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Depletion of Estrogen Receptor in Human Breast Tumor Cells by a Novel Substituted Indole that does not Bind to the Hormone Binding Domain

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Steroidal antiestrogens appear to have at least two major modes of action in breast cancer cells, direct antagonism of estrogen binding to its receptor and depletion of estrogen receptors (ER) due to inhibition of dimerization of the receptor and a resultant destabilization of the receptor protein. In a search for other classes of compounds which would act as dimerization inhibitors, a novel substituted indole (8-{2-[1-(4-chlorobenzoyl)-5-hydroxy-2-methyl-1H-indol-3-yl]-acetylamino}octanoic acid butyl-methyl amide, MDL 101,906) was synthesized. Binding of the ER to its consensus response element (ERE) was apparently decreased in nuclear extracts from MCF-7 human breast cancer cell treated with MDL 101,906. This decreased binding was found to be due to depletion of ER based on direct measurement of ER using an enzyme-linked immunoassay. Other transcription factors were apparently unaffected by MDL 101,906 treatment. Whereas depletion of ER with a steroidal antiestrogen was almost complete after 3 h of treatment of MCF-7 cells, the effect of MDL 101,906 took significantly longer to occur, suggesting a fundamental difference in the mechanisms of action of the two drugs. This was also evident in the lack of binding of MDL 101,906 to the hormone binding domain of ER. MDL 101,906 treatment also caused depletion of ER mRNA in MCF-7 cells. Depletion of ER mRNA was noted by 3 h of drug treatment and was apparently almost complete after 24 h of treatment. Depletion of ER from MCF-7 cells led to a dose-dependent decrease in the expression of luciferase by an ERE-driven luciferase reporter gene assay system. The mechanism of MDL 101,906 appears to be unique and additional studies with this chemical class seem to be warranted to assess the potential for therapeutic utility. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

Hormone therapy of breast cancer with the nonsteroidal antiestrogen tamoxifen is well established for the treatment of tumors containing estrogen receptors [1]. However, 30–40% of breast tumors will not respond to tamoxifen despite the presence of estrogen receptors [2] and of those tumors that do respond initially, a very high percentage develop resistance to tamoxifen [3] resulting in tumor recurrence and disease progression. Resistance to tamoxifen may result from a number of different changes in breast cancer cells including, but not limited to:

(1) mutations in the estrogen receptor which cause either decreased binding affinity for estrogens and antiestrogens [4, 5] or a constitutively active, ligand-independent receptor [6, 7];

(2) metabolism or isomerization of tamoxifen to less active or more estrogenic species [8, 9];

(3) reduced accumulation of tamoxifen in resistant cells [10];

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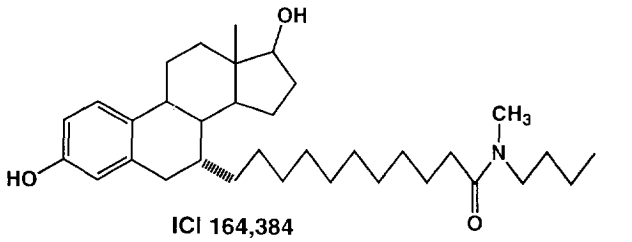
(4) the secretion of autocrine or paracrine growth factors within the tumor that support tumor cell growth in the absence of hormone [11–13]; and 5) increased levels of antiestrogen binding sites [14]. Tamoxifen treatment has also been associated with an increased incidence of endometrial cancer in breast cancer patients [15] and hepatic cancers in rats [16]. Because of these concerns, a search for new antiestrogens with divergent chemical structure continues with the aim of circumventing the problems of resistance and the liability of induction of liver and endometrial cancers.

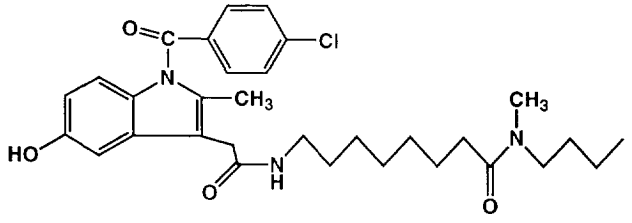
Steroidal, pure estrogen receptor antagonists, ICI 164,384 and ICI 182,780, have no estrogenic effects and are inhibitory to MCF-7 human breast tumors cells and xenografts [17, 18]. These compounds bind potently to the estrogen receptor and block the binding of endogenous hormone. They also cause depletion of the estrogen receptor protein from mouse uterus [19] and human breast cancer cells [20, 21] by inhibiting dimerization of the estrogen receptor [22], a process thought to be involved in binding of the receptor to its response element [23] and probably stabilization of the receptor. The steroidal antiestrogens are derivatives of estradiol which have in common a long alkyl sidechain at the 7a position which has the potential for additional binding to the estrogen receptor outside of the steroid binding site and is critical for the action of the compounds [24]. Although these compounds are effective against some tamoxifen-resistant cell lines and tumors [25], suggesting that some tamoxifen resistance can be circumvented by alternative estrogen receptor antagonists, they are not orally active [17, 18] and, therefore, are not ideal for chronic use.

We have synthesized a novel substituted indole (8-{2-[1-(4-chloro-benzoyl)-5-hydroxy-2-methyl-1H-indol-3-yl]-acetylamino}octanoic acid butyl-methyl-amide, MDL 101,906, Fig. 1) with the aim of finding a new pure antiestrogen that would mimic the activity of the steroidal antiestrogens, but with a more acceptable pharmacological profile. Compounds with structural homology to MDL 101,906, 2-phenylindoles with long alkyl sidechains at the 1-position, were synthesized previously by von Angerer *et al.* [26] and were found to be competitive with estradiol for binding to the estrogen receptor and disrupt estradiol-stimulated transcription. MDL 101,906 depletes estrogen receptor from human breast cancer cells but is not competitive with estradiol for binding to the receptor, thus representing a class of molecules with a potentially novel mechanism of action.

STEROIDAL ANTIESTROGEN AND INDOLE



ICI 164,384



MDL 101,906

Fig. 1. Structures of ICI 164,384 and MDL 101,906.

EXPERIMENTAL

Synthesis

8-(t-Butoxycarbonyl)amino-octanoic acid methylbutyl-amide. The t-butoxy carbonyl derivative of commercial 8-aminooctanoic acid was prepared by standard techniques (di-*t*-butyl dicarbonate, Et_3N, THF). Chromatography (1:1 ethylacetate-hexane, flash silica gel) provided a solid which was used as such. A mixture of 3.4 g (12 mmol) of the above solid, 3.9 ml (33 mmol) of methylbutylamine, 2.5 g (13 mmol) of 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride and 130 mg of hydroxybenzotriazole was stirred in 30 ml of dichloromethane. Aqueous workup yielded 4.5 g of crude product. Flash chromatography with 1:1 EtOAc-hexane yielded 2.1 g (50%) of the title amide as an oil, $R_f = 0.43$ with the column solvent.

