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## Molecular Characterization of the Microsomal Tamoxifen Binding Site\*

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Tamoxifen is a selective estrogen receptor modulator widely used for the prophylactic treatment of breast cancer. In addition to the estrogen receptor (ER), tamoxifen binds with high affinity to the microsomal antiestrogen binding site (AEBS), which is involved in ER-independent effects of tamoxifen. In the present study, we investigate the modulation of the biosynthesis of cholesterol in tumor cell lines by AEBS ligands. As a consequence of the treatment with the antitumoral drugs tamoxifen or PBPE, a selective AEBS ligand, we show that tumor cells produced a significant concentration- and time-dependent accumulation of cholesterol precursors. Sterols have been purified by HPLC and gas chromatography, and their chemical structures determined by mass spectrometric analysis. The major metabolites identified were  $5\alpha$ -cholest-8-en- $3\beta$ -ol for tamoxifen treatment and  $5\alpha$ -cholest-8-en- $3\beta$ -ol and cholesta-5,7-dien- $3\beta$ -ol, for PBPE treatment, suggesting that these AEBS ligands affect at least two enzymatic steps: the  $3\beta$ -hydroxysterol- $\Delta^8$ - $\Delta^7$ -isomerase and the  $3\beta$ -hydroxysterol- $\Delta^7$ -reductase. Steroidal antiestrogens such as ICI 182,780 and RU 58,668 did not affect these enzymatic steps, because they do not bind to the AEBS. Transient co-expression of human  $3\beta$ -hydroxysterol- $\Delta^8$ - $\Delta^7$ -isomerase and  $3\beta$ -hydroxysterol- $\Delta^7$ -reductase and immunoprecipitation experiments showed that both enzymes were required to reconstitute the AEBS in mammalian cells. Altogether, these data provide strong evidence that the AEBS is a hetero-oligomeric complex including  $3\beta$ -hydroxysterol- $\Delta^8$ - $\Delta^7$ -isomerase and the  $3\beta$ -hydroxysterol- $\Delta^7$ -reductase as subunits that are necessary and sufficient for tamoxifen binding in mammary cells. Furthermore, because selective AEBS ligands are antitumoral compounds, these data suggest a link between cholesterol metabolism at a post-lanosterol step and tumor growth control. These data afford both the identification of the AEBS and give new insight into a novel molecular mechanism of action for drugs of clinical value.

Tamoxifen is a selective estrogen receptor modulator (SERM) widely used for the treatment and the prevention of breast cancer (1). More than 20 years ago, Sutherland *et al.* (2) discovered that tamoxifen bound to a high affinity binding site that was different from the estrogen receptor (2). This site has been named the microsomal antiestrogen binding site (AEBS)<sup>1</sup> because it is localized in the microsomes of cells; it binds principally aryl aminoethoxy antiestrogens and has no affinity for estrogens (3). Two classes of selective ligands have been developed so far to selectively target the AEBS. The first class includes diphenylmethane derivatives of tamoxifen ((*z*)-2-[4-(1,2-diphenyl-1-butenyl)-phenoxy]-*N,N*-dimethylethanamine) such as *N,N*-diethyl-2-[4-(phenylmethyl)-phenoxy]-ethanamine-HCl (DPPE) and *N*-pyrrolidino-2-[4-(benzyl)-phenoxy]-ethanamine-HCl (PBPE) (4–6), while the second class includes oxygenated derivatives of cholesterol such as 7-ketocholestanol (7–9). We and others (5, 10–14) have shown that AEBS ligands inhibit the growth of tumor cell lines *in vitro* and *in vivo*, demonstrating that the AEBS was involved in the mediation of the effects of these structural classes of its cognate ligands. These compounds represent not only specific tools to study AEBS function but are also anticancer drug candidates because the selective AEBS ligand DPPE (Tesmifene) was brought up to phase II and III clinical trials for the treatment of breast and prostate cancer in association with doxorubicin (15–17). Two principal points remained unsolved: the first is the precise molecular nature of the AEBS and the second the explanation of the difference observed between the nanomolar affinity of AEBS ligands and their micromolar effectiveness for growth control and cytotoxicity.

We have been involved in the identification of the AEBS for several years. The AEBS can be found in most tissues in mammals and is abundant in microsomes of liver that contained 20–30× the amount found in tumor cell lines (18). For this reason, the liver have been chosen for the purification of the AEBS but the pharmacological profiles of the AEBS found in the liver and in tumor cell lines such as MCF-7 cells (a mam-

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<sup>1</sup> The abbreviations used are: AEBS, antiestrogen binding site; tamoxifen, *trans*-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-*N,N*-dimethylethanamine; PBPE, *N,N*-pyrrolidino-[4-(benzyl)-phenoxy]-ethanamine; mEH, microsomal epoxide hydrolase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; D8D7I,  $3\beta$ -hydroxysterol- $\Delta^8$ - $\Delta^7$ -isomerase; DHCR7,  $3\beta$ -hydroxysterol- $\Delta^7$ -reductase; DHCR24,  $3\beta$ -hydroxysterol- $\Delta^{24}$ -reductase; C5DS,  $3\beta$ -hydroxysterol-C<sup>5</sup>-desaturase; HA, hemagglutinin tag; zymosterol,  $5\alpha$ -cholesta-8,24-dien- $3\beta$ -ol; zymostenol,  $5\alpha$ -cholest-8-en- $3\beta$ -ol; lathosterol,  $5\alpha$ -cholest-7-en- $3\beta$ -ol; lanosterol, 4,4',14-trimethyl- $5\alpha$ -cholesta-8,24-dien- $3\beta$ -ol; 7-dehydrocholesterol, cholesta-5,7-dien- $3\beta$ -ol; desmosterol, cholesta-5,24-dien- $3\beta$ -ol; cholesterol, cholest-5-en- $3\beta$ -ol; cholestanol,  $5\alpha$ -cholestan- $3\beta$ -ol; RRT, relative retention times; *t*-BuPE, *N,N*-diethyl-2-[4-(tert-butyl)phenoxy]-ethanamine; HPLC, high performance liquid chromatography; *R<sub>f</sub>*, retention factor; E2, estradiol; Tx, tamoxifen; NTA, nitritriacetic acid; GC/MS, gas chromatography/mass spectrometry.

mary adenocarcinoma cell line) have been reported to be different, suggesting a possible difference in the composition of the AEBS in the two systems (19). We have recently reported that the AEBS from rat liver was a hetero-oligomeric multiprotein complex that contained subunits that were not directly involved in the binding of tamoxifen such as the microsomal epoxide hydrolase (mEH) (20), the carboxyl-esterase (ES-10), and the liver fatty acid-binding protein (FABP) (21). Each of these proteins have been implicated in lipid metabolism: mEH is a bile acid transporter (22), and carboxylesterase has cholesterol esterification properties (23) but were related to the AEBS found in normal liver because ES-10 and FABP were not found in tumor cell lines. The AEBS was also initially described as an intracellular receptor for histamine (HIC) by Brandes and co-workers (24). They have proposed that the AEBS/HIC might be a cytochrome p450 CYP 3A4. Recently, two different groups have reported that an enzyme involved in the biosynthesis of cholesterol, the  $3\beta$ -hydroxysterol- $\Delta^8$ - $\Delta^7$ -isomerase (D8D7I), when expressed in yeast, displayed a high affinity binding site for tamoxifen, suggesting that this site might be the AEBS (25, 26). D8D7I was first described as the emopamil binding site (EBP), or the SR 31747A binding protein 1 (SRBP1) because it bound the voltage-dependent calcium channel blocker emopamil and the immunosuppressive and antitumoral drug SR-31747A when expressed in yeast (25, 26). However D8D7I did not exhibit binding capacities for [ $^3$ H]tamoxifen when expressed in mammalian cells, suggesting that D8D7I was not sufficient by itself to constitute the AEBS (26). However the basic problem was that none of these proteins exhibit the molecular mass of 40 kDa that we have previously determined as being the size of the binding subunit of tamoxifen on the AEBS using photoaffinity labeling (27).

In this study, we report the consequences of the treatment by tamoxifen or PBPE, a selective AEBS ligand, on the biosynthesis of cholesterol in different tumor cell lines, including human adenocarcinoma cell line MCF-7. We have focused on cholesterol precursors of the C-27 series and have determined the amount and the structure of the metabolites that accumulated in cells when they were treated with these drugs. We report in this study that tamoxifen and the selective AEBS ligand PBPE produced a massive accumulation of  $5\alpha$ -cholest-8-en- $3\beta$ -ol and/or  $5\alpha$ -cholesta-5,7-dien- $3\beta$ -ol that suggests an inhibition of the D8D7I and the  $3\beta$ -hydroxysterol- $\Delta^7$ -reductase (DHCR7), two enzymes involved in the biosynthesis of cholesterol. Co-expression of D8D7I and DHCR7 in COS-7 cells produced a high affinity binding site for tritiated tamoxifen that displayed the pharmacological profile of the AEBS found in tumor cells. Immunoprecipitation followed by tamoxifen binding to the immunoprecipitate complex showed that D8D7I and DHCR7 are associated proteins necessary for the reconstitution of the AEBS.

