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METHYL AND BROMO DERIVATIVES OF ESTRADIOL ARE AGONISTIC LIGANDS FOR THE ESTROGEN RECEPTOR OF MCF-7 BREAST CANCER CELLS

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Summary—The binding affinity and relative estrogenic potency of 2-bromo-, 4-bromo-, 2-methyl- and 4-methylestradiol was evaluated in MCF-7 breast cancer cells. The relative binding affinities compared to estradiol were 47% for 2-methyl-, 25% for 4-methyl-, 37% for 4-bromo- and 17% for 2-bromoestradiol. However, both 2- and 4-methyl- as well as 2- and 4-bromoestradiol were able (a) to translocate the cytosolic estrogen receptor into the nucleus and (b) to induce the progesterone receptor in a concentration dependent manner. Finally, all ring-A substituted estrogens used in this study induced the pS2 mRNA as demonstrated by Northern-blotting. From these findings we conclude that 2-bromo-, 4-bromo-, 2-methyl- and 4-methylestradiol are agonistic ligands for the estrogen receptor in MCF-7 breast cancer cells.

INTRODUCTION

The catecholestrogens, 2- and 4- hydroxylated derivatives of estradiol, are major metabolites of estradiol in a variety of organs (for review see [1]). However, little is known about their estrogenic potency as a ligand for the estrogen receptor (ER), although their ability to bind to this molecule has been demonstrated in a variety of tissues [2–6]. There are two reasons for this: (a) metabolically they are converted into corresponding methoxy derivatives with a fairly high velocity and (b) *in vitro* they are relatively instable with regard to their decomposition by oxygenation.

Previous studies indicated that these experimental difficulties inherited by the natural ligands could be overcome if estrogens substituted by a methyl [7] or a halogen group [8–11] at C-atom 2 or 4 of the estradiol molecule are used. Therefore the aim of this study was to assess the estrogenic potency of estradiol substituted at C-atom 2 and 4 of the aromatic ring of the hormone. Additional interest in these components comes from earlier and most recently performed studies which suggest that estradiol substituted by a methyl group at C-atom 4 of the aromatic ring might be a potential antiestrogen [12, 13] as deduced from its missing uterotrophic capacity. Therefore, in this study we also attempted to test this hypothesis in MCF-7 breast cancer cells.

To demonstrate whether or not methylestradiol (ME) and bromoestradiol (BE) are potential ligands for the ER we tested the following criteria in MCF-7 breast cancer cells: (a) can they function as competitors for estradiol in a receptor binding assay?; (b) are they capable of downregulating the cytosolic ER?; (c) are they able to induce the ER mediated production of the progesterone receptor (PR) [14, 15]?: and finally; (d) can they stimulate the expression of the ER-dependent pS2 mRNA [16]?

MATERIALS AND METHODS

Hormones and probes

2-BE, 4-BE, 2-ME and 4-ME were synthesized as described previously [17, 18]. Prior to the experiments the hormone derivatives were purified by HPLC, so that contamination of the substituted hormones by estradiol was below 0.01%. As radioactive ligands for receptor binding assays we used [2,4,6,7-^{3}H]estradiol (sp. act. 85–110 Ci/mmol; Amersham, Braunschweig) for the quantitation of the ER and [^{3}H]ORG 2058 (16α-ethyl-21-hydroxy-19-nor[6,7-^{3}H]pregn-4-ene-3,20-dione, sp. act. 40–60 Ci/mmol; Amersham, Braunschweig) for the quantitation of the PR. The plasmid vectors

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containing cDNA-sequences of the pS2 and the 36B4 genes were kindly provided by Professor Chambon (Strassburg, France). These sequences have been subcloned into the vector pGEM 5 (Promega, Heidelberg, Fed. Rep. Germany) in order to generate antisense transcripts for use in the Northern hybridization procedure outlined below.

Cell culture

MCF-7 breast cancer cells clone M1 ([19]; kindly provided by Professor M. Dietel, Kiel, Fed. Rep. Germany) were maintained in 1:1 mixture of DMEM/F12 medium containing 10% fetal calf serum (FCS) and were routinely split in a 1:10 ratio. Two passages prior to the experiment cells were transferred into a steroid-free DMEM/F12 medium without phenol red and with 10% FCS that was stripped by dextran-coated charcoal (DCC).