8-[5-Methoxy-1-(4-chlorobenzoyl)-2-methyl-1H-indol-3-yl]-acetylamino]octanoic acid methyl-butyl-amide. To 3.23g (10 mmol) of the 8-(*t*-butoxycarbonyl)-protected amide from above in 5 ml of dioxane was added 10 ml of 4 M HCl in dioxane (Aldrich). After 0.5 h, ethyl ether was added. After stirring, an oily residue was allowed to settle. The ether was decanted and discarded. Repeated ether treatment and evaporation from dichloromethane yielded the crude hydrochloride as an oil. To this oil was added 5.3 ml (47 mmol) of *N*-methylmorpholine, 2 g (10 mmol) of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride, 90 mg of hydroxybenzo-triazole and 35 ml of dichloromethane. Stirring over-night followed by aqueous workup (1 M HCl, aqueous $NaHCO_3$, brine) gave a crude product. Chromatography (EtOAc, flash silica gel) yielded 5.1 g (94%) of the title compound ($R_f = 0.36$, EtOAc) as a solid. NMR consistent with structure.

8-[5-Hydroxy-1-(4-chlorobenzoyl)-2-methyl-1H-indol-3-yl]-acetylamino]octanoic acid methyl-butyl-amide (MDL 101,906). To 4.87 g (8.6 mmol) of the 5-methoxy-compound from the previous step, cooled to −40°C in 10 ml of dichloromethane was added 10 ml of 1 M BBr_3 in dichloromethane. The mixture was allowed to warm up to room temperature, stirred overnight, and poured into ice. Extraction with additional dichloromethane

yielded a crude product, which was chromatographed with 5% MeOH-95% dichloromethane to yield a solid. Recrystallization (EtOAc/hexane) yielded 2.15 g (45%) of MDL 101,906, mp 104–106°C (uncorrected).

IR(KBr): 1539 and 1309 cm^{-1}.

^{1}H-NMR (400 MHz, $CDCl_3$): 0.90 (*m*,3H), 1.09 (*m*,2H), 1.2–1.4 (*m*,8H), 1.6 (*m*,4H), 2.96 (S) and 3.02 (S; 3H), 3.22 (*q*, 2H, $\mathcal{J}$ = 5.9), 3.29 (*t*, 1H, $\mathcal{J}$ = 5.8, NH), 6.68 (*dd*, 1H; $\mathcal{J}$ = 8.8, 2.4), 6.84 (*d*, 1H, $\mathcal{J}$ = 8.8), 6.93 (*m*,1H), 7.48 (*d*, 2H, $\mathcal{J}$ = 8.6), 7.67 (*d*, 2H, $\mathcal{J}$ = 8.6).

Anal: Calculated for $C_{31}H_{40}N_3O_4Cl$: C 67.21, H 7.23, N 7.59; found C 66.29, H 7.14, N 7.48. MS: $(M+H)^+$ = 554.

Cell culture

MCF-7 (HTB 22, American Type Culture Collection) human breast tumor cells were grown in improved minimum essential medium (IMEM, Biofluids) without phenol red, supplemented with 4 μg/ml bovine insulin, 0.01 mg/ml gentamicin, 5 mM glutamine, and 5% charcoal stripped calf serum (Cocalico Biologicals). Cells were used between passages 20 and 40 in charcoal-stripped serum.

Nuclear extracts

Monolayers of MCF-7 cells were rinsed once with Hank's Balanced Salt Solution (HBSS) and the cells were scraped from the culture dishes into 15 ml conical tubes. The cells were sedimented by centrifugation at 250 ***g*** for 5 min and then suspended in 1 ml of HBSS. Cells were again sedimented at 1200 g for 5 min, 2 packed cell volumes (PCV) of lysis buffer (25 mM HEPES, pH 7.8, 50 mM KCl, 0.5% NP-40, and 0.5 mM dithiothreitol) were added and the cells were kept for 15 min on ice. The lysed cells were subjected to centrifugation for 3 min at 10,000 ***g***, after which the supernatant was decanted and kept as a cytosol fraction. The pellets were suspended in 2 PCV of extraction buffer (25 mM HEPES, pH 7.8, 500 mM KCl, 10% glycerol, and 0.5 mM dithiothreitol), mixed for 20 min by inversion at 4°C and subjected to centrifugation at 10,000 *g* for 20 min. The supernatant (nuclear extract) and cytosol were dialyzed against dialysis buffer (25 mM HEPES, pH 7.8, 50 mM KCl, 10% glycerol, and 0.5 mM dithiothreitol) for 2 h. All buffers used in the preparation of nuclear extracts contained a cocktail of protease inhibitors which included phenylmethylsulfonylfluoride (0.5 mM), leupeptin (0.05 mg/ml), aprotinin (0.05 mg/ml), pepstatin (0.025 mg/ml) and antipain (0.005 mg/ml). Protein concentrations were determined with a BIO-RAD kit. Nuclear extracts and cytosols were stored in aliquots at −80°C prior to use in mobility shift assays or for determination of estrogen receptors.

DNA mobility shift assays

DNA mobility shift assays were performed with double stranded oligonucleotides using modifications of published procedures [23]. To each reaction tube was added 10 μg nuclear or cytosol extract, 2 μg poly (dI · dC) (Pharmacia), 50 mM NaCl, 1 mM dithiothreitol and 10 mM Tris, pH 7.5 in a total volume of 10 μl and the mixture was kept at room temperature for 10 min. In some experiments, supershifting of the ER/ERE complex was shown by the addition of 1 μg of anti-ER antibody (human hinge region specific, StressGen #SRA-1000) to the incubation mixture. The binding reaction, initiated by adding a [^{32}P] 5'-end-labeled synthetic 35-bp oligonucleotide probe (5'-GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3') representing the wild type estrogen responsive element from the *Xenopus* vitellogenin gene [23], a 22-bp probe (5'-ATTCGATCGGGGCGGGGCGAGC-3') which binds the transcription factor SP1 [27], a 21-bp probe (5'-CGCTTGATGAGTCAGCCGGAA-3') which binds the AP-1 transcription factor [28], a 27-bp probe (5'-TCGACTGTACAGGATGTTCTAGCTACT-3') which binds glucocorticoid and progesterone receptors [29], and a 22-bp probe (5'-CCTTTGGCATGCTGCCAATATG-3') which binds the CTF/NF1 transcription factor [30], was incubated at room temperature for 20 min. After addition of 1 μl of electrophoresis sample buffer (50% glycerol, 0.02% xylene cyanol, 0.02% bromophenol blue, 10 mM Tris, pH 7.5) the samples were loaded onto a 6% nondenaturing polyacrylamide gel (Novex) and the bound and unbound oligonucleotide were separated by electrophoresis (20 mA, 15 min). The gels were vacuum dried for autoradiography using Kodak X-Omat film or phosphorimaging using a Molecular Dynamics imaging system. Quantitation of radioactive bands was performed using the phosphorimager.

Estrogen receptor determination

Estrogen receptors were measured in nuclear or cytosol extracts using an enzyme immunoassay kit (Abbott ER-EIA Monoclonal, Abbott Laboratories) containing monoclonal antibodies against the human estrogen receptor following the procedures outlined by the manufacturer. Typically 5–10 μl (10–30 μg of protein) of either nuclear or cytosol extract was sufficient for these assays. The assay has a sensitivity of approximately 1.5 fmol ER/ml in extracts with protein concentrations of 1 mg protein/ml.

Preparation of estrogen receptor and estrogen receptor binding

MCF-7 cells were rinsed with HBSS, scraped into HBSS containing 0.1% (v/v) monothioglycerol (MTG) and sedimented by centrifugation for 10 min at 800 ***g***.

