PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 87 (2003) 75–83

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Structure activity relationship studies on C2 side chain substituted 1,1-bis(4-methoxyphenyl)-2-phenylalkenes and 1,1,2-tris(4-methoxyphenyl)alkenes

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Received 11 April 2003; accepted 23 June 2003

Abstract

1,1-bis(4-Methoxyphenyl)-2-phenylalkenes (**1a**–**9a**) and 1,1,2-tris(4-methoxyphenyl)alkenes (**1b**–**9b**) with various C2-substituents (H (**1a**, **1b**), methyl (**2a**, **2b**), ethyl (**3a**, **3b**), propyl (**4a**, **4b**), butyl (**5a**, **5b**), 2-cyanoethyl (**6a**, **6b**), 3-cyanopropyl (**7a**, **7b**), 3-aminopropyl (**8a**, **8b**), 3-carboxypropyl (**9a**, **9b**)) were tested for cytotoxic effects on hormone dependent MCF-7 cells. The effects were correlated with agonistic and antagonist properties determined on the MCF-7-2a cell line stably transfected with the plasmid ERE_{wtc}luc. We demonstrated that the antiproliferative effects did not result from an interaction with the estrogen receptor (ER). The most cytotoxic compounds 5,5-bis(4-methoxyphenyl)-4-phenylpent-4-enylamine (**8a**) and 4,5,5-tris(4-methoxyphenyl)pent-4-enyl (**8b**) showed cytocidal effects without having significant agonistic and antagonistic properties.

Keywords: Triarylalkenes; Cytotoxicity; MCF-7 and MDA-MB 231 breast cancer cells

1. Introduction

Tamoxifen (TAM) is widely used in the treatment of breast cancer and is now available in the US for the chemoprevention of breast cancer in high-risk women [1,2]. The long term treatment, however, is limited by an increased incidence of endometrial or GI-tract cancers [3–5]. This liability has resulted in a large research effort to discover agents maintaining the benefits of TAM while avoiding its risks. Such compounds can be assigned to the class of selective estrogen receptor modulators (SERM).

For the characterisation of the agonistic and antagonistic potency of hormonally active compounds, many groups used hormone dependent MCF-7 breast cancer cells endowed with the estrogen receptor (ER). The classification is based on their ability to stimulate (agonist) or inhibit (antagonist) cell growth [6,7]. Detailed information about the hormonal profile of drugs can be obtained with MCF-7 cells stably transfected with the plasmid ERE_{wtc}luc (MCF-7-2a cells) [8]. The plasmid contains the "estrogen response element" (ERE) of the DNA as enhancer sequence and a reporter sequence which codes for luciferase. After binding of a hormonally active drug the ER/drug conjugates dimerize and are able to interact with the ERE of the plasmid leading to the activation of the luciferase gene. Therefore, the quantification of the luciferase expression allows not only a prediction of the agonistic but also of the antagonistic potencies of drugs.

In a structure activity study we investigated the effects of TAM, 4-hydroxytamoxifen (4OHT) and related 1,1-bis(4-hydroxyphenyl)-2-phenylalkenes and 1,1,2-tris(4-hydroxyphenyl)alkenes without basic side chain on the MCF-7-2a cell line [9,10]. We demonstrated that the endocrine properties are independent of the *N*,*N*′-dimethylaminoethane chain and depend only on the length and the kind of the C2-alkyl chain. All compounds showed high antiestrogenic activity without significant agonistic potency. The most active compounds were C2-ethyl or C2-propyl substituted and reduced the estradiol (E2) (1 nM) mediated luciferase expression more than TAM, comparable to 4OHT. Terminal groups at the C2-alkyl residue reduced the antagonistic properties [11].

Interestingly, the E2 antagonism did not influence the proliferation of MCF-7 cells. All of the dihydroxy and trihydroxy substituted 1,1,2-triarylalkenes were inactive, while TAM and 4OHT inhibited the cell growth significantly. In this structure activity relationship study we investigated 1,1-bis(4-methoxyphenyl)-2-phenylalkenes and 1,1,2-tris(4-methoxyphenyl)alkenes to evaluate whether the

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doi:10.1016/S0960-0760(03)00385-6

free hydroxy groups are responsible for diminishing the cytotoxic properties.

2. Materials and methods

2.1. General procedures

IR spectra (KBr pellets): Perkin-Elmer Model 580 A. ^{1}H NMR: ADX 400 spectrometer at 400 MHz (internal standard: TMS). Elemental analyses: Microlaboratory of Free University of Berlin; based on the C, H, and N analyses, all compounds were of acceptable purity (within 0.4% of the calculated values). Liquid Scintillation Counter: 1450 Microbeta^{TM} Plus (Wallac, Finland). Microplate Photometer: Labsystems Multiscan® Plus (Labsystems, Finland). Microlumat: LB 96 P (EG&G Berthold, Germany).

2.2. Syntheses

The 1,1,2-triarylalkenes **1a**–**8a** and **1b**–**8b** were synthesized as described previously [9–11]. The COOH derivatives **9a** and **9b** were obtained by alkaline hydrolysis of 6,6-bis(4-methoxyphenyl)-5-phenylhex-5-enenitrile **7a** and 5,6,6-tris(4-methoxyphenyl)hex-5-enenitrile **7b**:

The respective CN substituted compound dissolved in 5 ml of ethanol was combined with 1 ml NaOH (25%) and was heated to reflux. After 12 h, the half narrowed down solution was acidified with H_2SO_4 (20%) and the precipitate was sucked off. The crude product was dissolved in diethyl ether, washed with water and dried over Na_2SO_4. Subsequently, the solvent was removed under reduced pressure.

2.2.1. 6,6-bis(4-Methoxyphenyl)-5-phenylhex-5-enoic acid (9a)

From **7a** (0.1 g, 0.25 mmol). Yield: 0.08 g (0.21 mmol, 81.5%) of a red–brown solid (mp 120 °C) ^{1}H NMR ([D4]-methanol): δ = 1.66 (m, 2H, CH_2); 2.06 (t, 2H, CH_2); 2.5 (m, 2H, CH_2); 3.67 (s, 3H, OCH_3); 3.83 (s, 3H, OCH_3); 6.56 (AA′BB′ ^3J = 8.8 Hz, 2H, ArH-3, ArH-5); 6.77 (AA′BB′ ^3J = 8.8 Hz, 2H, ArH-2, ArH-6); 6.92 (AA′BB′ ^3J = 8.6 Hz, 2H, Ar′H-3, Ar′H-5); 7.05–7.16 (m, 7H, Ar′H-2/6, Ar″H-2/6). IR (KBr, cm^{-1}): 3493 s, br (OH); 3025 m (ArH); 2947m (CH_2); 1669 m (C=O); 1602 m (C=C); 1573 m (C=C); 1508 m (C=C). MS (EI, 190 °C): *m*/*z* (%) = 402 $[M]^{\bullet+}$ (100); 329 (59.9). CHN $C_{26}H_{26}O_4 \cdot 0.5H_2O$ (411.48): calc.: C 75.89%, H 6.61%; found: C 75.94%, H 6.56%.