Quantification of concentrations of ER and PR

To evaluate relative binding affinities, MCF-7 cells from three confluently grown 175 cm^2 tissue culture flasks (NUNC, Denmark) were harvested by trypsination. Cells were broken by three freeze thaw cycles in liquid nitrogen and the cytosols were prepared by ultracentrifugation (144,000 g; for 45 min at 4°C). The relative binding affinities of 2-ME, 4-ME, 2-BE and 4-BE were measured in a competitive ligand binding assay based on a Scatchard [20] analysis (for details see Table 1). In this assay eight different concentrations of tritiated estradiol ranging from 0.1–3 nM were used. Unspecific binding was determined by adding a 200 fold molar excess of unlabelled DES (diethylstilbestrol; Merck, Darmstadt, Fed. Rep. Germany) to an additional set of tubes containing the radioactive hormones. As a reference for the competition assay 1.5 nM unlabelled estradiol (0.5 fold excess relative to the highest concentration of radiolabelled estradiol used) was added to a third set of vials containing the radioactive ligands. Competition by BE and ME was determined in the presence of 1.5 and 3 nM concentrations (0.5 fold or equimolar excess of unlabelled hormone relative to the highest concentration of estradiol used in the Scatchard analysis) added to each concentration of radioactive hormone. Cytosols and ligands were incubated overnight (16–18 h) at 0°C. Thereafter bound and unbound hormone were separated using a DCC method. Calculations of results were performed as described in the legend of Table 1.

To measure the downregulation of the cytosolic ER and the induction of the PR 60,000–70,000 cells were seeded per well of a six-well culture plate (Costar, Cambridge, MA, U.S.A.) and cultured for three days in the presence of 10^{-12}–10^{-6} M concentrations of BE or ME. 10^{-8} M estradiol was used as a reference for maximal downregulation of the cytosolic ER and the induction of the PR. To analyse the receptor content, cells from three equally treated wells were harvested by trypsination and broken by three freeze thaw cycles in liquid nitrogen. Cytosols were prepared as described above. ER and PR contents were measured in a single point assay. We used 5 nM concentrations of estradiol for the quantitation of the ER and 5 nM concentrations of ORG 2058 for the quantitation of the PR. Unspecific binding was assessed in the presence of a 200 fold molar excess of unlabelled DES for ER determination and a 200 fold molar excess of unlabelled ORG 2058 (Amersham, Braunschweig) for

Table 1. Competitive Scatchard analysis

Set of tubes	[^{3}H]estradiol (nM)	Hormone		Fold molar excess	Parameter assayed
		Substance	nM		
(1)	0.1–3	—	—	—	Total binding
(2)	0.1–3	DES	600	—	Unspecific binding
(3)	0.1–3	Estradiol	1.5	15–0.5	Reference
(4)	0.1–3	BE or ME	1.5	15–0.5	Experiment 1
(5)	0.1–3	BE or ME	3	30–1	Experiment 2

All subsets of vials contained [^{3}H]estradiol (0.1–3 nM). In (1) total binding was assessed in the presence of radioactive estradiol only. In (2) unspecific binding was measured by adding 0.6 μM of DES to an additional subset of vials (200 fold excess to the highest concentration of radioactive estradiol used). As reference (3) we used the competition obtainable by 1.5 nM estradiol. In the experimental section of the analysis (4) and (5), we determined the competition obtainable by substituted ligands and added 1.5 nM (4) or 3 nM (5) of BE or ME as competitor. The molar excess of unlabelled hormone relative to the radiolabelled estradiol in the incubations of lines (3) to (5) is given. Binding parameters were calculated by subtraction of counting data of vials containing identical amounts of radioactive ligand: 1. *Specific-binding*: cpm (1) minus cpm (2). 2. *Reference*: cpm (3) minus cpm (2). 3. *Competition by substituted ligands*: cpm (4) or cpm (5) minus cpm (2). Each subset of binding data was transformed according to Scatchard ([20]; Fig. 1).

PR quantification. Samples were incubated overnight (16–18 h) at 0°C. Bound and unbound hormone were separated by a DCC method.