To extract total ER (cytosolic + nuclear), cells were suspended in 2 PCV of high salt extraction buffer [10% (v/v) glycerol, 500 mM KCl in 25 mM HEPES buffer, pH 7.8] [31, 32], subjected to three cycles of freezing/thawing and then mixed continuously for 30 min at 4°C. The extracts were centrifuged at 4°C for 30 min at 12,000 ***g*** and supernatants were stored at −80°C. Competitive binding assays were run in 96 well microtiter plates [33] with conical shaped well bottoms. Drugs (10 mM stock solutions in dimethyl sulfoxide) and nonradioactive estradiol (1 μM in ethanol) were diluted in TE buffer (10 mM TRIS, 1.5 mM EDTA, pH 7.4) containing 0.5% bovine serum albumin [34], 50% (v/v) dimethylformamide and 0.1% (v/v) MTG. Drug dilutions, TE buffer, [2,4,6,7-^{3}H]- estradiol (114 Ci/mmol Amersham) at a final concentration of 2 nM [^{3}H]-estradiol and ER were combined in a final volume of 100 μl, in triplicate, and incubated at 4°C for 16–18 hr. Receptor bound [^{3}H]-estradiol was separated from unbound [^{3}H]-estradiol by the addition of 100 μl of TE/MTG/BSA containing 0.05% dextran T70 and 0.5% Norit A, pH 7.4, and incubation at 4°C for 15 min, followed by 20 min centrifugation at 1200 ***g***. The mean net DPM were determined in 160 μl of supernatant by subtracting the mean of the non specific binding (DPM bound in the presence of 1 μM nonradioactive estradiol).

pVETLUC construction and transient transfection

The pVETLUC plasmid was kindly provided by Steven Busch and Gary Martin of Marion Merrell Dow Research Institute and was prepared as follows. The pGL2-basic vector (Promega) was digested with the restriction endonucleases, SmaI and XhoI, in the multicloning region of the plasmid. A DNA fragment containing two copies of the vitellogenin estrogen response element (ERE), 5'- AGC TTC TTA TCC AGG TCA GCG TGA CCG TCT TAT CCA GGT CAG CGT GAC CG-3', adjacent to a 180 bp fragment encoding the thymidine kinase (tk) promoter was inserted upstream to the luciferase gene [35].

MCF-7 cells were maintained in IMEM plus 5% fetal bovine serum. On the day of electroporation, cells were trypsinized and suspended in OptiMEM at 2×10^6 cells/ml. Plasmid DNA was added to the cell suspensions (50 mg/ml pVETLUC) in an electroporation chamber (GIBCO-BRL), then subjected to a charge (500 volts/cm, 800 microfarads, 0°C, low resistance). After a 1 min recovery period, the cells were suspended in growth medium and plated in 96-well plates at 2×10^4 cells/well. The next day, growth medium was replenished. Estradiol, either alone or with test compounds, was added to the wells and left in the cultures for 18–22 hr. The cells were harvested by washing once with HBSS, followed by addition of 120 ml lysis buffer (Promega). After 20 min agitation at room temperature, the lysates were analyzed for luciferase (Promega assay system) with a luminometer.

The plasmid pCMVβ (Clontech, Palo Alto, CA) encodes a full length β-galactosidase gene driven by the cytomegalovirus (CMV) immediate early gene promoter enhancer. This plasmid was introduced into MCF-7 cells using the same procedure as for pVETLUC. β-Galactosidase activity was measured using a chemiluminescent assay system (Galacto-Light, Tropixs, Bedford, MA).

RNA Isolation and Northern Blotting

Total RNA from MCF-7 cells was prepared using a *Rapid* Total RNA Isolation Kit (5 Prime → 3 Prime, Inc.). Cells which were approximately 90% confluent in a 100 mm culture dish were lysed directly with 4 M guanidium isothiocyanate and then extracted twice with phenol:chloroform:isoamyl alcohol and precipitated with isopropanol. The RNA pellets were washed several times with ethanol, dissolved in sterile water and stored frozen at −70°C until use. The absorbance 260 nm/280 nm ratio of the purified RNA was always in excess of 2.0. RNA was then fractionated by electrophoresis through a 1% agarose gel containing 7% formaldehyde, 20 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS), 1 mM EDTA and 8 mM sodium acetate (pH 7.0). RNA was transferred to nitrocellulose membranes (GIBCO/BRL), air dried and then baked for 2 h at 80°C in a vacuum oven. Prehybridization and hybridization was performed essentially as described by Jiang and Jordan [36]. The membrane was prehybridized at 47°C in a buffer (GIBCO/BRL) containing 6X SSC (pH 7.0), 5X Denhardt's solution, 100 mg sheared salmon sperm and 50% formamide for 2 h. Membranes were then hybridized overnight in the same buffer containing approximately 2×10^6 dpm/ml of radiolabeled probe. The ER probe was a 1.8 kb cDNA isolated by *Eco*RI digestion of the HEGO expression vector [37, 38], kindly provided by Dr Pierre Chambon, and the β-actin probe was a 2.0 kb cDNA purchased from Clontech (cat. # 9800-1). The cDNA probes were labeled with [^{32}P] using random primer extension (RadPrime DNA Labeling System, GIBCO/BRL) and [α-^{32}P]-deoxycytidine triphosphate (3000 Ci/mmol, New England Nuclear). Membranes were washed three times (1 h each) at room temperature in 2X SSC containing 0.2% sodium dodecyl sulfate (SDS) and 10 min at 65°C with 0.1X SSC containing 0.2% SDS. Quantitative analysis of the hybridized probes was performed using a Phosphorimager (Molecular Dynamics) and ImageQuant software.

RESULTS

Nuclear extracts made from MCF-7 cells contain high concentrations of estrogen receptors (ER) which bind to a 35 base pair, [^{32}P]-labelled consensus oligonucleotide (the estrogen response element, ERE) in