2.2.2. 5,6,6-tris(4-Hydroxyphenyl)hex-5-enoic acid (9b)

From **7b** (0.4 mmol, 0.17 g). Yield: 0.12 g (0.28 mmol, 69.4%) of a red–brown solid (mp 109 °C) ^{1}H NMR ([D4]-methanol): δ = 1.63 (m, 2H, CH_2); 2.09 (t, 2H, CH_2); 2.45 (m, 2H, CH_2); 3.66 (s, 3H, OCH_3); 3.72 (s, 3H, OCH_3); 3.80 (s, 3H, OCH_3); 6.55 (AA′BB′ ^3J = 8.8 Hz, 2H, ArH-3, ArH-5); 6.70 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar′H-3, Ar′H-5); 6.75 (AA′BB′ ^3J = 8.8 Hz, 2H, ArH-2, ArH-6); 6.89 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar″H-3, Ar″H-5); 7.03 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar′H-2, Ar′H-6); 7.11 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar″H-2, Ar″H-6). IR (KBr, cm^{-1}): 2952 m (CH_2); 2835 m (OCH_3), 1704 s (C=O), 1607 s (C=C); 1509 s (C=C). MS (EI, 155 °C): *m*/*z* (%) = 432 $[M]^{\bullet+}$ (100); 359 (34.8); 135 (30.8). CHN $C_{27}H_{28}O_5 \cdot H_2O$ (450.54): calc.: C 71.98%, H 6.71%; found: C 71.77%, H 6.84%.

2.3. Biological methods

2.3.1. Materials and reagents for bioassays

Dextran, 17β-estradiol, L-glutamine (L-glutamine solution: 29.2 mg/ml phosphate buffered saline (PBS)) and Minimum Essential Medium Eagle (EMEM): Sigma (Munich, Germany); Dulbecco's Modified Eagle Medium without phenol red (DMEM): Gibco (Eggenstein, Germany); fetal calf serum (FCS): Bio Whittaker (Verviers, Belgium); *N*-hexamethylpararosaniline (crystal violet) and gentamicin sulfate: Fluka (Deisenhofen, Germany); glutaric dialdehyde (25%): Merck (Darmstadt, Germany); trypsin (0.05%) in ethylenediaminetetraacetic acid (0.02%) (trypsin/EDTA): Boehringer (Mannheim, Germany); penicillin–streptomycin gold standard (10,000 IE penicillin/ml, 10 mg streptomycin/ml) and geneticin disulfate (geneticin solution: 35.71 mg/ml PBS): ICN Biomedicals GmbH (Eschwege, Germany); norit A (charcoal): Serva (Heidelberg, Germany); cell culture lysis reagent (5× diluted 1:5 with purified water before use) and the luciferase assay reagent: Promega (Heidelberg, Germany); optiphase HiSafe3 scintillation liquid: Wallac (Turku, Finland); NET-317-estradiol[2,4,6,7-^{3}H(*N*)] (17β-[^{3}H]estradiol): Du Pont NEN (Boston, Maryland); [D4]-methanol: Aldrich (Steinheim, Germany); PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4 \cdot 2H_2O$ and 0.2 g of KH_2PO_4 (all purchased from Merck or Fluka) in 1000 ml of purified water. Tris-buffer (pH = 7.5) was prepared by dissolving 1.211 g of trishydroxymethylaminomethan, 0.372 g of Titriplex III and 0.195 g of sodium azide (all from Merck or Fluka) in 1000 ml of purified water. Deionized water was produced by means of a Millipore Milli-Q Water System, resistivity >18 MΩ. T-75 flasks, reaction tubes, 96-well plates and 6-well plates were purchased from Renner GmbH (Darmstadt, Germany).

2.3.1.1. Estrogen receptor binding assay. The relative binding affinity (RBA) of the test compounds to the ER was determined in a competition test with 17β-[^{3}H]estradiol. For this purpose the test compounds dissolved in ethanol and diluted with Tris-buffer to six–eight appropriate concentrations (300 μl) were incubated shaking with calf uterine cytosol (100 μl) and 17β-[^{3}H]estradiol (0.723 pmol in Tris-buffer (100 μl); activity: 2249.4 Bq per tube) at 4 °C for 18–20 h. Five hundred microliters of a dextran–charcoal-suspension in Tris-buffer was added to each tube to stop the reaction. After shaking for 90 min at 4 °C and centrifugation 500 μl

HiSafe3 was mixed with 100 μl supernatant of each sample. The reactivity was determined by liquid scintillation spectroscopy. The same procedure was used to quantify the binding of 17β-[^{3}H]estradiol (0.723 pmol, control). Non-specific binding was calculated using 4 μmol of 17β-estradiol as the competing ligand. On a semilog plot the percentage of maximum bound labeled steroid corrected by the non-specifically bound 17β-[^{3}H]estradiol versus concentration of the competitor (log-axis) was plotted. At least six concentrations of each compound were chosen to estimate its binding affinity. From this plot those molar concentrations of unlabelled estradiol and of the competitors were determined which reduced the binding of the radioligand by 50%.

$$\text{RBA}\ (\%) = \frac{\text{IC}_{50}\ \text{estradiol}}{\text{IC}_{50}\ \text{sample}} \times 100$$

2.3.1.2. Cell lines and growth conditions. The MCF-7-2a cell line, the MCF-7 cell line and the MDA-MB 231 cell line were maintained as a monolayer culture at 37 °C in a humidified atmosphere (95% air, 5% CO_2) in T-75 flasks. Cell line banking and quality control were performed according to the seed stock concept reviewed by Hay [12]. Growth media: MCF-7-2a cell line: phenol red free DMEM with penicillin/streptomycin 1%, L-glutamine 1%, FCS 5% and geneticin solution 0.5%. MCF-7 cell line: L-glutamine containing EMEM supplemented with $NaHCO_3$ (2.2 g/l), sodium pyruvate (110 mg/l), gentamicin sulfate (50 mg/l) and FCS (100 ml/l). MDA-MB 231 cell line: McCoy's 5A medium supplemented with $NaHCO_3$ (2.2 g/l), sodium pyruvate (110 mg/l), gentamycin (50 mg/l) and 5% FCS.