Northern-blotting

Cells were seeded in 175 cm^2 culture flasks and cultured in steroid-free medium. Prior to harvesting cells were treated for 48 h either with 10^{-8} M estradiol, or 10^{-8} M 2-ME or 4-ME, or 10^{-7} M 2-BE or 4-BE or ethanol, used as a vehicle. The medium was changed and the hormonal treatment repeated after one day. Cells from equally treated flasks were harvested by trypsination and RNA consequently isolated by a guanidinium isothiocyanate/cesium chloride procedure [21]. After electrophoresis of 10–20 μg of total RNA on an agarose gel containing mercuric hydroxide, RNA was blotted onto a nylon membrane. For hybridization the filter was always first incubated with a radiolabelled, SP6 polymerase derived antisense pS2 RNA probe [22]. After autoradiography the pS2 probe was removed and a second hybridization using a 36B4 antisense RNA probe, which was either radiolabelled (methylestrogens) or digoxigenin-labelled (bromoestrogens; labelling kit purchased from Boehringer, Mannheim, Fed. Rep. Germany) was carried out. Digoxigenin-labelling was used for the detection of 36B4 mRNA in the experiments with BE, since during our experiments with ME radioactivity due to pS2-hybridizations could not always be removed completely on all blots. To overcome this difficulty in the cases of BE we used a digoxigenin-labelled probe to rehybridize filters in order to detect 36B4 mRNA. The expression of 36B4 was used as a control since it is constitutively expressed in MCF-7 cells [23]. The relative inductive capacity of ring-A substituted estrogens with respect to pS2 mRNA expression was assessed semiquantitatively by densitometric analysis.

RESULTS

To test if the ER occupied by estrogens substituted by a methyl or bromo group at C-atom 2 or 4 of the aromatic ring of the estradiol molecule is functional in MCF-7 breast cancer cells, we set up 4 different assays. In comparison to estradiol we performed (1) a competitive receptor binding analysis with the cytosolic ER prepared from MCF-7 cells, thereby attempting to evaluate the relative binding affinities of these ring-A derivatives of estradiol. (2) We tested if the substituted estrogens were capable of downregulating the cytosolic ER. (3) We investigated if these hormonal compounds were able to induce the PR, and (4) finally, we asked if they can induce the pS2 mRNA.

Relative binding affinities of ME and BE

The cytosolic ER of the M1 substrain of MCF-7 cells used in this study was characterized by Scatchard analysis. We found a mean K_d of $4.1 \pm 1.2 \times 10^{-11}$ M for estradiol and a mean amount of available cytosolic binding sites of 333 ± 129 fmol/mg protein in a culture medium containing no phenol red and only DCC treated serum. The relative binding affinities of 2-BE, 4-BE [Fig. 1(a)], and 2-ME and 4-ME [Fig. 1(b)] were measured by a competitive ligand binding assay based on a Scatchard analysis. As a reference we show the competition achieved by a 0.5 fold excess of unlabelled estradiol (Fig. 1). In each individual experiment we determined in parallel the relative competitive capacity of a 0.5 fold excess of unlabelled 2-ME and 4-ME (Fig. 1) or 2-BE and 4-BE (Fig. 1). The relative binding affinities (% binding affinity compared to estradiol) were calculated from the slopes of the curves obtained in the Scatchard analysis in the presence of unlabelled estradiol as compared to the substituted estradiol derivative. The mean values of the relative binding affinities from 8–10 experiments per ligand have been summarized in Table 2. As can be deduced from this table 2-ME had the highest relative binding affinity, 4-ME and 4-BE were less but similarly potent. Finally, the lowest relative binding affinity was measured for 2-BE.