#### EXPERIMENTAL PROCEDURES

**Chemicals**—PBPE, DPPE, *N*-morpholino-2-[4-(benzyl)-phenoxy]-ethanamine-HCl (MBPE), and *N,N*-diethyl-2-[4-(tert-butyl)-phenoxy]-ethanamine (*t*-BuPE) were synthesized as previously described (6). *trans*-1,4-Bis(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride (AY 9944) was synthesized in the laboratory, and its purity was greater than 98%. BD 1008 was kindly provided by Dr. W. D. Bowen (National Institutes of Health), BM 15,766 was from Roche Applied Science (Pernzberg, Germany). CI-628 was from Park Davis. ICI 164,384 was from Astra Zeneca Pharmaceutical (Wilmington, DE). Raloxifene, RU 58,668 and RU 39,411 were kindly provided by Dr. P. van de Velde from Aventis (Romainville, France). U-18,666A was from Upjohn (Kalamazoo, NJ). ICI 182,780 was from Tocris Cookson Ltd. 7-Ketocholesterol was from Steraloid. Tamoxifen, 4-OH-tamoxifen, clomiphene, cholesterol-oleate, lanosterol, cholesterol, lathosterol, desmosterol, 6-ketocholesterol, 7-ketocholesterol, and 7-dehydrocholesterol were from Sigma-Aldrich. All solvents were from Prolabo (Paris, France). *N,O*-bis

(trimethylsilyl)trifluoroacetamide was from Fluka. Other compounds and chemicals were from Aldrich-Sigma. Anti-hemagglutinin (anti-HA) mouse monoclonal antibody (16B10 clone) was from Eurogentec (Anger, France). The pCMV-LacZ reporter plasmid was kindly provided by H. Paris (Toulouse, France).

**Cell Culture**—MCF-7 cells (a human breast cancer cell line expressing estrogen receptors) were initially obtained from M. Rich (Michigan Cancer Foundation). These cells were adapted to grow in a chemically defined medium (28): in 5% CO<sub>2</sub> in RPMI 1640 supplemented with 2 g/liter sodium bicarbonate, 1.2 mM glutamine (pH 7.4 at 23 °C), 0.08 unit/ml human recombinant insulin, and 0.1 mg/ml human apo-transferrin and were called MCF-7<sup>WS</sup>. MDA-MB-231 (a human breast cancer cell line that does not express estrogen receptors) and SAOS-2 (a human osteosarcoma cell line) cells were from the American Tissue Culture Collection. These cells were grown in RPMI 1640 medium supplemented with 2 g/liter sodium bicarbonate, 1.2 mM glutamine, pH 7.4, at 23 °C, and 4% fetal calf serum in 5% CO<sub>2</sub>.

**Treatment of Cells with Drugs**—MCF-7<sup>WS</sup> were grown to 70% confluence. At day 0, tamoxifen, PBPE, ICI 182,780, RU 58,668 or 17 $\beta$ -estradiol at concentrations of 0.5  $\mu$ M, 0.5  $\mu$ M, 0.5  $\mu$ M, 0.5  $\mu$ M, and 10 nM, respectively were added in ethanolic solution. The final concentration of ethanol (0.1%, v/v) did not interfere with the biosynthesis of cholesterol in cells. After 48 h of incubation, cells were washed with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline, harvested, and counted. 0.1  $\mu$ Ci of [ $^{14}$ C]cholesterol (Isotopchim, specific activity is 1898 MBq/mmol) as internal standard and 0.05% butylated hydroxytoluene (5 mg/ml) were added to cells. Cells were then immediately processed for extraction of unsaponifiable lipids (29), and resuspended in 40  $\mu$ l of methanol. For each condition  $8 \times 10^7$  to  $10^8$  cells were used for analysis. Kinetics were performed by treatment of MCF-7<sup>WS</sup> with tamoxifen and PBPE for 0, 6, 12, 24, 48, 72, and 96 h. Dose response studies were done with increasing concentrations of tamoxifen and PBPE for 48 h of incubation with MCF-7<sup>WS</sup> cells. Tests on other cell lines were performed with 5  $\mu$ M tamoxifen or 10  $\mu$ M PBPE for 48 h. Comparative tests with other drugs were performed by a 48 h treatment of MCF-7 cells using 5  $\mu$ M antiestrogens or 10  $\mu$ M of the other drugs. Sterols were extracted as described above. The percentage recovery of sterols during their purification was 80–90% as judged by the amount of radioactivity recovered.

**Silver Nitrate Thin Layer Chromatography (TLC)**—TLC was carried out with silica gel 60 from Merck (Darmstadt, Germany). Plates were pulverized with a solution of 0.1% (w/v) silver nitrate in acetonitrile in the absence of light, and then wrapped in aluminum foil before drying in an oven under reduced pressure at 110 °C over 1 h. Sterols from the extracts were analyzed by silver nitrate thin layer chromatography with methanol/acetone (57:2, v/v) as the mobile phase (29, 30). Samples were detected by spraying with 50% sulfuric acid in methanol (v/v) and by heating the chromatogram on a hot plate. The standards used for calibration were cholesterol oleate, lanosterol, lathosterol, cholesterol, desmosterol, 7-dehydrocholesterol at 1 mg/ml in *n*-hexane or ethanol. Retention factors ( $R_f$ ) were determined for each spot on the TLC as the ratio between the distance of migration of the eluate from the deposit and the distance of the solvent from the deposit.

**High Performance Liquid Chromatography (HPLC)**—Samples were first passed through a Sep-Pack cartridge (Vac C18 1 cc, Waters) equilibrated with methanol. Reverse phase HPLC was carried out with a PerkinElmer system (series 200 DAD) coupled to a diode array detector. This system enable us obtain an in-line UV spectrum of the chromatographic peaks. The column, Lichrosorb C18 5  $\mu$ m (25 cm  $\times$  4 mm), fitted with a Lichrosorb C18 5  $\mu$ m (0.5 cm  $\times$  4 mm) guard cartridge, was developed isocratically, as described by Popjak *et al.* (31) with methanol/water (96:4, v/v) at a flow rate of 0.7 ml/min. The effluent was monitored at 210 nm or at 282 nm, and fractions were collected at 1-min intervals. The relative retention times (RRT) were measured by comparison with the retention time of cholesterol (RRT cholesterol is 1). Quantification of sterols were carried out using a calibration curve established with authentic corresponding sterols except for ymostenol for which the mass was estimated using the calibration curve for cholesterol because both compounds had a similar molar extinction coefficient at 210 nm.

**Gas Liquid Chromatography-Mass Spectrometry (GC/MS)**—Fractions that were collected from the HPLC column were reduced to dryness under a stream of nitrogen and treated with a mixture (0.1 ml) of *N,O*-bis (trimethylsilyl)trifluoroacetamide/pyridine (50:50, v/v) for 30 min at 60 °C. The reagents were evaporated under nitrogen flux, and the trimethylsilyl ether derivatives (TMS) were dissolved in hexane. GC/MS analyses were carried out using an HP 5935 instrument housing a fused silica column DB5 (25 m  $\times$  0.32 mm) coated with a 0.25  $\mu$ m layer of S.E.-30, DB-1 ending in the ion source. The oven temperature

was about 60 °C during the injection, after 3 min was rapidly increased to 200 °C, and was then programmed from 200 to 250 °C at a rate of 3 °C/min and from 250 to 300 °C at a rate of 6 °C/min.

**Subcloning Human cDNA for D8D7I and DHCR7**—Total RNA from Hela or MCF-7 cells was obtained by a rapid thiocyanate procedure (32). The cDNA encoding for D8D7I and for DHCR7 were obtained by reverse transcriptase-mediated polymerase chain reaction (RT-PCR) from total RNA by using the superscript preamplification system (Invitrogen Life Technologies, Inc.) with random hexamers for the reverse transcription step. For PCR, oligonucleotides with the EcoRI restriction site were used, matching the first 15 and last 16 bases, respectively, of the open reading frame of the cDNA encoding the human emopamil-binding protein (D8D7I) (33) or matching the first 15 and last 16 bases, respectively, of the cDNA encoding the human DHCR7 (34). The amplification products were cloned into the corresponding restriction sites of pSG5 vector (35) to give pSG5-D8D7I and pSG5-DHCR7. Constructs of HA N-terminal-fused D8D7I and DHCR7 were made as follows: the nucleotide sequence coding for a peptide epitope YPYDVPDYA from hemagglutinin (HA) of the influenza virus was fused to the N terminus of the D8D7I (HA-D8D7I) and DHCR7 (HA-DHCR7), using the polymerase chain reaction. The amplification product was cloned into the EcoRI restriction site of the pSG5 vector. Plasmids were sequenced by the dideoxy chain termination technique (36).