2.3.1.3. Transcriptional binding assay, luciferase assay. One week before starting the experiment, MCF-7-2a cells were cultivated in DMEM supplemented with L-glutamine, antibiotics and dextran/charcoal-treated FCS (ct-FCS, 50 ml/l). Cells from an almost confluent monolayer were removed by trypsinization and suspended to approximately 2.2×10^5 cells/ml in the growth medium mentioned above. The cell suspension was then cultivated in six well flat-bottomed plates (0.5 ml cell suspension and 2 ml medium per well) at growing conditions (see above). After 24 h, 25 μl of a stock solution of the test compounds was added to achieve concentrations ranging from 10^{-6} to 10^{-11} M and the plates were incubated for 50 h. Before harvesting, the cells were washed twice with PBS and then 200 μl of cell culture lysis reagent was added into each well. After 20 min of lysis at room temperature, the cells were transferred into reaction tubes and centrifuged. Luciferase was assayed using the Promega luciferase assay reagent. Fifty microliters of each supernatant was mixed with 50 μl of substrate reagent. Luminescence (in relative light units (RLU)) was measured for 10 s using a microlumat. Measurements were corrected by correlating the quantity of protein (quantified according to Bradford [13]) of each sample with the mass of luciferase. Estrogenic activity is expressed as percentage activation of a 10^{-8} M estradiol control (100%).

To evaluate the antagonistic activity, the cells were incubated with a constant concentration of E2 (10^{-9} M) and in combination with increased concentrations (10^{-11} to 10^{-6} M) of inhibitor. The percentage activation was calculated in relation to the luciferase expression of E2 alone. The IC_{50} value is taken from the concentration activation curve.

2.3.1.4. Determination of the antiproliferative activity against MCF-7 and MDA-MB 231 human breast cancer cells. Cells from an almost confluent monolayer were harvested by trypsinization and suspended to approximately 7×10^4 cells/ml. At the beginning of the experiment, the cell suspension was transferred to 96-well microplates (100 μl per well). After cultivating them for 3 days at growing conditions, the medium was removed and replaced by one containing the test compounds. Control wells (16 per plate) contained 0.1% of DMF that was used for the preparation of the stock solutions. The initial cell density was determined by addition of glutaric dialdehyde (1% in PBS, 100 μl per well). After incubation for 4–7 days, the medium was removed and glutaric dialdehyde (1% in PBS, 100 μl per well) was added for fixation. After 15 min, the solution of the aldehyde was decanted and 180 μl PBS per well were added. The plates were stored at 4 °C until staining. Cells were stained by treating them for 25 min with 100 μl of an aqueous solution of crystal violet (0.02%). After decanting, cells were washed several times with water to remove the adherent dye. After addition of 180 μl of ethanol (70%), plates were gently shaken for 4 h. Optical density of each well was measured in a microplate autoreader at 590 nm. The effectiveness of the compounds is expressed as:

$$T/C_{\text{corr}}\ (\%) = \frac{T - C_\text{o}}{C - C_\text{o}} \times 100,$$

where T (test) and C (control) are the optical densities of the crystal violet extract of the cells in the wells (i.e. the chromatin bound crystal violet extracted with ethanol 70%), and C_o is the density of the cell extract immediately before treatment. Cytocidal effect:

$$\tau\ (\%) = \frac{T - C_\text{o}}{C_\text{o}} \times 100.$$

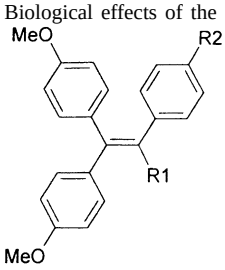
3. Results

The relative binding affinities (RBA values, see Table 1) were determined in a competition experiment with [^{3}H]-E2 and cytosol of calf uterine.[1]

The RBA values were low. Only **2b** (RBA = 1.05%), **3a** (RBA = 0.55%) and **6b** (RBA = 0.62%) possessed an RBA > 0.5%. Although no clear structure activity

[1] This ER source was used to get relative binding affinities, which can be compared with the data published earlier.

Table 1
Biological effects of the 1,1,2-triarylalkenes **1a**–**9a**, **1b**–**9b**



Compound	R1	R2	RBA[a] (%)	Antagonistic effect (%) at 1 μM[b]	Agonistic effect (%) at 1 μM[b]
1a	–H	H	0.05	29.4	23.9
1b	–H	OCH_3	0.06	11.5	13.1
2a	$–CH_3$	H	0.43	12.7	−35.1
2b	$–CH_3$	OCH_3	1.05	7.3	14.0
3a	$–CH_2–CH_3$	H	0.55	39.6	−66.1
3b	$–CH_2–CH_3$	OCH_3	0.26	49.0	9.9
4a	$–CH_2–CH_2–CH_3$	H	0.33	18.5	−67.9
4b	$–CH_2–CH_2–CH_3$	OCH_3	0.34	56.2	12.4
5a	$–CH_2–CH_2–CH_2–CH_3$	H	0.02	44.0	−68.6
5b	$–CH_2–CH_2–CH_2–CH_3$	OCH_3	0.09	38.8	10.4
6a	$–CH_2–CH_2–CN$	H	0.05	4.2	−10.5
6b	$–CH_2–CH_2–CN$	OCH_3	0.62	49.3	−19.9
7a	$–CH_2–CH_2–CH_2–CN$	H	0.01	28.5	−12.7
7b	$–CH_2–CH_2–CH_2–CN$	OCH_3	0.33	50.2	−11.9
8a	$–CH_2–CH_2–CH_2–NH_2$	H	0.05	26.3	13.6
8b	$–CH_2–CH_2–CH_2–NH_2$	OCH_3	0.01	32.3	5.7
9a	$–CH_2–CH_2–CH_2–COOH$	H	0.01	7.9	27.9
9b	$–CH_2–CH_2–CH_2–COOH$	OCH_3	0.01	13.7	−5.6
TAM			1.8	27.66 (IC_{50} = 500 nM)	−19.4
4OHT			15.6	−48.92 (IC_{50} = 7 nM)	−17.7

[a] Relative binding affinity (RBA) (%) = (IC_{50} (E2)/IC_{50} (ligand)) × 100; mean value of three independent determinations.
[b] Agonistic effects (percentage activation of luciferase expression) and antagonistic effects (inhibition of an E2 (1 nM) induced luciferase expression) were determined in hormone dependent MCF-7-2a cells in a concentration range of 10^{-11} to 10^{-6} M. The effects at 1 μM were taken from the respective concentration activity curve.

relationship can be deduced from the data listed in Table 1, it is obvious that hydrophilic terminal groups at the C2-alkyl chain disturb the ER interaction (NH_2: **8a** (RBA = 0.05%) and **8b** (RBA = 0.01%); COOH: **9a** (RBA = 0.01%) and **9b** (RBA = 0.01%)).