Downregulation of the cytosolic ER

We measured downregulation of ER and induction of PR in 10 experiments for 2-ME, 9 experiments for 4-ME, 5 experiments for 2-BE and 6 experiments for 4-BE either in a concentration dependent manner or at 10^{-8} M concentrations of the hormones. During these experiments cells were kept in a steroid-free medium for two passages. Thereafter they were cultured in the presence of estrogens for another 3 days. As a reference and for standardization, cells were grown in the presence of 10^{-8} M estradiol. For comparison the downregulation of the cytosolic ER, as measured in a single point analysis, caused by estradiol from 426 ± 140 fmol/mg in medium containing DCC-stripped serum only ($n = 22$) to 29 ± 21 fmol/mg in medium containing

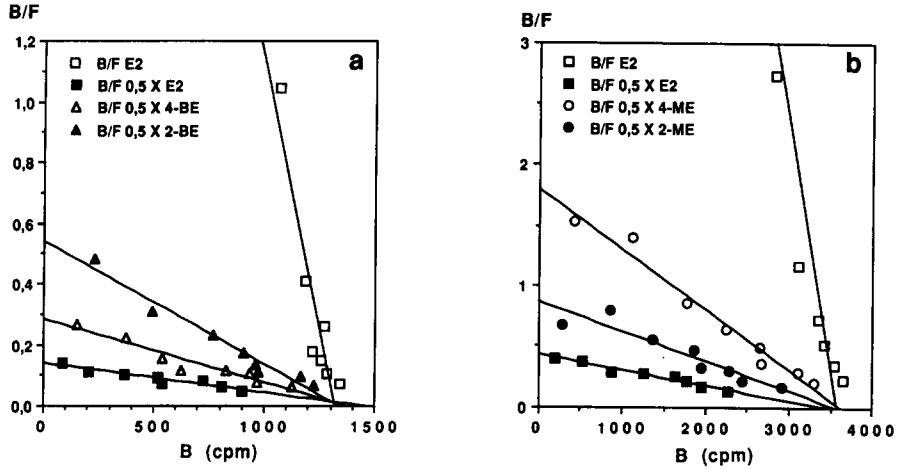

Fig. 1. Relative binding affinities of methyl- and bromoestrogens. MCF-7 breast cancer cells were maintained in a 1:1 mixture of DMEM/F12 medium containing 10% FCS and routinely split in a 1:10 ratio. Two passages prior to the experiment cells were transferred to a steroid-free DMEM/F12 medium without phenol red and 10% DCC stripped FCS. To evaluate relative binding affinities, cells from three 175 cm^2 tissue culture flasks were harvested by trypsination. Cells were broken by 3 freeze thaw cycles in liquid nitrogen and the cytosols were prepared by ultracentrifugation (144,000 *g*; for 45 min at 4°C). The relative binding affinities of 2-ME, 4-ME, 2-BE and 4-BE were measured in a competitive ligand binding assay based on a Scatchard analysis (a) and (b). As a reference we used the competition obtainable by a 0.5 fold excess of unlabeled estradiol (a) and (b).

additionally 10^{-8} M estradiol ($n = 22$; Fig. 2) was set to 100%. The downregulation for the substituted estrogens was expressed as % downregulation relative to values obtained for estradiol.

All ring-A substituted estrogens used in this study were able to downregulate the concentration of the cytosolic ER in a concentration dependent manner (Fig. 3). If the concentration of hormone needed for half maximal downregulation, which can be depicted from the curves in this figure, is used for comparison, 2-ME in this assay again was the most potent derivative. Both 4-ME and 4-BE were almost equally but less potent, whereas 2-BE had the lowest estrogenic potency in this assay. The same consecutive order in relative estrogenic strength is found if the relative downregulation of the cytosolic ER at 10^{-9} M concentrations of the derivatives is compared.

Table 2. Relative binding affinities of 2- and 4-BE and 2- and 4-ME

	Relative binding affinity	
Hormone derivative	*n*	%
2-BE	9	17.4 ± 9.3
4-BE	10	32.1 ± 12.7
2-ME	8	46.9 ± 5.3
4-ME	9	25.4 ± 4.1

In this table the mean values for the relative binding affinities of 2- and 4-BE, as well as 2- and 4-ME are summarized. The values are expressed as percent binding affinity relative to the natural ligand estradiol. *n* = number of experiments.