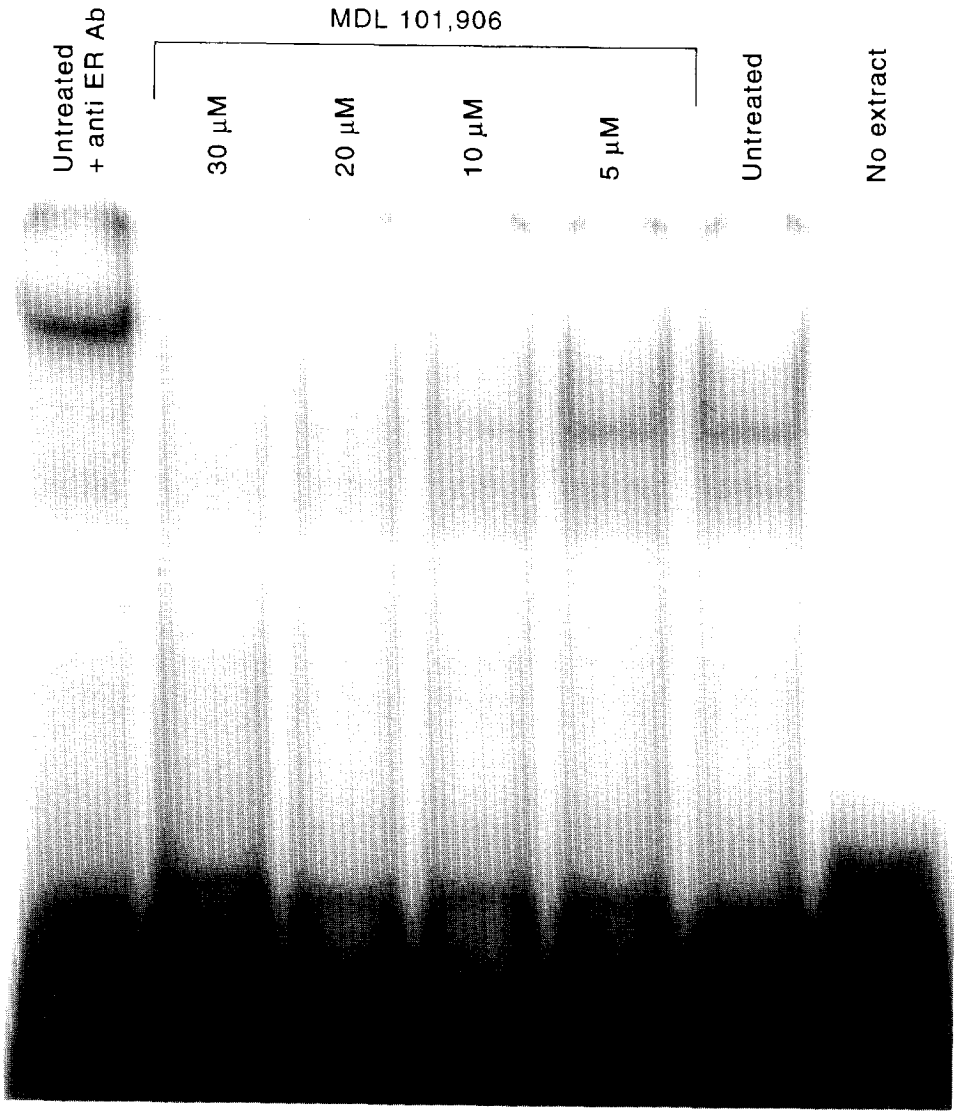


Fig. 2. Effect of MDL 101,906 Treatment on MCF-7 ER DNA Binding Activity. MCF-7 cells were incubated for 24 h either in the absence (untreated) or presence of MDL 101,906 (5-30 μM) followed by nuclear extract preparation. DNA mobility shift assays were performed as described in the Experimental section using 10 μg of nuclear protein for each incubation. Anti-ER antibody (1 μg) was added to one of the incubations to "supershift" the ER-ERE complex to determine which radioactive band was the specific complex. Autoradiography and quantitation were done by phosphorimage analysis.

DNA mobility shift assays (Fig. 2). Specificity of the binding was demonstrated using an anti-estrogen receptor monoclonal antibody to supershift the estrogen receptor/oligonucleotide complex. When the MCF-7 cells were incubated for 24 h in the presence of increasing concentrations (5–30 μM) of MDL 101,906 the specific estrogen receptor binding of the olignucleotide was decreased in a dose-dependent fashion. The ER/ERE band was quantified using phosphorimaging and inhibition was found to be 22, 53, 77 and 86% when 5, 10, 20 and 30 μM MDL 101,906, respectively, was added to the cell cultures. The calculated IC_{50} was 9.5 μM. Inhibition was greatest at 30 μM MDL 101,906, which was the highest concentration that could be added to MCF-7 cells without causing >10–20% cytotoxicity in clonogenic assays. MDL 101,906 had no direct inhibitory effect on the DNA mobility shift assay when added at concentrations up to 50 μM, demonstrating the need for preincubation of the MCF-7 cells with the drug to observe inhibition (data not shown).

The decrease in ER/ERE binding observed in the DNA mobility shift assays was apparently relatively specific. The binding of other radiolabeled consensus oligonucleotides to proteins from MCF-7 cell nuclear and cytosolic extracts was examined and found to be largely unaffected. The binding of the oligonucleotides specific for the transcription factors SP1, CTF/NF1, AP-1 and the gluococorticoid and/or progesterone receptors did not change in extracts from cells treated for 24 h with 20 μM MDL 101,906 (Fig. 3). In fact, the binding of AP-1 to its consensus oligonucleotide was increased slightly. Strong binding to the glucocorticoid response element (GRE) could only be shown