The possible gene activation resulting from ER interaction was evaluated in a luciferase assay using MCF-7-2a cells. These ER positive human breast cancer cells are stably transfected with the reporter plasmid ERE_{wtc}luc. The binding of ER/drug dimers at the estrogen response elements of the plasmid activates the luciferase gene. The quantification of the luciferase expression allows a prediction of the agonistic potency, the inhibition of the E2 (1 nM) induced activation correlates with the antagonistic effects of the compounds.

The triarylalkenes were tested for agonistic and antagonistic effects in a concentration range of 10^{-11} to 10^{-6} M. All triarylalkenes failed to evoke transcriptional activation of the luciferase gene, also in the highest used concentration (percentage activation at 1 μM, see Table 1). On the other hand, marginal antagonistic effects were determined for **3b**, **4b**, **6b** and **7b**, which reduced the E2 induced gene activation by about 50% in the highest used concentration of 1 μM (see Table 1). The amino (**8a**, **8b**) and COOH derivatives (**9a**, **9b**) were completely inactive.

Interestingly, **8a** and **8b** influenced the proliferation of MCF-7-2a cells. Therefore, we determined the cytotoxicity of the triarylalkenes **1a**–**9a** and **1b**–**9b** on the hormone dependent human MCF-7 breast cancer cell line in concentrations of 1, 5 and 10 μM over a time period up to 250 h. Time activity curves were created using the measured percentage *T*/*C* values at five different incubations times.

The compounds showed the maximum of activity after 75 h, which is diminished with exception of **8b** during the following time of incubation. Compound **8b** held its effects over 250 h (see Fig. 3).

The antiproliferative effects depend on both, the kind of terminal group at the C2-alkyl chain and the substituents in the aromatic rings (see Fig. 1). The 1,1-bis(4-methoxyphenyl)-2-phenylalkenes **2a**–**5a** showed significant cytotoxic effects and reduced the cell growth up to 50% in a concentration of 10 μM (see Fig. 1, **1a** was inactive). The CN substituted compound **7a** was more active in each of the used concentrations than the respective 1,1-bis(4-methoxyphenyl)-

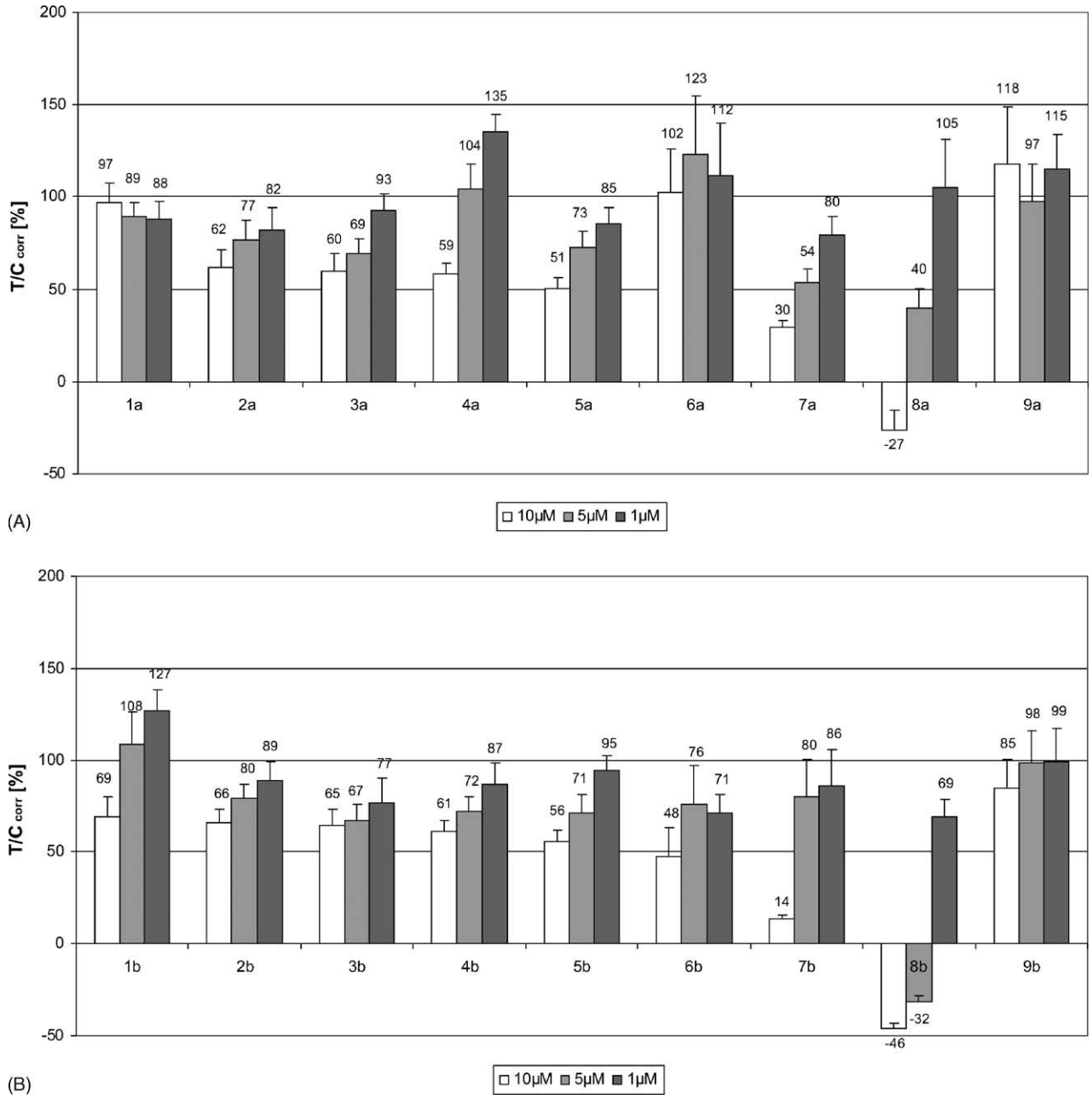


Fig. 1. Cytotoxicity of bismethoxy (A) and trismethoxy (B) substituted 1,1,2-triarylalkenes on the MCF-7 cell line after an incubation time of 75 h. The T/C_{corr} values represent usually the maximal inhibitory effects of the test compounds.

2-phenylpent-1-ene **4a**. Exchange of the CN function by NH_2 (**8a**) enhanced the cytotoxicity further. Compound **8a** showed cytocidal activity ($\tau = -27\%$) in a concentration of 10 μM (Fig. 1). Interestingly, **9a** endowed with a terminal COOH group was completely inactive.

The 4-OCH_3 substitution of the 2-phenyl ring increased the growth inhibitory effects especially of the compounds with terminal CN (**6b**, **7b**) and NH_2 groups (**8b**). The most active compound in this series, **8b** showed cytocidal effects ($\tau = -32\%$) even in a concentration of 5 μM and is therefore more potent than TAM ($T/C_{corr} = 12\%$ at 5 μM) and 4OHT ($T/C_{corr} = 27\%$ at 5 μM) (see Fig. 3).