Induction of the PR

The induction of the cytosolic PR was measured in the identical subset of cultures as was the downregulation of the cytosolic ER content. Treatment with 10^{-8} M estradiol increased the PR content from 391 ± 156 fmol/mg ($n = 22$) measured in culture medium containing only DCC treated serum to 1007 ± 265 fmol/mg ($n = 22$; Fig. 2) in the presence of 10^{-8} M estradiol. All ring-A substituted compounds used

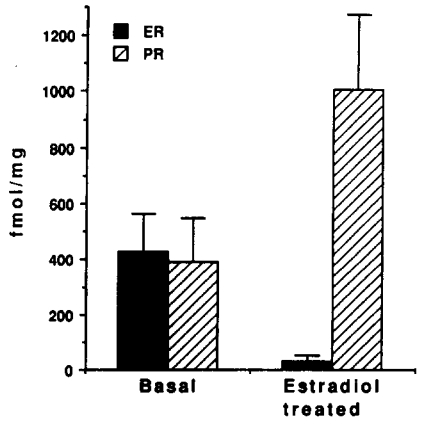

Fig. 2. Regulation of the receptor content by estradiol. In these control experiments we measured the basal levels of ER and PR in medium containing only DCC-stripped serum and after treatment of MCF-7 cells by 10^{-8} M estradiol. The mean values and standard deviations of 22 independent single point determinations are given.

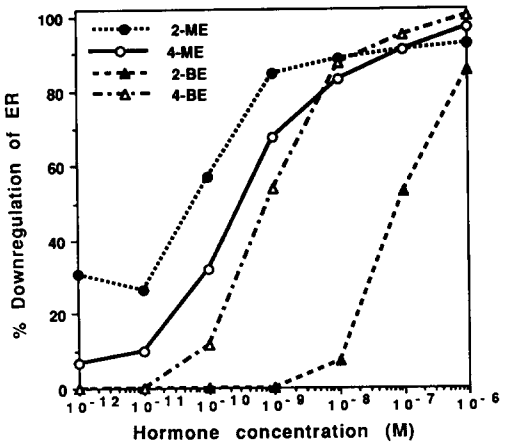

Fig. 3. Downregulation of the cytosolic ER. 60,000–70,000 cells were seeded per well of a six-well culture plate. The cells were kept in a steroid-free culture medium for three days, thereafter they were cultured in the presence of hormones for another three days. Medium and hormones were changed daily. For ER analysis cells from three equally treated wells were collected and the respective cytosol was prepared as described in the legend of Fig. 1. For quantification of the ER content, cytosols were incubated with 5 nM concentrations of tritiated estradiol with and without an 200 fold excess of unlabelled DES.

were capable of inducing the cytosolic PR in a concentration dependent manner (Fig. 4). The highest inductive capacity was observed for 2-ME. Both 4-substituted derivatives were almost equally effective, but less potent. By far the highest amount of hormone was needed

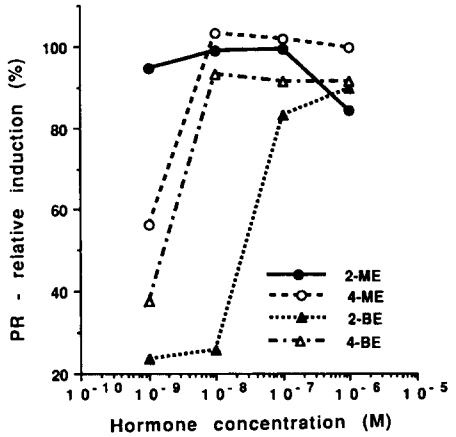

Fig. 4. Induction of the PR. 60,000–70,000 cells were seeded per well of a six-well culture plate. The cells were kept in a steroid-free culture medium for three days, thereafter they were cultured in the presence of hormones for another three days. Medium and hormones were changed daily. For PR analysis cells from three equally treated wells were collected and the respective cytosol was prepared as described in the legend of Fig. 1. For quantification of the PR content, cytosols were incubated with 5 nM concentrations of tritiated ORG 2058 with and without a 200 fold excess of unlabelled hormone. The inductive capacity of the various concentrations of 2-ME, 4-ME, 2-BE and 4-BE is expressed as percentage of maximal induction of the PR by 10^{-9} M estradiol.

in the case of 2-BE to significantly induce the PR.