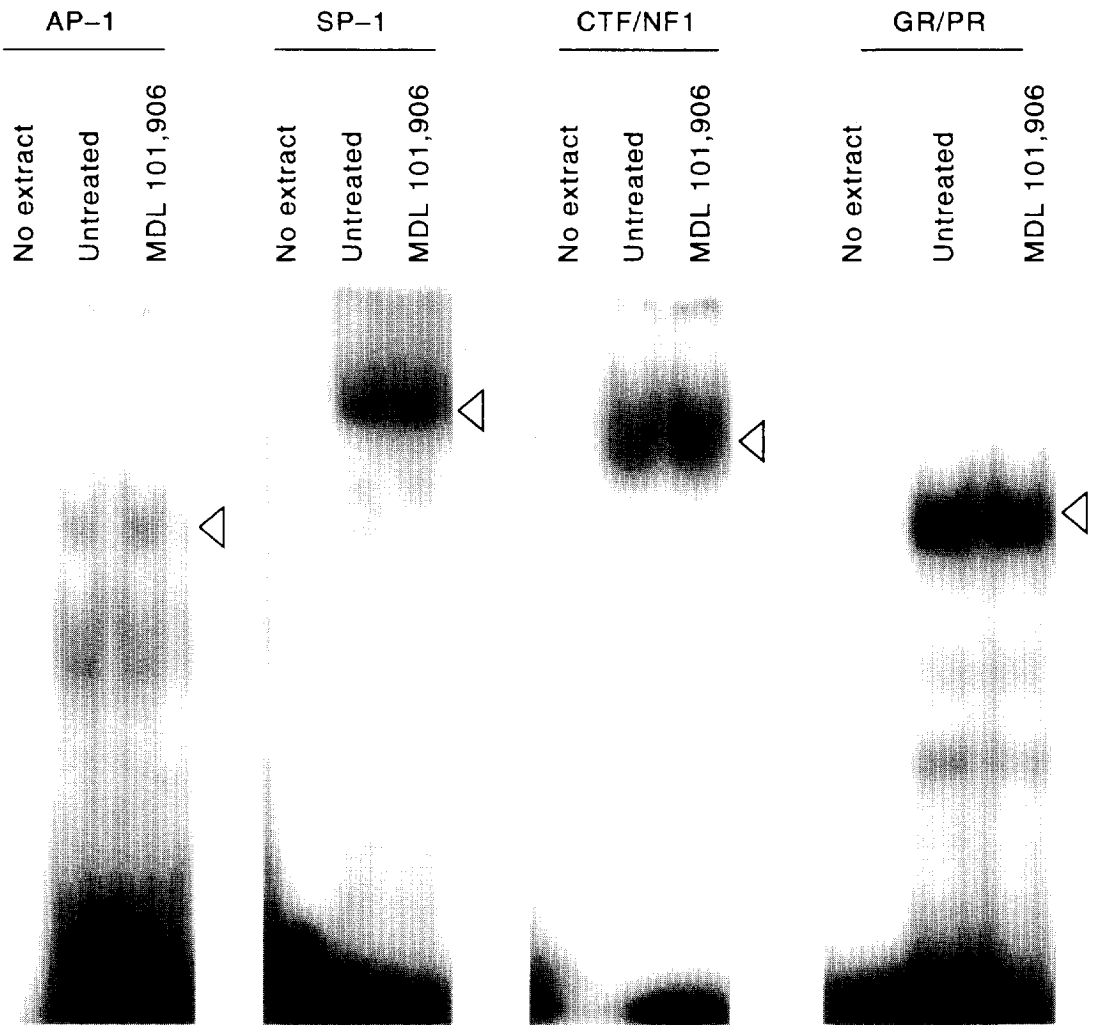


Fig. 3. Lack of Effects of MDL 101,906 on Transcription Factor Binding to DNA. The binding of the AP-1, SP-1, CTF/NF1 and progesterone/glucocorticoid receptor transcription factors to their consensus DNA response elements was examined using a DNA mobility shift assay as described in the Experimental section. MCF-7 cells were treated with 20 μM MDL 101,906 for 24 h prior to preparation of nuclear and cytosolic extracts. Each assay contained 10 μg of nuclear (AP-1, SP-1, CTF/NF1) or cytosolic (progesterone/glucocorticoid receptors) protein. Autoradiography was done using Kodak X-OMat film followed by densitometric imaging using a Personal Densitometer from Molecular Dynamics.

with cytosolic extracts. Binding with nuclear extracts was much less for the GRE and there were no differences in the binding between treated and control extracts.

The effects of MDL 101,906 on ER/ERE binding in nuclear extracts from MCF-7 cells was compared to the effects of ICI 164,384 in an experiment which examined the time course of inhibition. Inhibition of ER/ERE binding was rapid with 1 μM ICI 164,384, being observed at 3 h after addition of the drug to the cell cultures, whereas 30 μM MDL 101,906 had virtually no effect on the binding even at 6 hr (Fig. 4). Binding was inhibited maximally by MDL 101,906 at 24 h. The effects of both drugs on nuclear extracts were dependent on incubation of the cells with the drugs and subsequent preparation of nuclear extracts, because neither MDL 101,906 nor ICI 164,384 was inhibitory to ER/ERE binding when added directly to the DNA mobility shift assays (not shown).

It was shown previously that ICI 164,384 depletes ER from cells treated with the drug. Because this mechanism of action might explain our data with MDL 101,906 as well, the ER levels were measured in nuclear extracts from drug-treated MCF-7 cells using an ER-enzyme immunoassay procedure (ER-EIA). Nuclear ER decreased markedly in nuclear extracts from cells treated with increasing doses of MDL 101,906 (Fig. 5) and the decrease correlated well with the inhibition of ER/ERE binding shown in the DNA mobility shift assay above. A time course for changes in ER levels was also performed (inset to Fig. 5) and paralleled the results of the DNA mobility shift assay, i.e. MDL 101,906 was markedly inhibitory at 24 hr but not at earlier time points at which ICI 164,384 already had almost maximum effect.

MDL 101,906 was examined for its affinity for the ER in a competitive binding assay. Whereas, unlabelled estradiol competed for [^{3}H]-estradiol binding to MCF-7

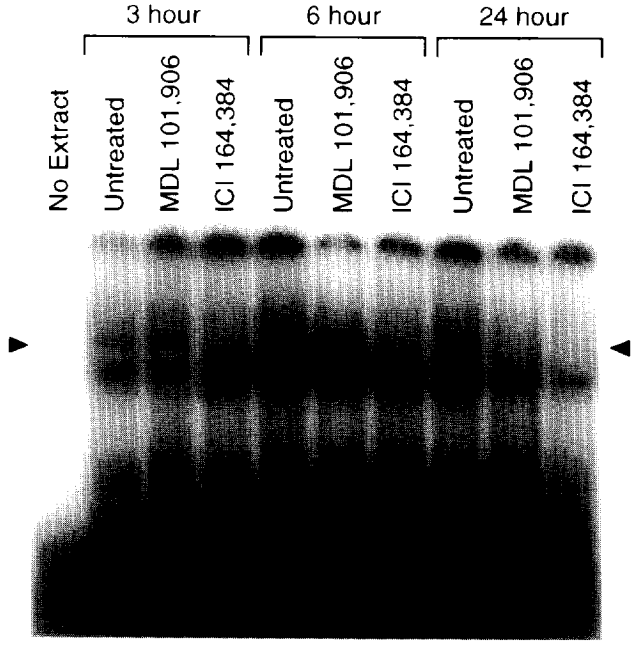


Fig. 4. Time Course of Inhibition of ER Binding to DNA During Treatment of MCF-7 Cells with MDL 101,906. MCF-7 cells were incubated either in the absence of or presence of 30 μM MDL 101,906 for either 3, 6 or 24 h and nuclear extracts were prepared. DNA mobility shift assays were performed as described in the Experimental section with 10 μg of nuclear protein added in each incubation. Autoradiography was performed using a Phosphorimager.

ER (IC_{50} = 7 nM), MDL 101,906 at concentrations up to 20 μM had no effect on [^{3}H]-estradiol (Fig. 6). The steroidal antiestrogen ICI 164,384 binds to the ER [16] and MDL 101,906 is thus differentiated from this other compound which has similar biochemical effects.

Because of the great differences in the time course of inhibition of ER/ERE binding observed between

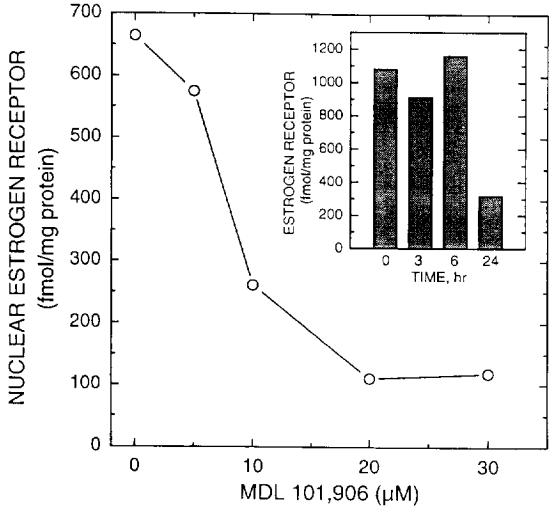


Fig. 5. Dose–Response and Time–Course for Depletion of ER Protein by Treatment of MCF-7 Cells with MDL 101,906. MCF-7 cells were incubated for 24 h in the absence or presence of the concentrations of MDL 101,906 indicated (5-30 μM). ER protein was measured in nuclear extracts using an ER-EIA. The inset graph shows the time course of ER depletion in response to addition of 30 μM MDL 101,906 to MCF-7 cells.