**Expression of HA-D8D7I and HA-DHCR7 in COS-7 Cells**—COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. A dose response curve was first carried out to estimate the optimum amount of DNA required for the best transfection. Plasmids were transfected into COS-7 cells using the polyethylenimine methodology (37). COS-7 cells were seeded in 100-mm plates at a density of  $5 \times 10^5$  cells per dish and transfected under identical conditions with a constant quantity of plasmid (8 µg/dish). In experiments, cells were transfected with 3.75 µg of each plasmid encoding for protein and completed, if necessary, with empty vector (pSG5) to keep the total amount of DNA constant. 3.75 µg of each plasmid/dish gave maximal expression of both proteins as measured by Western blotting of the total protein extract with an anti-HA antibody. For Western blotting, COS-7 cells were transfected with plasmids encoding for HA-DHCR7 or HA-D8D7I. For co-immunoprecipitation, COS-7 cells were transfected with plasmids encoding for HA-DHCR7 and/or D8D7I. For binding experiments, COS-7 cells were transfected with plasmids coding for DHCR7 and/or D8D7I with or without the HA tag. For all experiments, COS-7 cells were also transfected with 50 ng of pCMV-lacZ to measure the efficiency of transfection and mock-transfected cells were transfected with 8 µg of pSG5 plasmid that lacked a DNA insert.  $\beta$ -Galactosidase activity was measured by the luminescence derived from 10 µl of each sample incubated in 200 µl of 1 mg/ml *O*-nitrophenyl- $\beta$ -D-galactopyranoside and used to correct transfection efficiency among the different treatment groups (luminescent  $\beta$ -galactosidase detection kit, Clontech).

**Western Blotting**—The transfected cell pellet was thawed with 100 µl of cold phosphate-buffered saline, pH 7.4, in the presence of a mixture of protease inhibitors (Sigma) and was homogenized by sonication. The homogenate was centrifuged for 10 min at  $13,000 \times g$  at 4 °C. The protein content was determined by the Bradford method (38). The proteins were incubated in one volume of 2 $\times$  Laemmli gel loading buffer and incubated at 60 °C for 20 min, and then 40 µg of protein were separated by electrophoresis through a 12% SDS-polyacrylamide gel at constant current. The separated proteins were electroblotted onto polyvinylidene difluoride membranes. The membranes were then saturated with saline buffer (10 mM Tris, 140 mM NaCl, pH 7.4, containing 5% (w/v) nonfat dried milk) and incubated overnight at 4 °C with the anti-HA (16B10 clone) antibody. Membranes were washed three times with saline buffer containing 1% nonfat dried milk and then incubated with a goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody (Amersham Biosciences). Visualization was achieved with an ECL plus kit (Amersham Biosciences) and fluorescence measured either by autoradiography or using a PhosphorImager (Storm 840, Amersham Biosciences).

**Binding Assay**—48 h after transfection, cells were scraped and suspended in 100 µl of cold phosphate-buffered saline, pH 7.4, 0.15–0.3 TIU/ml aprotinin (Sigma), 1 mM benzamide, 1 µg/ml pepstatin, and 2 µg/ml leupeptin. Cells were homogenized by 6 successive freeze/thaw cycles and microsomal fractions prepared as described earlier (20). Binding experiments were conducted as described previously (6). Microsomes (10 µg) were incubated in a binding buffer (20 mM Tris-HCl, 2.5 mM EDTA, pH 7.4, 2.5 mM thioglycerol) with various concentrations of [<sup>3</sup>H]tamoxifen (specific activity: 84.0 Ci/mmol; Amersham Biosciences) from 0.1 to 25 nM for 18 h at 4 °C. After incubation, bound and

free radioligands were separated using Sephadex-LH20 gel filtration (1.5 ml), and the radioactivity of the flow-through was counted in a  $\beta$  counter. Competition assays with tamoxifen, PBPE, CI-628, clomiphene, 4-OH-tamoxifen, ICI 164,384, 7-ketocholestanol, *t*-BuPE, BD 1008, U-18,666A, cholestanol, cholesterol, lathosterol, zymosterol (5 $\alpha$ -cholesta-8-en-3 $\beta$ -ol), desmosterol, 7-dehydrocholesterol, and 17 $\beta$ -estradiol on whole cell lysates of transfected COS-7 cells were performed using eight concentrations of unlabeled test ligand ranging from 0.1 to 10,000 nM with a single concentration of [<sup>3</sup>H]tamoxifen of 3 nM. Incubation and separation of bound and free radioligand were performed as described above. Binding and competition assays were performed in duplicate in at least three separate experiments. Nonspecific binding was determined in the presence of 1 µM tamoxifen and was always less than 20% of total binding. Binding data were determined using the Graphpad Prism program (version 3). 5 $\alpha$ -Cholest-8-en-3 $\beta$ -ol (zymostenol) was purified by HPLC following the procedure described above after MCF-7<sup>wt</sup> treatment with 1 µM tamoxifen. HPLC peaks with a RRT of 0.93 from five injections were pooled, concentrated, and then submitted to a second HPLC purification under the same conditions. The product was evaporated to dryness and then dissolved in absolute ethanol. The concentration of 5 $\alpha$ -cholest-8-en-3 $\beta$ -ol was measured by UV analysis. Binding on the AEBs from MCF-7 was conducted exactly as described previously (6). MCF-7 microsomes were incubated with 3 nM [<sup>3</sup>H]tamoxifen and 12 concentrations of unlabeled test ligands ranging from 0.1 to 100 nM or 1 to 10,000 µM. Assays included 1 µM 17 $\beta$ -estradiol. IC<sub>50</sub> values were determined using the iterative curve fitting program GraphPad Prism. IC<sub>50</sub> values were converted into the apparent  $K_i$  using the Cheng-Prusoff equation and the  $K_d$  values of tamoxifen (39). Metabolism experiments were conducted in triplicate, and the values presented in the tables were taken from one representative of three independent experiments.

**Production of Polyclonal Antibodies against D8D7I**—The pSG5-D8D7I plasmid was digested by BamHI and XhoI and the D8D7I cDNA was subcloned into the prokaryotic expression vector pQE31 (Qiagen) and the resulting pQE31-D8D7I plasmid was used to produce His<sub>6</sub>-tagged recombinant D8D7I (6His-D8D7I). 6His-D8D7I was expressed in *Escherichia coli* (TG1 strain, Fiona Sait MRCC Cambridge). The production and affinity purification of 6H-D8D7I was as follows: 50 ml of pQE31-D8D7I-transformed cells were grown at 37 °C until the optical density at 600 nm reached 0.6. Isopropyl-1-thio- $\beta$ -D-galactopyranoside was added to a final concentration of 1 mM to induce the expression of recombinant protein for 4 additional hours. Bacteria were collected by centrifugation (5,000  $\times g$ , 20 min, 4 °C) and suspended in 2 ml of lysis buffer (6.3 M urea, 30 mM CHAPS, 0.3% SDS, 10 mM thioglycerol, 10% glycerol). Cells were broken by three consecutive freeze/thaw cycles. The lysate was then diluted with 1 ml of Nonidet P-40 5% (Roche Applied Science) and 8 ml of dilution buffer (10 mM Tris-HCl, pH 7.8, 300 mM NaCl) and centrifuged (10,000  $\times g$ , 20 min, 4 °C). The protein concentration was measured by the method of Bradford (38). Recombinant proteins were purified on Ni-NTA agarose beads (Qiagen). The lysate was incubated in batches with the Ni-NTA agarose beads by gentle agitation overnight at 4 °C, and the suspensions were then loaded onto columns (polyprep chromatography column, Bio-Rad). The flow-through was collected, and the columns were washed twice with 2 ml of washing buffer supplemented with CHAPS (30 mM). His<sub>6</sub>-protein was then eluted with: (a) 2  $\times$  100 µl of an elution solution of 300 mM NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 30 mM CHAPS, 50 mM imidazole; (b) 2  $\times$  100 µl of the same buffer with 250 mM imidazole and finally 2  $\times$  100 µl with 500 mM imidazole in same buffer. The fractions were frozen in liquid nitrogen and stored at -80 °C. Production and purification of recombinant protein were monitored by 12% SDS-PAGE. The gel was run according to standard procedure (Bio-Rad) and was stained with Coomassie Blue. 50 µg of purified protein from the elution (b) above was emulsified with Freund's complete adjuvant, and the mixture was injected subcutaneously into rabbits. Animals were boosted twice at monthly intervals with 100 and 400 µg of purified recombinant protein. The immune serum was tested by dot blotting. The rabbits were then bled 10 days after the last injection, and the immune serum was recovered and stored at -80 °C.

**Dot Blot Analysis**—Recombinant D8D7I was spotted onto a nitrocellulose membrane. The membrane was saturated with 5% (w/v) nonfat dry milk in TBST (25 mM Tris borate, pH 7.8, 140 mM NaCl, 0.1% (v/v) Tween 20), then incubated overnight with immune serum anti-D8D7I (diluted 1:1,000). The membrane was washed thoroughly with TBST and incubated for 1 h with goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad). Detection was performed with enhanced chemiluminescence kits (ECL, Amersham Biosciences), and the fluorescence was measured on a Storm 840 apparatus (Amersham Biosciences).

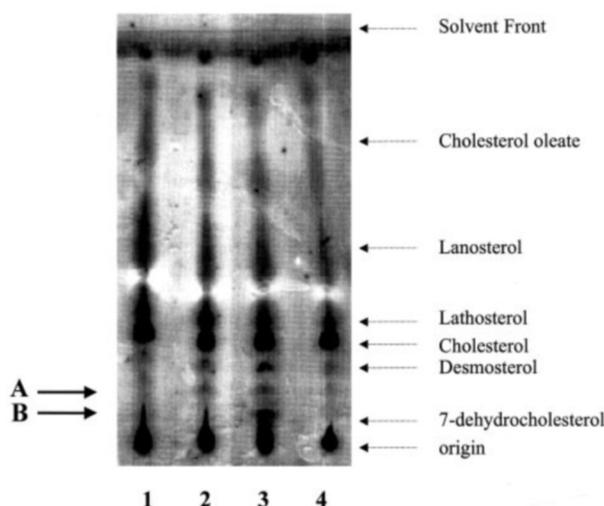


FIG. 1. AEBS ligands induce accumulation of putative cholesterol precursors in MCF-7<sup>us</sup> cells. Cells were grown for 2 days in the presence (lane 1) or absence of 0.5  $\mu$ M tamoxifen (lane 2), 0.5  $\mu$ M PBPE or 10 nM 17 $\beta$ -estradiol. The lipids were extracted and separated by silver nitrate impregnated TLC plates as described under "Experimental Procedures." TLC was developed with a H<sub>2</sub>SO<sub>4</sub>/methanol mixture and compared with commercial standards. The positions of identified sterols are indicated.