Interestingly, the ether cleavage reduced the cytotoxicity. The hydroxy substituted NH_2 derivatives **8a**-OH, **8b**-OH did not influence the growth of the MCF-7 cells (see Fig. 2). The same loss of activity was observed for compounds with terminal CN group (**7a**-OH, **7b**-OH) and their parent compounds **5a**-OH, **5b**-OH, although they represent true antiestrogens [11] and should interfere with the hormonal regulation of the cell growth.

Due to these data we propose a mode of action of **7a**, **7b** and **8a**, **8b** which is ER independent. To demonstrate this, we investigated the effects of **8a** and **8b** on the hormone independent MDA-MB 231 breast cancer cell line (Fig. 3). Both

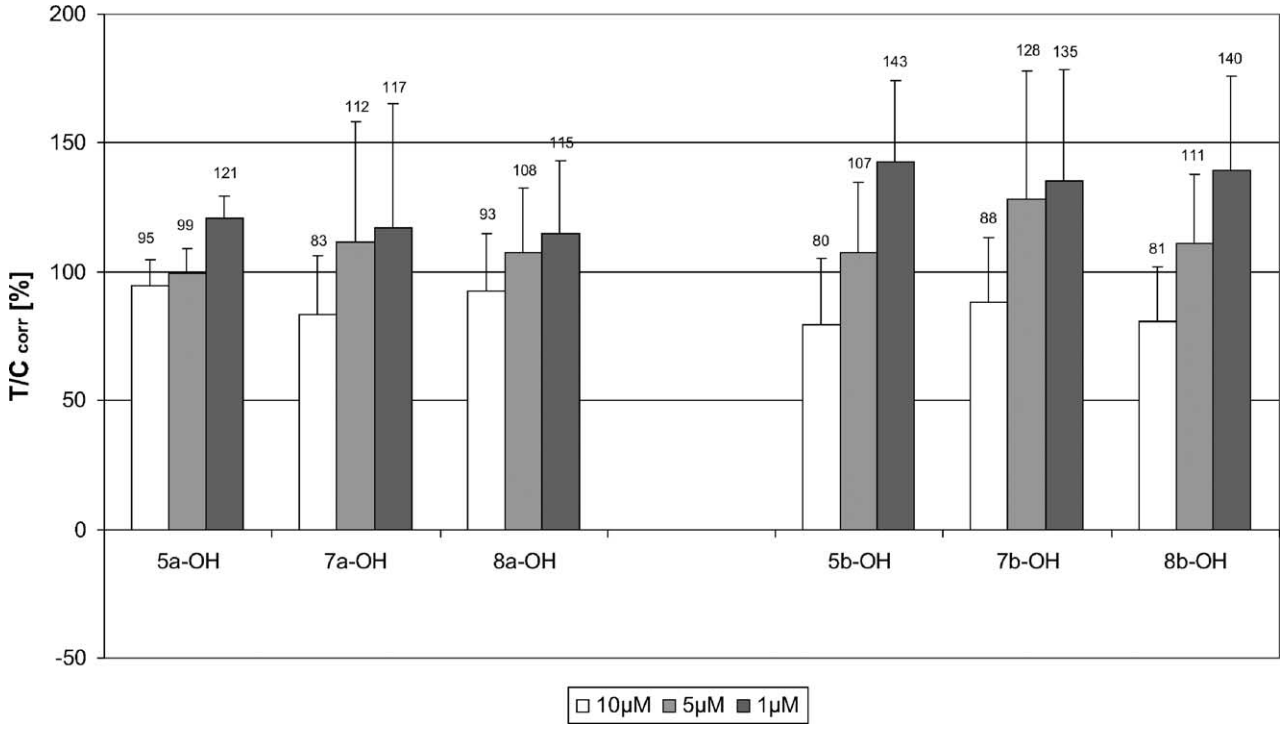


Fig. 2. Effects of selected bishydroxy and trishydroxy substituted 1,1,2-triarylalkenes on the MCF-7 cell line. The T/C_{corr} values relate to an incubation time of 75 h.