Induction of the pS2 mRNA

After two passages in steroid-free culture medium MCF-7 cells were treated for 48 h either with 10^{-8} M estradiol as a reference, or 10^{-8} M 2-ME or 4-ME, or 10^{-7} M 2-BE or 4-BE, or ethanol used as a vehicle for control. As shown in Fig. 5 both ME and BE were potent inducers of the pS2 mRNA, as can be deduced from the densitometric scanning of the pS2 and the 36B4 signals. To measure induction, densitometric units of pS2 signals in untreated controls and hormone treated cultures were compared. 36B4 signals within a given blot were used to adjust variations of RNA content of various lanes within one blot. Densitometric analysis of autoradiograms revealed that 10^{-8} M estradiol induces the pS2 mRNA about 12 fold, 2-ME 18 fold and 4-ME about 11 fold, whereas 10^{-7} M concentration of 4-BE only induced pS2 about 10 fold and the same concentration of 2-BE about 8 fold.

DISCUSSION

With this study we were able to demonstrate that the ER occupied by 2-ME, 4-ME, 2-BE or 4-BE is functional in MCF-7 cells. We demonstrated that these hormones compete with estradiol for ER-binding. These hormone derivatives were capable of downregulating the concentration of the cytosolic ER of MCF-7 cells and thereby inducing the production of the cytosolic PR. Finally, all estrogenic ligands studied induced the pS2 mRNA. Taken together, these experiments provide evidence that 2-ME, 4-ME, 2-BE and 4-BE are potent ligands to occupy the ER. We measured higher relative binding affinities of BE to the ER of MCF-7 cells than Petrovskaia and Ivanenko [10] for BE to the ER of rat uterus, but significantly lower than Katzenellenbogen *et al.* [8, 9] did for the binding of halogenated, nonsteroidal hexestrol derivatives to the ER of the lamb uterus. However, as is also clearly indicated by our results, the estimation of the relative binding affinity of respective hormone derivatives to their receptor is not sufficient to predict its relative estrogenic potency. As had been shown, according to the relative binding affinities 4-BE is the more potent receptor ligand if compared to 4-ME, although this difference is not statistically significant as can be deduced from the relative high