**Immunoprecipitation of D8D7I**—Immunoprecipitation of the recombinant enzymes was performed as described previously for seven transmembrane receptor-G protein complexes (40). 250  $\mu$ g of protein extracted from the D8D7I-transfected cells with lysis buffer (50 mM Hepes, 150 mM NaCl, 10 mM EDTA, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 100 mM NaF, 1.5% CHAPS, 0.15–0.3 TIU/ml aprotinin (Sigma), 1 mM benzamidin, 1  $\mu$ g/ml pepstatin, and 2  $\mu$ g/ml leupeptin) were incubated in the presence or not of 50  $\mu$ l of immune serum (anti-D8D7I) overnight at 4 °C in lysis buffer in which the CHAPS concentration was brought to 1%. Then 20  $\mu$ l of sheep anti-rabbit IgG coupled to magnetic beads (Dyna-beads M-280; Dynal, Great Neck, NY) was added. After 4 h of incubation at 4 °C, the beads were washed three times with 1 ml of washing buffer (30 mM Hepes, 30 mM NaCl, 0.1% Triton X-100). The pellet was then resuspended in 2 $\times$  Laemmli buffer before SDS-PAGE. SDS-PAGE and Western blotting were performed as described above, and the D8D7I protein was revealed with anti-HA (16B10 clone) antibodies. Binding experiments on immunoprecipitated proteins were performed as follows: proteins were suspended in 200  $\mu$ l of a buffer containing 0.1% bovine serum albumin, 20 mM Tris-HCl, pH 7.4, CHAPS 2.5 mM, KCl 0.4 M. 100  $\mu$ l of this suspension was incubated with 20 nM [<sup>3</sup>H]tamoxifen, with or without 1  $\mu$ M cold tamoxifen, and incubated for 18 h at 4 °C. Specific binding was measured using the Sephadex<sup>TM</sup>-LH20 methodology as described above.

## RESULTS

**Tamoxifen and PBPE Are Modulators of the Biosynthesis of Cholesterol in MCF-7<sup>us</sup> Cells**—We wanted to address the question of what was the effect of tamoxifen and a selective AEBS ligand (PBPE) on the biosynthesis of cholesterol in a tumor cell line. We used a technique that allowed the separation of positional isomers of cholesterol and polyunsaturated sterols (30). This methodology is based on silver nitrate impregnated silica gel plates TLC. The characterization of sterols was done by reference to commercially available standards. 5 $\alpha$ -Cholest-8-en-3 $\beta$ -ol (zymostenol) and 5 $\alpha$ -cholesta-8,24-dien-3 $\beta$ -ol (zymosterol) were not commercially available standards, but their presence and their  $R_F$  was obtained from the published data of Shefer *et al.* (30). Fig. 1 shows the sterol content of 4.10<sup>7</sup> MCF-7<sup>us</sup> cells treated for 48 h with the solvent vehicle (lane 1), 0.5  $\mu$ M tamoxifen (lane 2), 0.5  $\mu$ M PBPE (lane 3), and 10 nM 17 $\beta$ -estradiol (lane 4). In the control lane, the major spot was cholest-5-en-3 $\beta$ -ol (cholesterol) ( $R_F$  = 0.25). Among the sterols from the C-27 series that were of interest, 2 spots co-migrated with 5 $\alpha$ -cholest-7-en-3 $\beta$ -ol (lathosterol) ( $R_F$  = 0.33) and cho-

lesta-5,24-dien-3 $\beta$ -ol (desmosterol) ( $R_F$  = 0.18). A diffuse spot can be seen with a  $R_F$  = 0.48 corresponding to lanosterol that might correspond to other material than lanosterol that was stained in our conditions. In lane 2, tamoxifen treatment induced the appearance, in addition to cholesterol, of a spot (A, lane 2) with a  $R_F$  = 0.14. According to Shefer *et al.* (30), this spot might be 5 $\alpha$ -cholest-8-en-3 $\beta$ -ol (zymostenol). The spot that co-migrated with lathosterol ( $R_F$  = 0.33) was more intense than in the control, and suggested an accumulation of a compound at this  $R_F$ . The treatment of cells with PBPE induced a profound modification of the sterol profile. Cholesterol was the major peak. As seen with tamoxifen a new spot with a  $R_F$  = 0.14 appeared, suggesting the possible appearance of 5 $\alpha$ -cholest-8-en-3 $\beta$ -ol. The spot that co-migrated with desmosterol was more intense than in lanes 1 and 2, suggesting the accumulation of a new product at this  $R_F$  with PBPE treatment. PBPE induced the appearance of a new spot (B, lane 3) that co-migrated with cholesta-5,7-dien-3 $\beta$ -ol (7-dehydrocholesterol;  $R_F$  = 0.07). This suggests that the effect of PBPE was slightly different from that of tamoxifen at this concentration of drug. Treatment of cells with 10 nM 17 $\beta$ -estradiol gave a TLC profile (lane 4) comparable to that of the control (lane 1) with a diminution of the spot corresponding to desmosterol. Interestingly, co-treatment of MCF-7 with 0.5  $\mu$ M tamoxifen or 0.5  $\mu$ M PBPE and 10 nM 17 $\beta$ -estradiol gave the same sterol profile as the one obtained with the single treatment with tamoxifen or PBPE (data not shown). These observations suggested that the participation of the estrogen receptor is not involved in this effect. This experiment showed that sterol metabolism might be affected on the C27 series of sterol in MCF-7 cells by treatment with AEBS ligands.

**Analysis of the Structure of Cholesterol Metabolites That Accumulated in MCF-7<sup>us</sup> over 48 h of Treatment with Tamoxifen or PBPE**—The second set of experiments was performed to determine the structure of the metabolites that were produced by cells when they were treated with tamoxifen or the selective AEBS ligand, PBPE. Neutral lipids were extracted from cells, and were separated by HPLC according to the methodology of Popjak *et al.* (31). The collected fractions were evaporated to dryness, trimethylsilylated, and analyzed by GC/MS for structural analysis according to Gerst *et al.* (41). The diode array detector on the HPLC system enabled UV characterization of homodiene compounds such as 7-dehydrocholesterol to be carried out. The separation of sterols such as cholesterol and lathosterol, which were not separated by HPLC, was achieved by GC of trimethylsilylated derivatives. The mass fragmentation profile of trimethylsilylated derivatives of sterols helped to solve their chemical structures. HPLC chromatograms are shown in Fig. 2. MCF-7 cells treated for 2 days with solvent vehicle gave two major peaks: one peak at 43.5 min (RRT = 1.00) and another peak at 31.6 min (RRT = 0.71) that co-migrated with commercial cholesterol/lathosterol and desmosterol, respectively (Fig. 2A) (41). The subsequent analyses of the RRT = 1.00 peak showed that it contained exclusively cholesterol. This result indicated that the spot at  $R_F$  = 0.33 seen in TLC in the above section was not lathosterol (Fig. 1, lane 1), and the major spots with  $R_F$  = 0.25 and 0.18 were cholesterol and desmosterol, respectively. Furthermore, GC/MS analyses of the fraction collected between 20 and 55 min of the chromatograms did not reveal the presence of any known sterols other than cholesterol, lathosterol, and desmosterol. In particular lanosterol was not detected in our conditions showing that the diffuse spots that co-migrated with lanosterol in TLC experiments were not lanosterol. Tamoxifen treatment induced a profound modification of the HPLC profile similar to the silver nitrate TLC profile (Fig. 2B). Two supple-