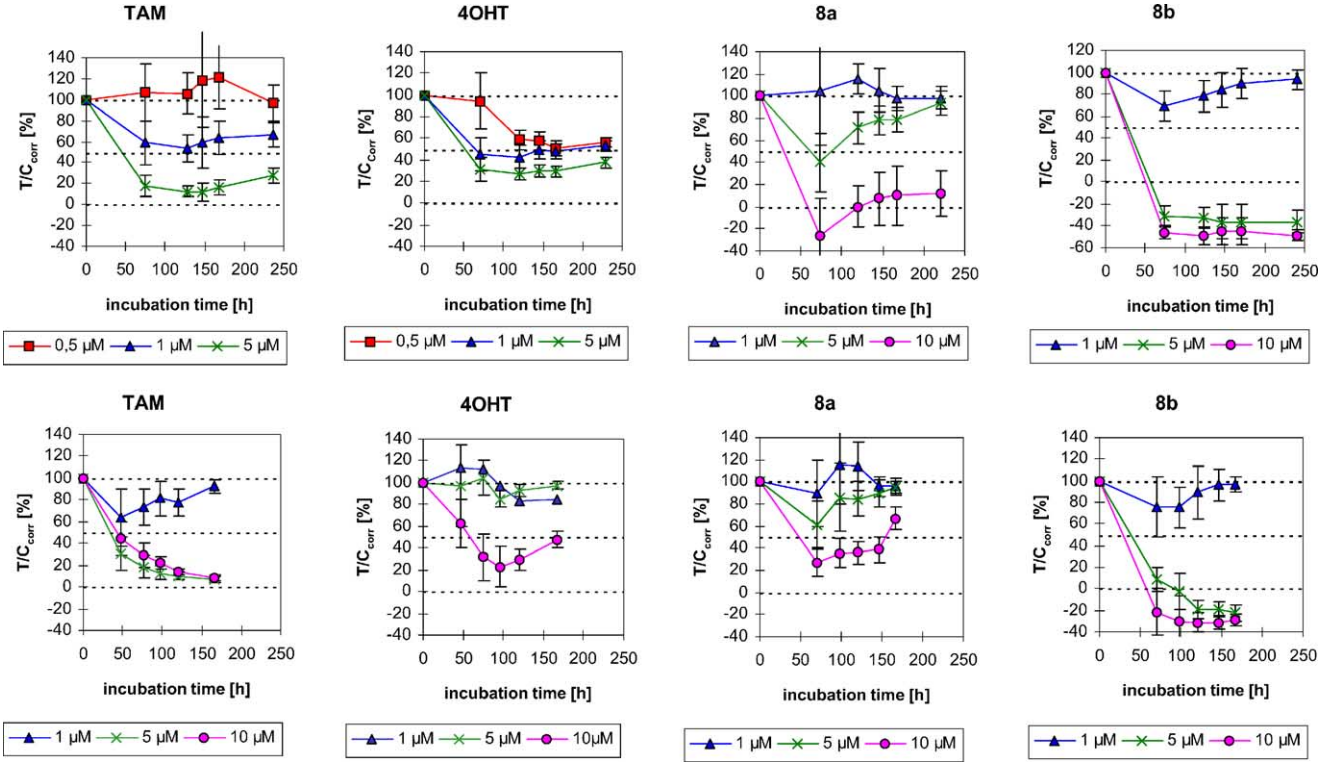


Fig. 3. Antiproliferative effects of TAM, 4OHT, **8a** and **8b** on the hormone dependent MCF-7 (upper) and hormone independent MDA-MB 231 (lower) breast cancer cell line. The cytocidal effects are expressed as $\tau\ (\%) = ((T - C_o)/C_o) \times 100$.

compounds reduced the cell growth, whereby **8a** equaled the effect of 4OHT and **8b** was even more active than both 4OHT and TAM.

4. Discussion

TAM, droloxifene or teremifene are drugs, investigated for the treatment of the hormone dependent breast cancer. They have tissue selective estrogen agonistic or antagonistic effects and are commonly referred to as selective estrogen receptor modulators. They possess antagonistic properties in breast malignancy and agonistic properties in the skeleton, uterus, and cardiovascular system [14]. As prerequisite for this mode of action a basic side chain located in the 4-position of the C1-phenyl ring is supposed.

To get more insight into the structural requirement for the antagonistic properties of TAM in tumor cells, we investigated the E2 antagonism of related dihydroxy and trihydroxy substituted 1,1,2-triarylalkenes in hormone dependent MCF-7-2a breast cancer cells. Interestingly, their antagonistic profile is mainly determined by the kind of side chain at the C2-atom. A dimethylaminoethane substituent is dispensable [9–11].

The influence on the cell growth, however, is only marginal despite their capability to interfere with the hormonal regulation of the cell growth. After O-methylation, the hormonal potency decreased drastically, but now the compounds are able to reduce the cell growth. As most active compounds the amino derivatives **8a** and **8b** showed cytocidal effects without having significant agonistic and antagonistic properties. The nearly identical effects on both, the hormone dependent MCF-7 and the hormone independent MDA-MB 231 cell line, indicate an ER independent mode of action, which is quite unclear. However, there are many investigations on TAM and related compounds suitable for discussion.

It is well known that the carcinogenesis of TAM results from the formation of DNA adducts after the activation by the cytochrome P450 mixed function oxidases [15–17]. The metabolic activation involves α-hydroxylation [18] followed by a hydroxy steroid sulfotransferase mediated sulfate conjugation [19]. Loss of this sulfate moiety leaves a carbocation, promoting nucleophilic attack by the N^2-aminogroup of deoxyguanosine to form α-(deoxyguanosine-N^2-yl)tamoxifen [20]. This DNA-damage can also be the reason for the ER independent antiproliferative effects of TAM on the hormone dependent MCF-7 and hormone independent MDA-MB 231 breast cancer cell lines.

Fan et al. [21] investigated the oxidation of TAM and toremifene in MCF-7 cells and confirmed the building of quinone methides and the reaction with electrophiles in the cells. However, the reaction is slow compared to other *p*-chinone methides and the formed conjugates are not stable.

Kuramochi [22] studied the stability of reactive intermediates of TAM and its 4-OH metabolite 4OHT by molecular mechanics, molecular dynamics and quantum mechanics calculation and confirmed a close relationship between the stability of the intermediates and their DNA adduct formation. He demonstrated a higher stability of the carbocation intermediates of 4OHT compared to TAM. Deprotonation of the 4-OH group neutralizes the carbocation and a quinone methide with lower electrophilic reactivity is built. The same effect was confirmed for toremifene and 4-hydroxytoremifene, however with a considerably higher amount of the deprotonated form. This correlates very well with the much lower reactivity and the lower DNA adduct formation of 4-hydroxytoremifene.

The oxidation of 1,1-bis(4-hydroxy/methoxyphenyl)-2-phenylalkenes and 1,1,2-tris(4-hydroxy/methoxyphenyl) alkenes by P450 enzymes might be a possible activation step. However, the inactivation by deprotonation seems to be favored in the case of the dihydroxy and trishydroxy substituted derivatives and could be the reason for the low antiproliferative effects. This reaction is impossible after O-methylation and the α-cation should possess sufficient reactivity for a covalent binding to nucleobases of the DNA.

The α-cation gets a satisfactory stabilization either by inductive effects of the C2-alkyl chain or by anchimeric assistance of functional groups. Terminal NH_2 and the COOH groups are nucleophiles reacting with the α-C-atom to azetidin and a γ-butyrolactone intermediate (see Fig. 4). Nucleobases such as guanosine can open both rings to be amidated and alkylated, respectively. The stability of amides is much lower compared to the related alkyl derivative under in vitro conditions and could be the reason for the different cytotoxicity of **8a**, **8b** and **9a**, **9b**.

The cytotoxicity of 4OHT indicates however that also further targets must be taken into consideration. It was demonstrated that TAM and other 1,1,2-triarylalkenes interact with the antiestrogen binding site (AEBS) protein [23], the P-glycoprotein efflux pump [24], calmodulin [25] and the regulatory domain of protein kinase C (PKC) [26]. PKC is an activator for phospholipase D (PLD) which plays a mechanistic role in cellular transformation, especially of human breast adenocarcinoma cells, colon adenocarcinoma cells and human renal cancers [27,28].

Lavie et al. [29] reported that TAM elicited isozyme-specific membrane association of PKC-ε in MCF-7 cells. In SAR studies, they evaluated the chemical requirements for PKC-ε translocation. TAM, droloxifene and clomiphene bearing basic side chains interact with PKC-ε, while compounds such as related hydroxylated 1,1,2-triarylbut-1-enes are inactive. This suggests that PKC activity can be inhibited by different routes depending on the 1,1,2-triarylalkene structure.

