of "nontransformed" estrogen receptor by biohormonal affinity chromatography. *J. Biol. Chem.* **260** (1985) 3996–4002.
30. Green N. M., Konieczny L., Toms E. J. and Valentine R. C.: The use of bifunctional biotinyl compounds to determine the arrangement of subunits in avidin. *Biochem. J.* **125** (1971) 781–791.