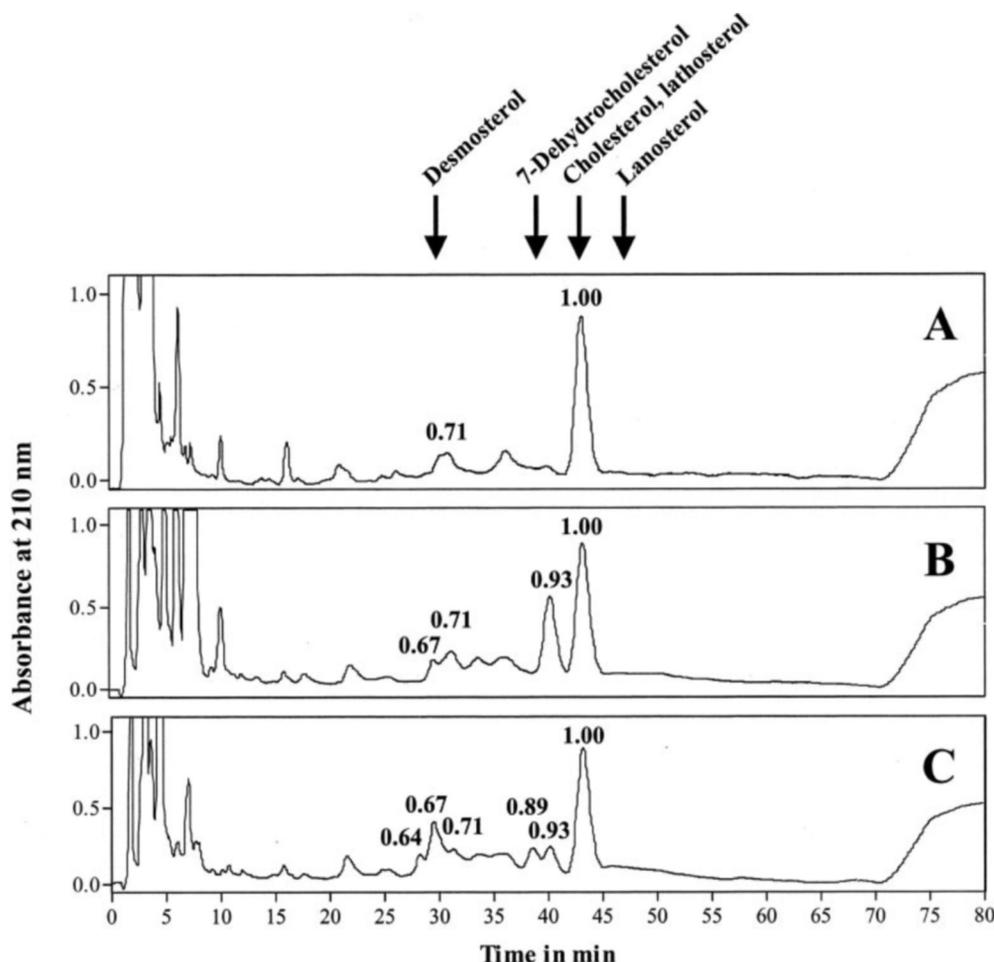


FIG. 2. HPLC profiles of sterols from MCF-7<sup>wt</sup> cells treated with AEBS ligands. The treatment of cells and the extraction of lipids were performed as described under "Experimental Procedures." Sterols were analyzed by HPLC using a reverse-phase column (Lichrosorb C18) run at room temperature with a flow rate of 0.7 ml/min. The profiles of sterols were determined at 210 nm after elution with methanol/H<sub>2</sub>O (96:4, v/v). MCF-7<sup>wt</sup> cells were treated for 48 h with solvent vehicle (A), 0.5  $\mu$ M tamoxifen (B), or 0.5  $\mu$ M PBPE (C). Numbers indicate the RRT. Arrows indicate peaks corresponding to commercial standard sterols: desmosterol, 7-dehydrocholesterol, cholesterol, lanosterol.

mentary peaks appeared with retention times of 29.5 min (RRT = 0.67) and 40.5 min (RRT = 0.93). GC/MS analysis of the fractions at 43.5 min (RRT = 1.00) corresponding to cholesterol, gave two peaks in GC/MS at 19.4 min and 20.1 min. The 19.4 min peak gave ions that are characteristic of trimethylsilylated derivative of cholesterol (Table I) with a molecular ion of 458 (51%), a base peak of 129 (100%) and other fragments corresponding to commercial cholesterol (41). The 20.1 min peak from the GC gave in MS a molecular peak of 458 (95%) and a base peak of 255 (100%) and other fragments corresponding to commercial lathosterol that had been trimethylsilylated (41). Analyses in GC/MS of the fraction at 40.5 min (RRT = 0.93) gave one peak with a molecular ion of 458 (100%) that was the base peak. The fragment profile (Table I) was consistent with the fragment profile of zymosterol (41). The peak at 31.6 min (RRT = 0.71) in the HPLC gave one peak in the GC with a molecular ion of 456 (15%) and a base peak of 129 (100%) with a fragment profile characteristic of commercial desmosterol that had been trimethylsilylated (41). The peak at 29.5 min (RRT = 0.67) gave one peak in GC with a molecular ion of 456 (100%) that corresponded to the base peak. The fragmentation profile (Table I) was characteristic of zymosterol (41). Altogether, these data show that at this concentration, tamoxifen induced the accumulation of precursors of cholesterol in MCF-7 cells. This accumulation might reflect a blockage at the D8D7I step and to a lesser extent at the  $3\beta$ -hydroxysterol- $\Delta^{24}$ -reductase (DHCR24) and the  $3\beta$ -hydroxysterol-C<sup>5</sup>-desaturase

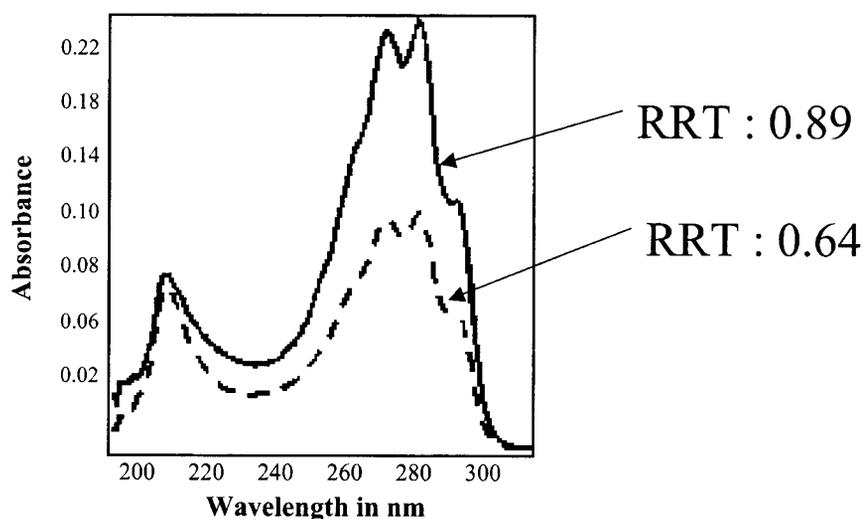
(C5DS) steps. This illustrates that tamoxifen may affect multiple steps in the cholesterol biosynthesis pathway, and this partly confirms the previous observations of Cho *et al.* (42) showing that the D8D7I and DHCR24 steps were inhibited by tamoxifen in normal rat liver microsomes homogenates. Fig. 2C shows the HPLC profile obtained with neutral lipids extracted from MCF-7 cells treated with the selective AEBS ligand PBPE. This compound induced considerable modifications of the post-lanosterol cholesterol. Six major peaks could be detected by HPLC. A GC/MS analysis (Table I) of the peak with a retention time of 43.5 min (RRT = 1.00) in HPLC shows that it contained cholesterol but no trace of lathosterol. The peak at 40.5 min (RRT = 0.93) corresponded to zymosterol, which gave the same fragment profile as the peak at 40.5 min in Fig. 2B. The peak at 38.2 min (RRT = 0.89) gave a UV spectrum that was characteristic of a homo-annular-conjugated diene (Fig. 3) and gave the same UV spectrum as authentic 7-dehydrocholesterol ( $\lambda_{\max 1} = 272 \mu\text{m}$ ,  $\epsilon_1 = 11,250$ ;  $\lambda_{\max 2} = 282 \mu\text{m}$ ,  $\epsilon_2 = 11,900$ ;  $\lambda_{\max 3} = 293 \mu\text{m}$ ,  $\epsilon_3 = 6,650$ ). It gave one peak in GC. Analyses of the fragmentation profile (Table I) gave a molecular ion of  $m/z = 456$  (19%), a base peak of 345 and fragment characteristic of 7-dehydrocholesterol. The peak at 31.6 min (RRT = 0.71) corresponded to desmosterol as judged by GC/MS analyses. The peak at 29.5 min (RRT = 0.67) gave one peak in GC analyses. The fragmentation profile is given in Table I. The  $m/z$  of the molecular ion is 456 (100%), which was also the base peak. The fragmentation profile is consistent with the one of

TABLE I  
GC/MS analysis of trimethylsilylether derivatives of sterols

The sterol composition was analyzed by gas chromatography with a silica column DB5 and helium as carrier gas as described under "Experimental Procedures." The structure of purified sterols obtained by HPLC from MCF-7 cells was determined based on comparison of their relative retention times to standards in GC and further confirmed by mass spectrometry fragmentation.