Another mode of action can be gathered from the investigations of Jonnalagadda et al. [30]. They studied the influence of ring halogenation, methoxylation and benzyloxylation of *Z*-1,1-dichloro-2,3-diphenylcyclopropane (analog II), which is an effective anti-breast cancer agent

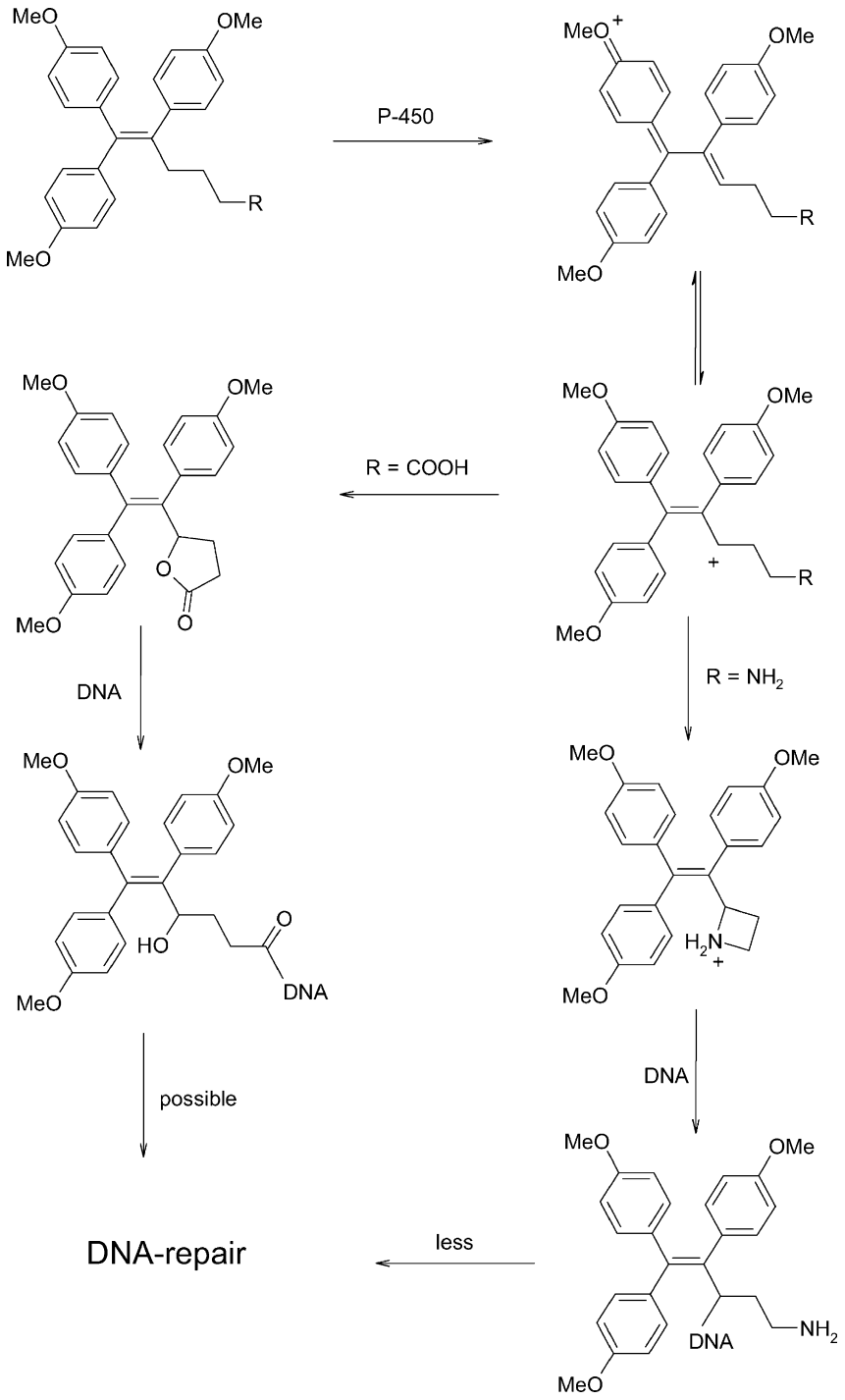


Fig. 4. Postulated activation of the methoxy substituted 1,1,2-triarylalkenes **8a** and **8b**.

in rodents and in cell culture. The cytostatic activities of the compounds against MCF-7 and MDA-MB 231 human breast cancer cells were tested. The *Z*-1,1-dichloro-2-(4-methoxyphenyl)-3-phenylcyclopropane was found to be more active than analog II. Both compounds inhibited tubulin assembly in vitro and caused microtubule loss in breast cancer cells, leading to an accumulation in the G2/M portion of the cell cycle.

5. Conclusion

The compounds assayed in this study show only modest estrogen receptor binding affinity and antiestrogenicity as well as no estrogenic effects. The amines **8a** and **8b** exert a strong cytotoxic effect on the MCF-7 cell line. According to our findings, this cytotoxicity cannot be estrogen receptor mediated. Further studies must be carried

out to investigate the mechanism of cytotoxicity and tissue selectivity.

Acknowledgements

The technical assistance of S. Bergemann and I. Schnautz is acknowledged. The presented study was supported by Grant Gu285/3-1 and Gu285/3-2 from the Deutsche Forschungsgemeinschaft.