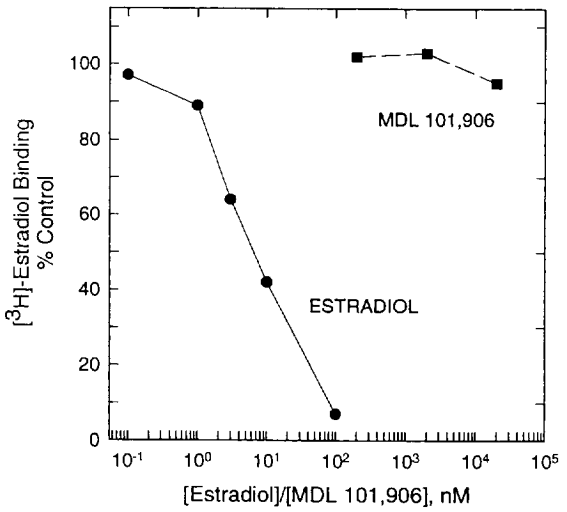


Fig. 6. Lack of Binding of MDL 101,906 to the hormone binding site bof ER. The binding of [^{3}H]-estradiol to ER was measured in whole cell extracts of MCF-7 cells using a standard competitive binding assay as described in the Experimental section. MDL 101,906 was added to the binding assays at 0.2, 2 and 20 μM. Unlabelled estradiol was added to the assays at concentrations of 0.1-100 nM. Assays were incubated overnight and unbound [^{3}H]-estradiol was separated from bound [^{3}H]-estradiol with charcoal.

MDL 101,906 and ICI 164,384 and the lack of competition of MDL 101,906 for [^{3}H]-estradiol binding to the ER, the possibility that MDL 101,906 has a fundamentally different mode of action from that of ICI 164,384 was considered. Northern blotting of total RNA extracted from MCF-7 cells treated with MDL 101,906 and subsequent hybridization with a probe for ER resulted in finding that the levels of ER mRNA were greatly reduced by treatment with 30 μM MDL 101,906 (Fig. 7). In this experiment levels of mRNA were found to be inhibited 72, 74 and 92% at 3, 6 and 24 h, respectively, by quantitative phosphorimaging. The β-actin mRNA remained essentially at control levels at 3 and 6 hr (97% and 98% of control, respectively) and dropped slightly to 71% of control levels at 24 hr. Thus the time course for inhibition of ER mRNA by MDL 101,906 was much more rapid than depletion of the ER protein itself, suggesting that the decrease in ER protein was primarily a result of decreased ER message rather than a destabilization of ER.

To show that there were functional consequences of depletion of the ER by MDL 101,906, we examined the effects of drug treatment on ER responsive luciferase reporter gene expression in transfected MCF-7 cells. Inhibition of the expression of luciferase was dependent on the dose of MDL 101,906 and the IC_{50} was approximately 9.5 μM (Fig. 8), similar to the values obtained for the inhibition of DNA mobility shift assays and the depletion of ER measured by ER-EIA. In a similar type of experiment, a non ER responsive plasmid vector, pCMV-β-gal, was transfected into MCF-7 cells. Its expression was found to be unaffected by MDL 101,906 treatment (inset to Fig. 8).

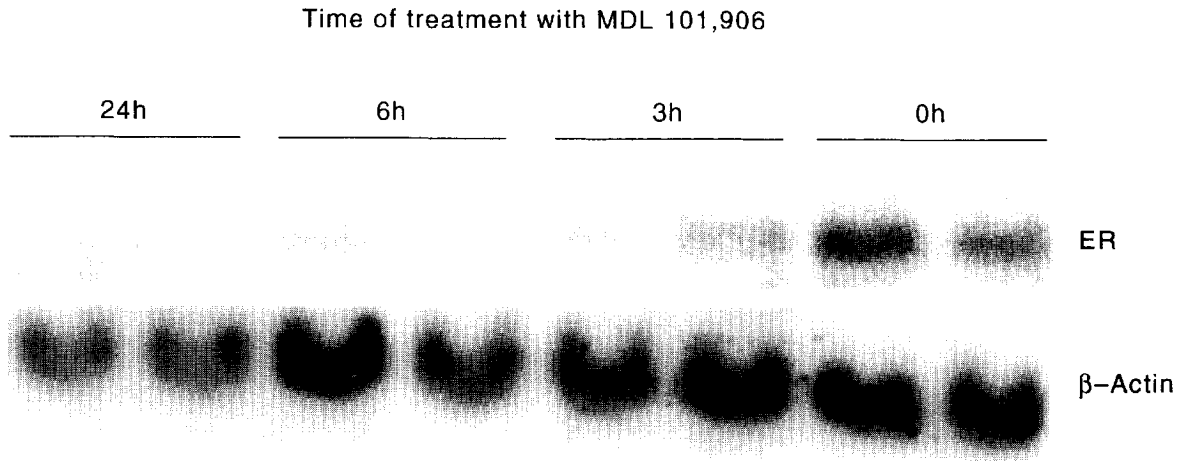


Fig. 7. Northern Blot Analysis of ER mRNA. MCF-7 cells were incubated for either 0, 3, 6 or 24 h with 30 μM MDL 101,906. Total RNA was isolated and 20 μg was separated on a formaldehyde gel and then blotted onto a nitrocellulose membrane. The membrane was hybridized with [32P] labelled cDNA probes for ER and β-actin mRNA as described in the Experimental section. Autoradiography was performed using a Phosphorimager.

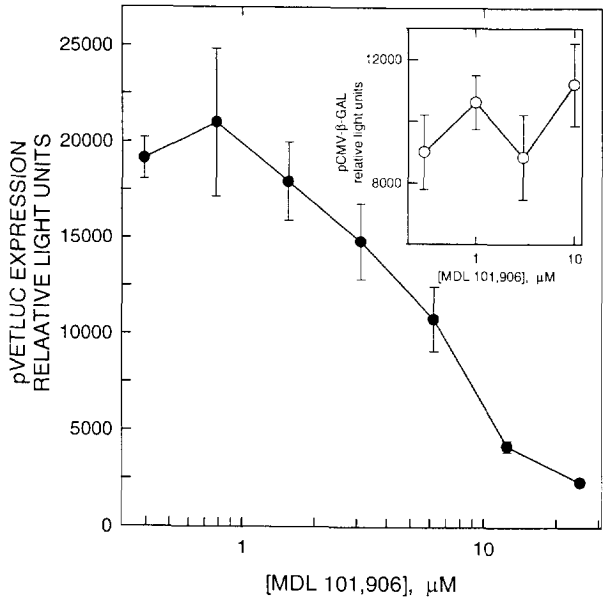


Fig. 8. Inhibition of Luciferase Expression in MCF-7 Cells by MDL 101,906. MCF-7 cells were transiently transfected with either an estradiol-responsive luciferase expression plasmid, pVETLUC, or an estradiol-independent plasmid which expresses β-galactosidase, pCMV-β-GAL (Inset). After 24 h 1 μM estradiol either alone or with MDL 101,906 (0.4-25 μM) was added to the cells and incubation was continued for an additional 24 h. Luciferase and β-galactosidase activities were both measured with a luminometer. Luciferase activity in cells treated only with estradiol was 20,000 ± 3420 relative light units. Control β-galactosidase activity was 9565 ± 495 units.

DISCUSSION

In this paper we have described a novel indole derivative, MDL 101,906, which causes rapid reduction in the ER mRNA and protein levels in the MCF-7 human breast cancer cell nucleus. The functional consequence of reduction in ER is that genes normally regulated by ER, some of which are necessary for growth of breast cancer cells, would no longer be transcribed actively and growth inhibition would result. In fact, MCF-7 cell growth was inhibited by incubation with MDL 101,906 ($IC_{50} = 5.0 \pm 1.8$ μM, n = 4) in a seven day antiproliferative assay. However, the ER-negative cell line, MDA-MB-231 was also inhibited by MDL 101,906 ($IC_{50} = 4.7$ μM, n = 1), suggesting that there is an inhibitory effect of the compound unrelated to the ER. One goal of future studies will be to optimize the compound for potential use in ER-dependent diseases by separating the nonspecific cytotoxic effects of MDL 101,906 from the ER-depleting effects of the compound. The inhibitory effect on gene transcription was shown using an estradiol/ER-responsive luciferase expression vector transiently transfected into MCF-7 cells which was markedly reduced by the incubation of MDL 101,906 in a dose-dependent manner. Presumably endogenous genes dependent on estrogen/ER regulation would also be down-regulated.