HPLC RRT	1.00	1.00	0.93	0.71	0.67	0.89
GC $R_t$ in min	19.4	20.1	19.6	19.9	20.08	20.02
<i>m/z</i>				<i>m/z</i>		
458	51	95	100	456	15	100
443	10	18	26	441	11	26
368	97	10	9	372	20	5
353	35	22	26	366	19	28
345	2	10	9	351	23	36
329	79	2	2	343	65	10
275	6	2	4	327	25	1
260	4	6	8	325	4	2
255	27	100	20	253	58	7
247	23	5	5	245	14	3
229	5	30	30	229	4	18
213	16	40	34	227	6	6
143	18	11	12	213	16	22
142	2	3	6	211	9	9
129	100	12	14	129	100	17
Unsaturation position	$\Delta 5$	$\Delta 7$	$\Delta 8$	$\Delta 5,24$	$\Delta 8,24$	$\Delta 5,7$

FIG. 3. UV spectrum of fractions eluted from the HPLC profile of sterols from cells treated with a specific AEBS ligand. UV spectrum of peaks at RRT 0.89 (38.2 min) (solid line) and at RRT 0.64 (28.2 min) (dashed line) from the HPLC chromatogram shown in Fig. 2C.



zymosterol as described by Schroepfer *et al.* (41). Finally, the peak at 28.2 min (RRT = 0.64) gave a UV spectrum with the same characteristics as the peak at 31.6 min that corresponded to a homo-annular-conjugated diene such as 7-dehydrocholesterol (Fig. 3). This peak was not analyzable in GC/MS and did not give a fragmentation profile, probably because the quantity of product was too low. However, taking into account its UV spectrum and the accumulation of cholesterol and zymosterol and their  $\Delta 24$  derivatives, and the accumulation of 7-dehydrocholesterol, it is reasonable to suppose that this compound might be the cholesta-5,7,24-trien-3 $\beta$ -ol (7-dehydrodesmosterol). The same HPLC profiles were observed with the culture medium of the treated cells, but the amounts found were 1:10 (in mass) of the amounts found in cells (data not shown). Altogether, these data showed that PBPE produced the accumulation of sterols that might indicate a blockage at the D8D7I, the DHCR7, and to a lesser extent, the DHCR24 levels.

**Quantitative Analysis of the Modulation of Cholesterol Biosynthesis with Various Antiestrogens and Selective AEBS Ligands on MCF-7<sup>ws</sup> Cells**—We have performed the structural identification of the sterols that accumulated in MCF-7 cells during treatment with various compounds such as E2, tamox-

ifen, PBPE, ICI 182,780, and RU 58,668, and we have estimated the quantity of the sterol species produced with respect to the amount of cholesterol that accumulated (Table II). Solvent vehicle and untreated cells produced 91  $\mu\text{g}$  of cholesterol and 0.3  $\mu\text{g}$  of desmosterol per  $4 \times 10^7$  cells. Treatment with tamoxifen produced 88  $\mu\text{g}$  of cholesterol, 79  $\mu\text{g}$  of zymosterol, and 1.3  $\mu\text{g}$  of desmosterol per  $4 \times 10^7$  cells. As expected, pure antiestrogens such as ICI 182,780 and RU 58,668 did not significantly modify the post-lanosterol profile of cells; these compounds have no affinity for the AEBS. 80  $\mu\text{g}$  of cholesterol were found in cells treated with PBPE and the accumulation of 29  $\mu\text{g}$  of zymosterol, 1.3  $\mu\text{g}$  of desmosterol, 0.8 ng of 7-dehydrocholesterol, and 0.4 ng of 7-dehydrodesmosterol could be measured. Tamoxifen was more potent than PBPE in inhibiting D8D7I. The amount of  $\Delta^{24}$ -sterol obtained by PBPE treatment was 1.5 $\times$  the amount obtained with tamoxifen, suggesting a weak inhibition of DHCR24 with PBPE but no inhibition at this concentration with tamoxifen. Interestingly, the HPLC profile obtained after co-treatment of MCF-7 cells with 0.5  $\mu\text{M}$  tamoxifen and a 0.5  $\mu\text{M}$  concentration of PBPE was similar qualitatively and quantitatively to that of PBPE alone. PBPE displayed a 5 $\times$  higher affinity than tamoxifen for the AEBS in

TABLE II

Determination and quantification of sterols accumulated in MCF-7<sup>ws</sup> during 48 h of treatment with tamoxifen and PBPE

Analyses were performed by HPLC as described in the legend to Fig 2. The identification of sterols was done as reported in Table I. Quantification of sterol intermediates in cells treated with AEBS ligands was calculated as a percent by weight of total sterol. The amount of sterol in each peak was quantified by reference to an external standard.

Treatment	Sterol amount/total sterol					
	$\Delta 8,24$	$\Delta 8$	$\Delta 5,7,24$	$\Delta 5,7$	$\Delta 7$	$\Delta 5,24$
	%					
Control						0.4
Tx	0.5	34.6			0.1	0.8
PBPE	2.1	21.7	0.001	0.004		1.01
Tx + PBPE	1.9	23.3	0.002	0.003		0.9
E2						0.4
E2 + Tx	0.6	33.9			0.08	0.8
E2 + PBPE	2.0	22.1	0.002	0.006		0.9
ICI 182,780						0.5
RU 58,668						0.4

MCF-7 suggesting that the sterol profile obtained resulted from the binding of PBPE to the AEBS.

Fig. 4A shows that tamoxifen and the selective AEBS ligand PBPE induced a time-dependent accumulation of 5 $\alpha$ -cholest-8-en-3 $\beta$ -ol (zymostenol) in MCF-7. The accumulation of zymostenol reached a plateau 24 h after the start of treatment and decreased strongly after 48 h. This showed that for the same number of cells the total mass of sterols had almost doubled in cells. When cells were scraped after treatment with the drugs, they were immediately worked up for sterol analysis, otherwise the signal corresponding to zymostenol diminished strongly. Zymostenol is an unstable sterol, and presumably very sensitive to oxidation. This probably paralleled the diminution of zymostenol content observed during the kinetics and the convergent appearance of more polar and yet unidentified compounds. Even when cells were worked up in the presence of the antioxidant butylated hydroxytoluene, we observed a time-dependent decrease in the zymostenol content. This problem was less apparent in the case of other unsaturated sterols such as desmosterol and 7-dehydrocholesterol that were detectable up to 96 h in cells, and after several months of conservation at -80 °C.

Fig. 4B shows a dose response curve for the accumulation of zymostenol in MCF-7 cells treated for 48 h with tamoxifen or PBPE. Both drugs induced a concentration-dependent accumulation of zymostenol with an ED<sub>50</sub> of 163 and 630 nM, respectively. The accumulation of zymostenol reached a plateau at a mean value of 75  $\mu$ g for 4  $\times$  10<sup>7</sup> cells for tamoxifen and PBPE. These results showed that AEBS ligands induced a time- and concentration-dependent accumulation of zymostenol, and revealed that the ED<sub>50</sub> for the inhibition of the enzyme was 32.6 and 630 $\times$  lower than their affinity for the AEBS. These data showed that the treatment of cells with tamoxifen led to the doubling of the amount of sterol species in cells. This accumulation was followed by a synchronization of cells in G<sub>0</sub>-G<sub>1</sub> phase of the cell cycle (data not shown) showing the parallel between growth control and the accumulation of sterol species in cells.

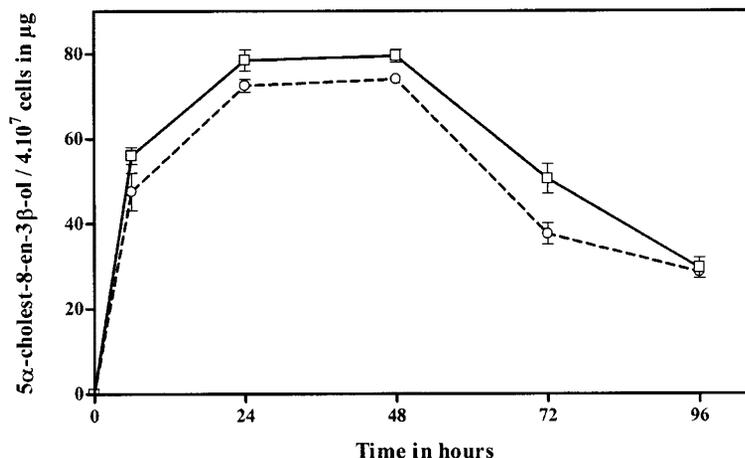
*Modulation of the Biosynthesis of Cholesterol in Wild-type MCF-7, MDA-MB-231, and SAOS-2 Cells Treated for 48 h with Antiestrogens and AEBS Ligands*—In the next set of experiments we treated wild-type MCF-7 cells that were grown in the presence of 4% fetal calf serum. The cholesterol profiles are described in Table III. Table III shows that MCF-7 treated with 5  $\mu$ M tamoxifen or 10  $\mu$ M PBPE gave a sterol profile that was similar to that obtained with MCF-7<sup>ws</sup>. The sensitivity of wild-type MCF-7 cells to the accumulation of sterols induced by AEBS ligands was 10 $\times$  less than for MCF-7<sup>ws</sup>. The same con-

centrations of drugs were used to treat MDA-MB-231 (an estrogen receptor-negative human adenocarcinoma mammary cell line) and SAOS-2 cells (a human osteosarcoma cell line), and we obtained an HPLC profile comparable to that of MCF-7<sup>ws</sup>. GC/MS analysis of HPLC-purified sterols confirmed the identity of the different sterols that accumulated in cells. This shows that *de novo* biosynthesis of cholesterol was highly active in these proliferative cells despite the presence of serum lipids in the medium. This accumulation of sterols did not seem to be dependent on the estrogen receptor status of cells.