References

[1] V.C. Jordan, A current view of tamoxifen for the treatment and prevention of breast cancer, Br. J. Pharmacol. 110 (1993) 507–517.
[2] B. Fisher, J.P. Costantino, D.L. Wickerham, C.K. Redmond, M. Kavanah, W.M. Cronin, V. Vogel, A. Robidoux, N. Dimitrov, J. Atkins, M. Daly, S. Wieland, E. Tan-Chiu, L. Ford, N. Wolmark, National surgical adjuvant breast and bowel project P-1 study, J. Natl. Can. Inst. 90 (1998) 1371–1388.
[3] B. Fisher, J.P. Costantino, C.K. Redmond, E.R. Fisher, D.L. Wickerham, W.M. Cronin, Endometrial cancer in tamoxifen treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14, J. Natl. Can. Inst. 86 (1994) 527–537.
[4] L.E. Rutqvist, H. Johansson, T. Signomklao, U. Johansson, T. Fornander, N. Wilking, Adjuvant tamoxifen therapy for early stage breast cancer and second primary malignancies, J. Natl. Can. Inst. 87 (1995) 645–651.
[5] V.C. Jordan, Tamoxifen and tumorigenicity: a predictable concern, J. Natl. Can. Inst. 87 (1995) 623–626.
[6] A.D. Palkowitz, A.L. Glasebrook, K.J. Thrasher, K.L. Hauser, L.L. Short, D.L. Phillips, B.S. Muehl, M. Sato, P.K. Shetler, G.J. Cullinan, T.R. Pell, H.U. Bryant, Discovery and synthesis of [6-hydroxy-3-[4-[2-(1-piperidinyl)ethoxy]phenoxy]-2-(4-hydroxyphenyl)]benzo[*b*]thiophene: a novel, highly potent, selective estrogen receptor modulator, J. Med. Chem. 40 (1997) 1407–1416.
[7] T.A. Grese, S. Cho, D.R. Finley, A.G. Godfrey, C.D. Jones, C.W. Lugar III, M.J. Martin, K. Matsumoto, L.D. Pennington, M.A. Winter, M.D. Adrian, H.W. Cole, D.E. Magee, D.L. Phillips, E.R. Rowley, L.L. Short, A.L. Glasebrook, H.U. Bryant, Structure–activity relationships of selective estrogen receptor modulators: modifications to the 2-arylbenzothiophene core of raloxifene, J. Med. Chem. 40 (1997) 146–167.
[8] F. Hafner, E. Holler, E. von Angerer, Effect of growth factors on estrogen receptor mediated gene expression, J. Steroid Biochem. Mol. Biol. 58 (1996) 385–393.
[9] V. Lubczyk, H. Bachmann, R. Gust, Investigations on estrogen receptor binding. The estrogenic, antiestrogenic and cytotoxic properties of C2-alkyl-substituted 1,1-bis(4-hydroxyphenyl)-2-phenylalkenes, J. Med. Chem. 45 (2002) 5358–5364.
[10] V. Lubczyk, H. Bachmann, R. Gust, Antiestrogenically active 1,1,2-tris(4-hydroxyphenyl)alkenes without basic side chain: synthesis and biological activity, J. Med. Chem. 46 (2003) 1484–1491.
[11] R. Gust, V. Lubczyk, Investigations on the influence of terminal groups at the C2-propyl side chain of 1,1-bis(4-hydroxyphenyl)-2-phenylpent-1-ene and 1,1,2-tris(4-hydroxyphenyl)pent-1-ene on the estrogen receptor binding and the estrogenic and anti-estrogenic properties, J. Steroid Biochem. Mol. Biol. 86 (2003) 57–70.
[12] R.J. Hay, The seed stock concept and quality control for cell lines, Anal. Biochem. 171 (1988) 225–237.
[13] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[14] B.H. Mitlak, F.J. Cohen, Selective estrogen receptor modulators. A look ahead, Drugs 57 (1999) 653–663.
[15] X. Han, J.G. Liehr, Induction of covalent DNA adducts in rodents by Tamoxifen, Cancer Res. 52 (1992) 1360–1363.
[16] C. Mani, D. Kupfer, Cytochrome P450 mediated action and irreversible binding of the antiestrogen tamoxifen to proteins in rat and human liver: possible involvement of the flavin-containing monooxygenases in tamoxifen action, Cancer Res. 51 (1991) 6052–6058.
[17] I.N.H. White, F. De Matteis, A.H. Gibbs, C.K. Lim, C.R. Wolf, C. Henderson, L.L. Smith, Species differences in the covalent binding of [^{14}C]tamoxifen to liver microsome and the forms of cytochrome P450 involved, Biochem. Pharmacol. 49 (1995) 1035–1042.
[18] D.H. Phillips, P.L. Carmichael, A. Hewer, K.J. Cole, G.K. Poon, Alpha-hydroxytamoxifen, a metabolite of tamoxifen with exceptionally high DNA-binding activity in rat hepatocytes, Cancer Res. 54 (1994) 5518–5522.
[19] S. Shibutani, L. Dasaradhi, I. Terashima, E. Banoglu, M.W. Duffel, Alpha-hydroxytamoxifen is a substrate of hydroxysteroid (alcohol) sulfotransferase, resulting in tamoxifen DNA adducts, Cancer Res. 58 (1998) 647–653.
[20] C. Sanchez, S. Shibutani, L. Dasaradhi, J.L. Bolton, P.W. Fan, R.A. McClelland, Lifetime and reactivity of an ultimate tamoxifen carcinogen: the tamoxifen carbocation, J. Am. Chem. Soc. 120 (1998) 13513–13514.
[21] P.W. Fan, F. Zhang, J.L. Bolton, 4-Hydroxylated metabolites of the antiestrogens tamoxifen and toremifene are metabolized to unusually stable quinone methides, Chem. Res. Toxicol. 13 (2000) 45–52.
[22] H. Kuramochi, Conformational studies and electronic structures of tamoxifen and toremifen and their allylic carbocations proposed as reactive intermediates leading to DNA adduct formation, J. Med. Chem. 39 (1996) 2877–2886.
[23] C. Chouvet, S. Saez, High affinity cytosol binding site(s) for antiestrogens in two human breast cancer cell lines and in biopsy specimens devoid of estrogen receptors, J. Steroid Biochem. Mol. Biol. 21 (1984) 755–761.
[24] F. Leonessa, M. Jacobson, B. Boyle, J. Lippman, M. McGarvey, R. Clarke, Effect of tamoxifen on the multidrug-resistant phenotype in human breast cancer cells: isobologram, drug accumulation, and M(r) 170,000 glycoprotein (gp170) binding studies, Cancer Res. 54 (1994) 441–447.
[25] M.C. Lopes, M.G. Vale, A.P. Carvalho, Ca2(+)-dependent binding of tamoxifen to calmodulin isolated from bovine brain, Cancer Res. 50 (1990) 2753–2758.
[26] E. Bignon, M. Pons, J.C. Dore, J. Gilbert, T. Ojasoo, J.F. Miquel, J.P. Raynaud, A. Crastes de Paulet, Influence of di- and tri-phenylethylene estrogen/antiestrogen structure on the mechanisms of protein kinase C inhibition and activation as revealed by a multivariate analysis, Biochem. Pharm. 42 (1991) 1373–1383.
[27] G. Fiucci, M. Czarny, Y. Lavie, D. Zhao, B. Berse, J.K. Blusztajn, M. Liscovitch, Changes in phospholipase D isoform activity and expression in multidrug-resistant human cancer cells, Int. J. Cancer 85 (2000) 882–888.
[28] Y. Zhao, H. Ehara, Y. Akao, M. Shamoto, Y. Nakagawa, Y. Banno, T. Deguchi, N. Ohishi, K. Yagi, Y. Nozawa, Increased activity and intranuclear expression of phospholipase D2 in human renal cancer, Biochem. Biophys. Res. Commun. 278 (2000) 140–143.
[29] Y. Lavie, Z. Zhang, H. Cao, T. Han, R.C. Jones, Y. Liu, M. Jarman, I.R. Hardcastle, A.E. Giuliano, M.C. Cabot, Tamoxifen induces selective membrane association of protein kinase C epsilon in MCF-7 human breast cancer cells, Int. J. Cancer 77 (1998) 928–932.
[30] S.S. Jonnalagadda, E. ter Haar, E. Hamel, C.M. Lin, R.A. Magarian, B.W. Day, Synthesis and biological evaluation of 1,1-dichloro-2,3-diarylcyclopropanes as antitubulin and anti-breast cancer agents, Bioorg. Med. Chem. 5 (1997) 715–722.