Tamoxifen and other non-steroidal estrogen antagonists cause an increase in the estrogen receptor in breast cancer cells [39]. Our data with the steroidal antiestrogen, ICI 164,384, are consistent with previous work which showed that the compound causes rapid depletion of ER protein [20, 21]. This was shown to occur by inhibition of ER dimerization resulting in ER protein destabilization. In our studies, ER depletion with ICI 164,384 was already maximum by three hours, the earliest time point examined. In contrast, MDL 101,906 did not cause significant depletion of ER protein until 24 h. However, MDL 101,906 caused rapid depletion of ER mRNA (72% inhibition within 3 h) whereas, ICI 164,384 was shown previously to have no effect on ER message [19, 20]. Unlike direct estrogen receptor antagonists, MDL 101,906 does not bind to the estrogen receptor at the hormone binding domain, as determined with competitive binding assays. However, we cannot rule out that MDL 101,906 binds to an allosteric site that does not interfere with binding

of estradiol that somehow causes an increase in the turnover of the receptor protein. MDL 101,906 also differs significantly from a series of 2-phenylindoles with long alkyl side chains synthesized by von Angerer *et al.* [26]. These compounds competitively inhibit the binding of estradiol to the ER and inhibit transcription from an ER-dependent luciferase reporter vector, but it is not known whether they can also cause depletion of ER mRNA. The 2-phenylindoles are substituted on the indole nitrogen whereas MDL 101,906 is substituted at the 3 position of the indole ring. This change in orientation of the side chains is obviously of great importance to the biochemical effects of the drugs.

There have been relatively few studies on the stability of ER mRNA and, to our knowledge, the only reports of drug-induced down regulation of ER message levels, are studies which showed that estradiol [40] and 12-*O*-tetradecanoylphorbol-13-acetate [41] decrease expression of ER mRNA by post transcriptional mechanisms. It is not known whether MDL 101,906 blocks transcription of the ER gene or whether the stability of the ER mRNA is modified. These are questions that will be answered in future studies designed to elucidate the mechanism of action of MDL 101,906 more fully.

In conclusion, we have synthesized a novel indole derivative which causes rapid depletion of ER mRNA leading to disappearance of ER protein in human breast cancer cells. New approaches to blocking the function of the ER could prove useful in such ER-dependent diseases as breast cancer, because tamoxifen resistance is a major problem with current therapy and, although the steroidal antiestrogens have been shown to be effective in some tamoxifen resistant breast tumors [42], the potential for development of resistance to these drugs has not yet been widely investigated. Therefore compounds such as MDL 101,906, with alternative sites and modes of action are of interest for further development.

REFERENCES

1. Henderson I. C., Harris J. R., Kinne D. W. and Hellman S.: Cancer of the Breast. In: *Cancer: Principles and Practice of Oncology* 3rd Edition, (Edited by V. T. DeVita, Jr., S. Hellman and S. A. Rosenberg). J. B. Lippincott Co., Philadelphia, PA (1989) pp. 1197–1268.
2. Arafah B. M. and Pearson O. H.: Endocrine Treatment of Advanced Breast Cancer. In: *Estrogen/Antiestrogen Action and Breast Cancer Therapy* (Edited by V. C. Jordan) University of Wisconsin Press, Madison, WI (1986) pp. 417–430.
3. Jordan V. C. and Murphy C. S.: Endocrine Pharmacology of Antiestrogens as Antitumor Agents. *Endocrine Rev.* **11** (1990) 578–610.
4. Katzenellenbogen B. S.: Antiestrogen Resistance: Mechanisms by Which Breast Cancer Cells Undermine the Effectiveness of Endocrine Therapy. *J. Natl. Cancer Inst.* **83** (1991) 1434–1435.
5. Graham M. L., Krett N. L., Miller L. A., Leslie K. K., Gordon D. F., Wood W. M., Wei L. L. and Horwitz K. B.: T47DCO Cells, Genetically Unstable and Containing Estrogen Receptor Mutations, Are a Model for the Progression of Breast Cancers to Hormone Resistance. *Cancer Res.* **50** (1990) 6208–6217.
6. Fuqua S. A. W., Fitzgerald S. D., Chamness G. C., Tandon A. K., McDonnell D. P., Nawaz Z., O'Malley B. W. and McGuire W. L.: Variant Human Breast Tumor Estrogen Receptor with Constitutive Transcriptional Activity. *Cancer Res.* **51** (1991) 105–109.
7. Castles C. G., Fuqua S. A. W., Klotz D. M. and Hill S. M.: Expression of a Constitutively Active Estrogen Receptor Variant in the Estrogen Receptor-negative BT20 Human Breast Cancer Cell Line. *Cancer Res.* **53** (1993) 5934–5939.
8. Osborne C. K., Coronado E., Allred D. C., Wiebe V. and DeGregorio M.: Acquired Tamoxifen Resistance: Correlation with Reduced Breast Tumor Levels of Tamoxifen and Isomerization of *trans*-4-Hydroxytamoxifen. *J. Natl. Cancer Inst.* **83** (1991) 1477–1482.
9. Osborne C. K., Wiebe V. J., McGuire W. L., Ciocca D. R. and DeGregorio M. W.: Tamoxifen and the Isomers of 4-Hydroxytamoxifen in Tamoxifen-Resistant Tumors from Breast Cancer Patients. *J. Clin. Oncol.* **10** (1992) 304–310.
10. Johnston S. R. D., Haynes B. P., Smith I. E., Jarman M., Sacks N. P. M., Ebbs S. R. and Dowsett M.: Acquired Tamoxifen Resistance in Human Breast Cancer and Reduced Intra-tumoral Drug Concentration. *The Lancet* **342** (1993) 1521–1522.
11. King R. J. B., Wang D. Y., Daly R. J. and Darbre P. D.: Approaches to Studying the Role of Growth Factors in the Progression of Breast Tumours from the Steroid Sensitive to Insensitive State. *J. Steroid Biochem.* **34** (1989) 133–138.
12. Kern F. G., Cheville A. L. and Liu Y.: Growth Factor Receptors and the Progression of Breast Cancer. *Sem. Cancer Biol.* **1** (1990) 317–328.
13. King R. J. B.: Receptors, Growth Factors, and Steroid Insensitivity of Tumours. *J. Endocrinol.* **124** (1990) 179–181.
14. Pavlik E. J., Nelson K., Srinivasan S., Powell D. E., Kenady D. E., DePriest P. D., Gallion H. H. and van Nagell J. R. Jr.: Resistance to Tamoxifen with Persisting Sensitivity to Estrogen: Possible Mediation by Excessive Antiestrogen Binding Site Activity. *Cancer Res.* **52** (1992) 4106–4112.
15. Leeuwen F. E., Benraadt J., Coebergh J. W. W., Kiemeney L. A. L. M., Gimbrere C. H. F., Otter R., Schouten L. J., Damhuis R. A. M., Bontenbal M., Diepenhorst F. W., vanden Belt-Dusebout A. W. and van Tinteren H.: Risk of Endometrial Cancer after Tamoxifen Treatment of Breast Cancer. *The Lancet* **343** (1994) 448–452.
16. Hard G. C., Iatropoulos M. J., Jordan K., Radi L., Kaltenberg O. P., Imondi A. R. and Williams G. M.: Major Difference in the Hepatocarcinogenicity and DNA Adduct Forming Ability between Toremifene and Tamoxifen in Female Crl: CD(BR) Rats. *Cancer Res.* **53** (1993) 4534–4541.
17. Thompson E. W., Katz D., Shima T. B., Wakeling A. E., Lippman M. E. and Dickson R. B.: ICI 164,384, a Pure Antagonist of Estrogen-stimulated MCF-7 Cell Proliferation and Invasiveness. *Cancer Res.* **49** (1989) 6929–6934.
18. Wakeling A. E., Dukes M. and Bowler J.: A Potent Specific Pure Antiestrogen with Clinical Potential. *Cancer Res.* **51** (1991) 3867–3873.
19. Gibson M. K., Nemmers L. A., Beckman W. C. Jr., Davis V. L., Curtis S. W. and Korach K. S.: The Mechanism of ICI 164,384 Antiestrogenicity Involves Rapid Loss of Estrogen Receptor in Uterine Tissue. *Endocrinol.* **129** (1991) 2000–2010.
20. Dauvois S., Danielian P. S., White R. and Parker M. G.: Antiestrogen ICI 164,384 Reduces Cellular Estrogen Receptor Content by Increasing Its Turnover. *Proc. Natl. Acad. Sci. U.S.A.* **89** (1992) 4037–4041.
21. Reese J. C. and Katzenellenbogen B. S.: Examination of the DNA-Binding Ability of Estrogen Receptor in Whole Cells: Implications for Hormone-Independent Transactivation and the Actions of Antiestrogens. *Mol. Cell. Biol.* **12** (1992) 4531–4538.
22. Fawell S. E., White R., Hoare S., Sydenham M., Page M. and Parker M. G.: Inhibition of Estrogen Receptor-DNA Binding by the 'Pure' Antiestrogen ICI 164,384 Appears to be Mediated by Impaired Receptor Dimerization. *Proc. Natl. Acad Sci. U.S.A.* **87** (1990) 6883–6887.
23. Kumar V. and Chambon P.: The Estrogen Receptor Binds Tightly to Its Responsive Element as a Ligand-Induced Homodimer. *Cell* **55** (1988) 145–156.
24. Bowler J., Lilley T. J., Pittam J. D. and Wakeling A. E.: Novel Steroidal Pure Antiestrogens. *Steroids* **54** (1989) 71–99.
25. Brunner N., Frandsen T. L., Holst-Hansen C., Bei M., Thompson E. W., Wakeling A. E., Lippman M. E. and Clarke R.: MCF7/LCC2: A 4-Hydroxytamoxifen Resistant Human