*The Reconstitution of the AEBS by Co-expression of D8D7I and DHCR7*—To assess the relationship between the metabolism of cholesterol and the nature of the AEBS, we have expressed the HA-D8D7I and/or the HA-DHCR7 in COS-7 cells. As COS-7 cells have been previously tested by Moebius *et al.* (26) without success for the expression of D8D7I (EBP), we therefore expressed both proteins in these cells. Western blotting analyses of the transfected cells showed that anti-HA antibody recognized bands on SDS-PAGE with molecular masses corresponding to the expected size of both recombinant proteins of 28 kDa for HA-D8D7I and 40 kDa for HA-DHCR7 (Fig. 5). This result was consistent with published observations (26, 34). Scatchard analysis as presented in Fig. 6A showed that COS-7 cells had a basal expression of the AEBS with a  $K_d = 4.2 \pm 1.2$  nM and a  $B_{max} = 2.02 \pm 0.5$  pmol/mg for tamoxifen. In COS-7 transfected with pSG5-HA-D8D7I (Fig. 6A) the  $K_d$  and the  $B_{max}$  of [<sup>3</sup>H]tamoxifen were  $7.2 \pm 0.9$  nM and  $1.82 \pm 0.6$  pmol/mg protein, respectively. The  $B_{max}$  was unchanged, and the affinity was decreased by a factor of 1.7. In COS-7 transfected with pSG5-HA-DHCR7 (Fig. 6A) the  $K_d$  and the  $B_{max}$  for [<sup>3</sup>H]tamoxifen were  $7.47 \pm 0.6$  nM and  $5.9 \pm 0.5$  pmol/mg protein. The  $B_{max}$  of [<sup>3</sup>H]tamoxifen increased by a factor of 2.95 with respect to the control. As seen in Fig. 6B the co-expression of both enzymes gave a  $K_d = 6.4 \pm 1.6$  nM and a  $B_{max} = 52.6 \pm 5.2$  pmol/mg protein. This value corresponds to an increase of the  $B_{max}$  by a factor of 26.3 and illustrated the appearance of a high affinity and high capacity binding site for tamoxifen. The same experiments, performed using proteins without the HA tag, gave similar results showing that the fusion with HA had no effects on the binding activity of [<sup>3</sup>H]tamoxifen. Pharmacological profiles of cells transfected with both HA-D8D7I and HA-DHCR7 are shown in Fig. 7. The order of affinity of various AEBS ligands for the reconstituted [<sup>3</sup>H]tamoxifen binding site was: clomiphene ( $K_i = 1.09 \pm 0.4$  nM) > PBPE ( $K_i = 1.46 \pm 0.2$  nM) > tamoxifen ( $K_i = 1.93 \pm 0.2$  nM) > CI-628 ( $K_i = 1.85 \pm 0.4$  nM) > 7-ketocholestanol ( $K_i = 2.42 \pm 0.7$  nM) > 4-OH-tamoxifen ( $K_i = 9.99 \pm 0.6$  nM) > BD 1008 ( $K_i = 59.47 \pm 0.8$  nM) > U-18,666A ( $K_i = 70.96 \pm 1.1$  nM) > *t*-BuPE ( $K_i = 228.61 \pm 2.4$  nM). Cholestanol, cholesterol, lathosterol, zymostenol, 7-dehydrocholesterol, desmosterol, lanosterol, 17 $\beta$ -estradiol, and pure antiestrogen ICI 164,384 did not compete for [<sup>3</sup>H]tamoxifen binding. This pharmacological profile corresponds to that of the AEBS defined in the AEBS from MCF-7 cell microsomes (5–7, 12, 19). We also performed [<sup>3</sup>H]tamoxifen binding experiments in detergent solution, after solubilization with CHAPS of the recombinant proteins. These conditions solubilized insoluble sterols, but again cholestanol, cholesterol, lathosterol, zymostenol, 7-dehydrocholesterol, desmosterol, lanosterol, 17 $\beta$ -estradiol, and pure antiestrogen ICI 182,780 had no detectable affinity for the AEBS (data not shown).

*Production of a Polyclonal Antibody against D8D7I*—The above results suggested that the AEBS required D8D7I and DHCR7 as subunits. To validate this hypothesis we produced a polyclonal antibody against D8D7I in order to conduct immunoprecipitation studies to show whether D8D7I and DHCR7

A



B

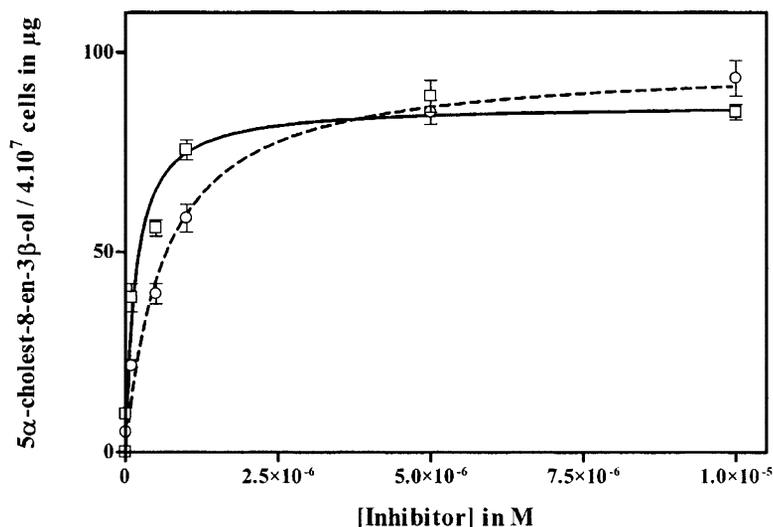


FIG. 4. Kinetics and dose response study of the production of 5 $\alpha$ -cholest-8-en-3 $\beta$ -ol by MCF-7 treated with tamoxifen or PBPE. A, kinetics of the accumulation of 5 $\alpha$ -cholesta-8-en-3 $\beta$ -ol (zymostenol) in MCF-7<sup>ws</sup> after treatment with tamoxifen ( $\square$ ) and PBPE ( $\circ$ ) for 0, 6, 12, 24, 48, 72, and 96 h. B, dose response studies were done with increasing concentrations of tamoxifen ( $\square$ ) and PBPE ( $\circ$ ) for 48 h of incubation of MCF-7<sup>ws</sup> cells.  $8 \times 10^7$  to  $10^8$  cells were used per condition. Analysis of 5 $\alpha$ -cholesta-8-en-3 $\beta$ -ol was done by HPLC as described in the legend to Fig. 2.

TABLE III  
Determination and quantification of sterols accumulated in MCF-7, SAOS-2, and MDA-MB-231 during 48 h of treatment with tamoxifen or PBPE

Analysis was performed as described in Table II.

Cells	Treatment	Sterol amount/total sterol					
		$\Delta 8,24$	$\Delta 8$	$\Delta 5,7,24$	$\Delta 5,7$	$\Delta 7$	$\Delta 5,24$
%							
MCF-7	Control						0.4
	Tx	0.4	31.5			0.1	0.6
	PBPE	1.2	20.8	0.001	0.004		0.8
SAOS-2	Control						0.6
	Tx	1.0	28.2				1.2
	PBPE	1.8	18.5	0.002	0.003		1.0
MDA-MB-231	Control						0.6
	Tx	0.3	22.2				0.8
	PBPE	0.7	19.5	0.002	0.003		0.8

were associated within the same complex. Results related to the expression and the affinity purification of the recombinant D8D7I expressed in *E. coli* are presented in Fig. 8. Bacteria

were solubilized and the extract purified to near homogeneity in one step by the desorption of proteins by a step gradient of imidazole using Ni-NTA agarose as an affinity gel. The size of

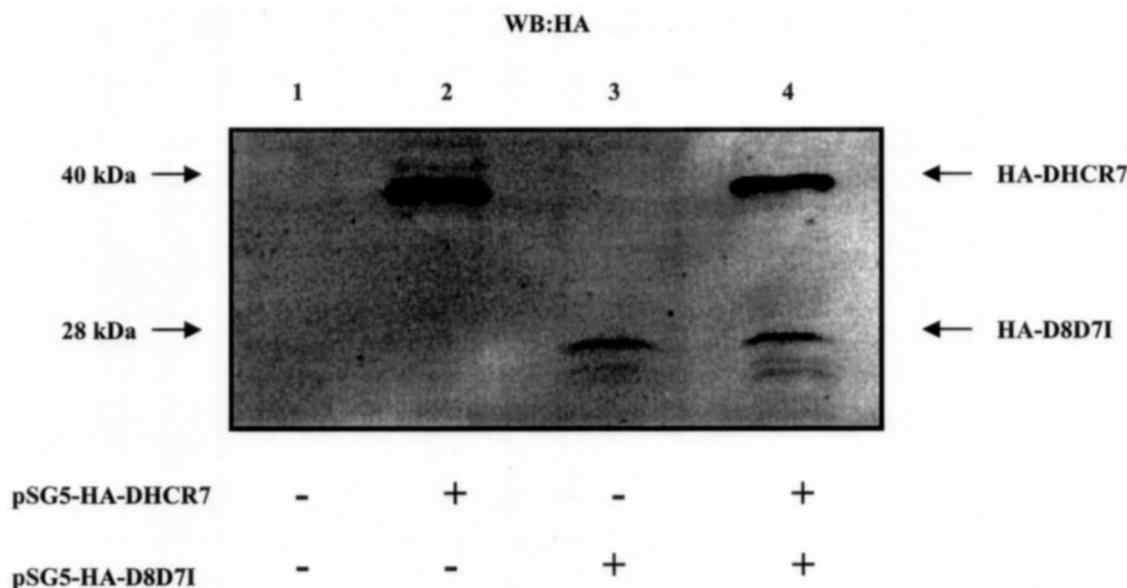


FIG. 5. Western blot analysis of D8D7I and HA-DHCR7 expressed in transfected COS-7 cells. Cells were transiently transfected with Mock (pSG5) (lane 1), pSG5-HA-DHCR7 (lane 2), pSG5-HA-D8D7I (lane 3), or both pSG5-HA-DHCR7 and pSG5-HA-D8D7I (lane 4). For all experiments, COS-7 cells were also transfected with 50 ng of pCMV-lacZ to measure the efficiency of transfection.  $\beta$ -Galactosidase activity was measured as described under "Experimental Procedures" and used to control the transfection efficiency. 40  $\mu$ g of total protein were subjected to SDS-PAGE and Western blotting. Expressed proteins were detected with an anti-HA antibody (16B10 clone).

the His<sub>6</sub>-tagged D8D7I was 28 kDa (Fig. 8A, lanes 4–7). These purified proteins were used to immunize rabbits. Dot blots are represented in Fig. 8B, and it can be seen that the serum recognized the bacterial affinity purified recombinant protein. Recombinant D8D7I was detected up to a dilution of 10,000 for 5 ng of recombinant protein. The specificity of the recognition was confirmed by the absence of recognition of recombinant microsomal epoxide hydrolase (data not shown).