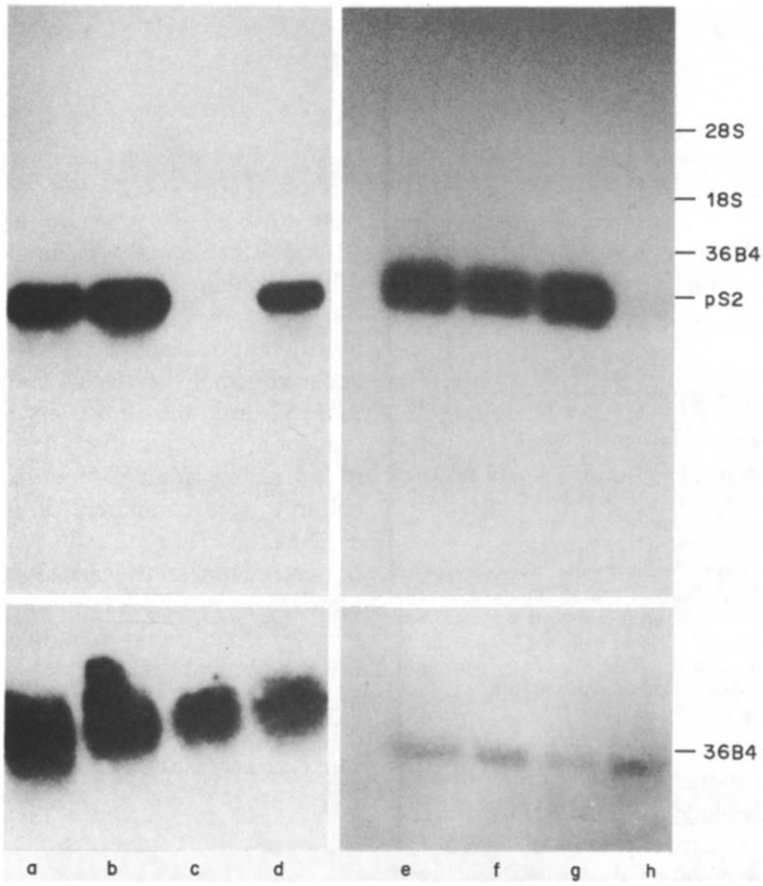

Fig. 5. Induction of the pS2 mRNA. After two passages in steroid-free culture medium the cells were seeded in 175 cm^2 flasks and incubated in medium containing 10% DCC treated FCS. They were incubated with estrogens for 2 days. Controls received the respective amount of ethanol, used as vehicle. The medium was changed and the hormonal treatment repeated after one day. Following trypsination of the cells the RNA was isolated by a guanidinium isothiocyanate/$CsCl_2$ procedure. Total RNA (15–20 μg/lane) was applied to a agarose gel containing methylmercuric hydroxide. After electrophoresis the RNA was transferred to a nylon membrane. The filter was then hybridized with a SP6 polymerase derived antisense pS2 RNA probe. After autoradiography of the filter the pS2 probe was removed and the filter rehybridized to a SP6 derived 36B4 antisense RNA probe, which was either radiolabelled (ME) or digoxigenin-labelled (BE) and which was used as a control. The upper part of the figure shows a pS2 blot. Prospective positions of 36B4 and ribosomal 18S and 28S RNAs are indicated by arrows. The lower part of the figure shows a 36B4 blot. ME: pS2 mRNA from cells treated with 10^{-8} M 4-ME (a) and 10^{-8} M 2-ME (b), from untreated controls (c) and from cells treated with 10^{-8} M estradiol (d) is shown. BE: The figure shows the pS2 mRNA of the 10^{-7} M 4-BE (e) and 10^{-7} M 2-BE treated cells (f), 10^{-8} M estradiol treated cells (g), and untreated cells (h).

standard deviation in experiments with 4-BE. However, in all other assays performed 4-ME was more potent than 4-BE. For the 2-derivatives data for the relative binding affinities and the data for all other parameters measured correlate. Maximal effects at lowest hormone concentrations were demonstrated for the best binder namely 2-ME. Inversely, 2-BE had poor binding affinities to the ER and we needed the highest hormone concentrations to induce maximal effects with this hormone.

In MCF-7 cells the action of 2-BE, 4-BE, 2-ME and 4-ME appears to be purely agonistic and not antagonistic as emphasized recently for 4-ME in uterine tissue [13] and for 2-hydroxyestrone in MCF-7 cells [24]. In addition, preliminary unpublished results of our group indicate that ME and BE exert a mitotic effect on MCF-7 cells grown in steroid-free culture medium. As a conclusion from these results and as a hypothesis we postulate that the natural 2- and 4-hydroxy metabolites of estradiol are also potent estrogenic ligands and most likely have an agonistic function in MCF-7 cells after their binding to the ER. This hypothesis is further evidenced by the observation that catecholestrogens bind to the ER of various organs with high affinity [2–6]. The question whether or not there additionally exists a specific receptor for catecholestrogens as

postulated earlier [25, 26], can not be clarified with this study.

Another point that has to be discussed is the different potency of BE and ME as receptor ligands. Taken together the results of all parameters analysed we found that ME-derivatives were the more potent estrogenic ligands for the ER in MCF-7 cells than BE-derivatives were. If this finding is due to the smaller size of the ligands or if their lower polarity accounts for this effect remains to be elucidated.

Another interesting finding shows that if the ring-A of the estrogen molecule is substituted by the small methyl group the 2-derivative rather than the 4-derivative is the more potent estrogen. This finding is in accordance with findings on the relative estrogenic potency of ME-derivatives in rat pituitary gland, hypothalamus and uterus [7, 12, 28, 29]. On the other hand substitution by the large bromo group leads to a more potent 4-substituted estrogen. Since both 4-derivatives of estradiol have been found to be almost equally potent receptor ligands, it can be deduced that the substitution of the 2-position of the estradiol molecule by a large substituent somehow must lead to a more pronounced steric hindrance in the interaction of the receptor with the ligand than substitution in the 4-position does.

Taken together, different derivatization of the aromatic ring of estradiol leads to significantly different biological activities of the resulting molecules. In our study 2-ME, 4-ME and 4-BE appeared to be potent ligands for the ER of MCF-7 cells. In addition, they should have the capacity to act as long lasting estrogens since the positions of major metabolic conversion of the molecule is already blocked by a metabolically inert substituent.

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