Breast Cancer Variant That Retains Sensitivity to the Steroidal Antiestrogen ICI 182,780. *Cancer Res.* **53** (1993) 3229–3232.
26. von Angerer E., Biberger C., Holler E., Koop R. and Leichtl S.: 1-Carbamoylalkyl-2-phenylindoles: Relationship Between Side Chain Structure and Estrogen Antagonism. *J. Steroid Biochem. Molec. Biol.* **49** (1994) 51–62.
27. Anderson F. M. *et al.*: *Current Protocols in Molecular Biology*, Vol. 2, John Wiley and Sons, New York (1989) 12.2.
28. Lee W., Mitchell P. and Tjian R.: Purified Transcription Factor AP-1 Interacts with TPA-Inducible Enhancer Elements. *Cell* **49** (1987) 741–752.
29. Evans R. M.: The Steroid and Thyroid Hormone Receptor Superfamily. *Science* **240** (1988) 889–895.
30. Chodosh L. A., Baldwin A. S., Carthew R. W. and Sharp P. A.: Human CCAAT-Binding Proteins Have heterologous Subunits. *Cell* **53** (1988) 11–24.
31. Sutherland R. L. and Baulieu E. E.: Quantitative estimates of cytoplasmic and nuclear oestrogen receptors in chick oviduct. Effect of oestrogen on receptor concentration and subcellular distribution. *Eur. J. Biochem.* **70** (1976) 531–541.
32. Wakeling A. E.: Anti-hormones and other steroid analogues. In: *Steroid Hormones: A Practical Approach* (Edited by B. Green and R. E. Leake) IRL Press, Oxford (1987) pp. 219–236.
33. 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.
34. Murphy L. C. and Sutherland R. L.: Antitumor activity of clomiphene analogs *in vitro*; relationship to affinity for the estrogen receptor and another high affinity antiestrogen-binding site. *J. Clin. Endocrinol. and Metab.* **57** (1983) 373–379.
35. McKnight S. L. and Kingsbury R.: Transcriptional control Signals of a Eukaryotic Protein-Coding. *Gene Science* **217** (1982) 316–324.
36. Jiang S.-Y. and Jordan V. C.: Growth Regulation of Estrogen Receptor-Negative Breast Cancer Cells Transfected with Complementary DNAs for Estrogen Receptor. *J. Natl. Cancer Inst.* **84** (1992) 580–591.
37. Tora L., Mullick A., Metzger D., Ponglikitmongkol M., Park I. and Chambon P.: The Cloned Human Oestrogen Receptor Contains a Mutation Which Alters Its Hormone Binding Properties. *EMBO J.* **8** (1989) 1981–1986.
38. Berry M., Metzger D. and Chambon P.: Role of the Two Activating Domains of the Oestrogen Receptor in the Cell-Type and Promoter-Context Dependent Agonistic Activity of the Anti-oestrogen 4-Hydroxytamoxifen. *EMBO J.* **9** (1990) 2811–2818.
39. Kiang D. T., Kollander R. E., Thomas T. and Kennedy B. J.: Up-Regulation of Estrogen Receptors by Nonsteroidal Anti-estrogens in Human Breast Cancer. *Cancer Res.* **49** (1989) 5312–5316.
40. Saceda M., Lippman M. E., Chambon P., Lindsey R. L., Ponglikitmongkol M., Puente M. and Martin M. B.: Regulation of the Estrogen Receptor in MCF-7 Cells by Estradiol. *Mol. Endocrinol.* **2** (1988) 1157–1162.
41. Saceda M., Knabbe C., Dickson R. B., Lippman M. E., Bronzert D., Lindsey R. K., Gottardis M. M. and Martin M. B.: Post-transcriptional Destabilization of Estrogen Receptor mRNA in MCF-7 Cells by 12-*O*-Tetradecanoylphorbol-13-acetate. *J. Biol. Chem.* **266** (1991) 17809–17814.
42. Lykkesfeldt A. E., Madsen M. W. and Briand P.: Altered Expression of Estrogen-regulated Genes in a Tamoxifen-resistant and ICI 164,384 and ICI 182,780 Sensitive Human Breast Cancer Cell Line, MCF-7/TAM^{R}-1. *Cancer Res.* **54** (1994) 1587–1595.