**D8D7I and DHCR7 Formed a Complex That Bound Tamoxifen**—In this series of experiments we have tested whether D8D7I and DHCR7 are associated within a complex. Transfection of COS-7 cells with pSG5 alone, pSG5-D8D7I or/and pSG5-HA-DHCR7, and 50 ng of pCMV-lacZ to measure the efficiency of transfection were performed. Extracts from transfected cells were immunoprecipitated with the immune serum directed against the D8D7I. Extracts were analyzed for  $\beta$ -galactosidase activity, and the variation of activity was less than 4% for the different conditions. Immunoprecipitated proteins were analyzed by SDS-PAGE and electrotransferred onto a polyvinylidene difluoride membrane. Immunoblotting with anti-HA antibodies were then performed. Fig. 9A shows that in the absence of HA-DHCR7 no band could be detected (Fig. 9A, lanes 1 and 2). In contrast, expression of HA-DHCR7 or co-expression of HA-DHCR7 and D8D7I led to the appearance of a band with the expected size of 40 kDa (Fig. 9A, lanes 3 and 4). In lane 4, the band corresponding to DHCR7 was more intense than in lane 3 because of the expression of the recombinant DHCR7. Fig. 9B shows [<sup>3</sup>H]tamoxifen binding experiments with immunoprecipitated proteins in each of the above conditions. Specific binding was detected in the case of the single expression of HA-DHCR7 or with both D8D7I and HA-DHCR7. In the first case the specific binding was one-tenth of that bound in the case of co-expression experiments. This paralleled [<sup>3</sup>H]tamoxifen binding experiments described in the above section in which the  $B_{\max}$  for co-expression experiments with D8D7I and DHCR7 was 10 $\times$  the values obtained with the single DHCR7 expression experiment.

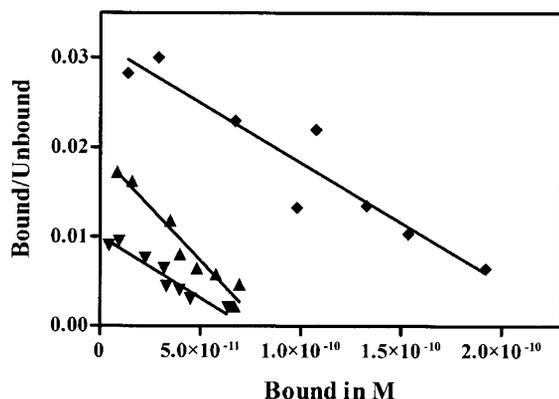
**Binding to the AEBS Is Not Correlated with the Inhibition of D8D7I or DHCR7 in MCF-7 Cells**—First, we have shown that two ligands of the AEBS (tamoxifen and PBPE) inhibited the

catalytic activities of D8D7I or/and DHCR7, and second that the overexpression of D8D7I and DHCR7 resulted in the reconstitution of the [<sup>3</sup>H]tamoxifen binding site on the AEBS. We have also shown that sterols that were substrates and products of the catalytic activities of both D8D7I and DHCR7 were not ligands of the AEBS suggesting that the binding site of drugs on the AEBS was different from the catalytic sites of enzymes. In order to get more insight into this point we have evaluated the potentiality of other AEBS ligands to inhibit D8D7I or DHCR7 in MCF-7 cells. Tables IV and V show the affinity of various compounds for the AEBS extracted from MCF-7 cells, and the nature of the sterol metabolites that accumulated in MCF-7 after 48 h treatment with these compounds. All these compounds were apparent competitive inhibitors of [<sup>3</sup>H]tamoxifen binding to the AEBS, as measured by Scatchard analysis, because the presence of these inhibitors diminished the affinity of tamoxifen for the AEBS as judged by an increase of the  $K_d$  value in tamoxifen without diminishing the  $B_{\max}$  (data not shown).

First antiestrogens were evaluated as shown in Table IV. Non-phenolic triphenyl ethylenic compounds such as tamoxifen, CI-628 (nitromiphen), and clomiphene (Clomid) gave a similar sterol profile; the major metabolite that accumulated in cells was zymosterol. Interestingly, the major metabolite accumulated with raloxifene treatment was zymosterol, suggesting that this compound inhibited DHCR24 and D8D7I. 4-OH-Tamoxifen and RU 39,411 produced the selective accumulation of desmosterol, showing that D8D7I and DHCR7 were not inhibited by these compounds despite their high affinity for the AEBS. Pure antiestrogens such as ICI 164,384, ICI 182,780, and RU 58,668 had no impact on post-lanosterol biosynthetic enzymes.

Diphenylmethane compounds such as PBPE, DPPE, and MBPE were inhibitors of D8D7I and DHCR7 (Table V) and were almost equipotent at 10  $\mu$ M and gave a similar profile of sterol precursors in MCF-7. Similar results were obtained with the cumylphenol derivatives PCPE and MCPE. *t*-BuPE had no effect on sterol metabolism on the C27 series in MCF-7, despite the fact that it is an AEBS ligand with moderate affinity ( $K_i$ , 200 nM). 7-Ketocholestanol is a high affinity ligand for the AEBS and was a selective inhibitor of D8D7I. 7-Ketocholesterol

A



B

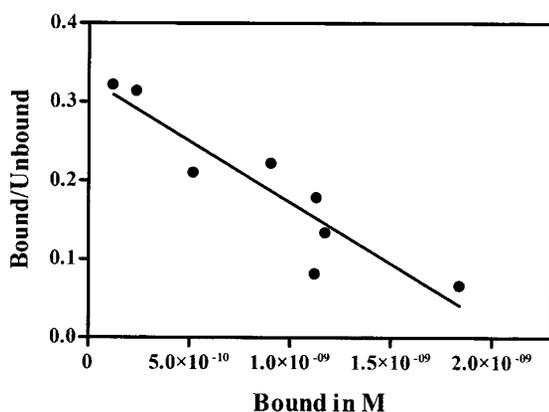


FIG. 6. Scatchard analysis of tamoxifen binding in COS cells transfected with pSG5-DHCR7 and pSG5-D8D7I. Scatchard plots of specifically bound tamoxifen to lysates of COS-7 cells transfected with control vector pSG5 (▼), pSG5-D8D7I (▲), pSG5-DHCR7 (◆) (A), or pSG5-D8D7I and pSG5-DHCR7 (●) (B). For all experiments, COS-7 cells were also transfected with 50 ng of pCMV-lacZ to measure the efficiency of transfection.  $\beta$ -Galactosidase activity was measured as described under "Experimental Procedures" and used to control the transfection efficiency. Microsomes from transfected cells were prepared as described under "Experimental Procedures." 10  $\mu$ g of microsomal protein were incubated in a binding buffer (20 mM Tris-HCl, 2.5 mM EDTA, pH 7.4, 2.5 mM thioglycerol) with various concentrations of [ $^3$ H]tamoxifen (84.0 Ci/mmol; Amersham Biosciences) from 0.1 to 25 nM  $\pm$  1  $\mu$ M of cold tamoxifen for 18 h at 4  $^{\circ}$ C. After incubation, bound and free radioligands were separated using Sephadex-LH20 gel filtration (1.5 ml), and the flow-through was counted for radioactivity in a  $\beta$  counter. Binding assays were performed in duplicate in at least three separate experiments.

had a 53.6 $\times$  lower affinity than tamoxifen for the AEBS and is an inhibitor of D8D7I. BD 1008 and U-18,666A displayed an affinity in the same range for the AEBS. BD 1008 was an inhibitor of both D8D7I and DHCR7 but U-18,666A, which had the same affinity as BD 1008 for the AEBS did not inhibit these enzymes. Finally, we have tested two prototypical inhibitors of DHCR7, AY-9944, and BM 15,667. Both compounds were competitive ligands of weak affinity for the AEBS and, as expected, inhibitors of DHCR7 in MCF-7. Interestingly, AY-9944 was also an inhibitor of D8D7I. These data showed that binding to the AEBS was not systematically associated with inhibition of D8D7I or DHCR7 illustrating that the binding site for tamoxifen on the AEBS was different from the catalytic sites of D8D7I and DHCR7.

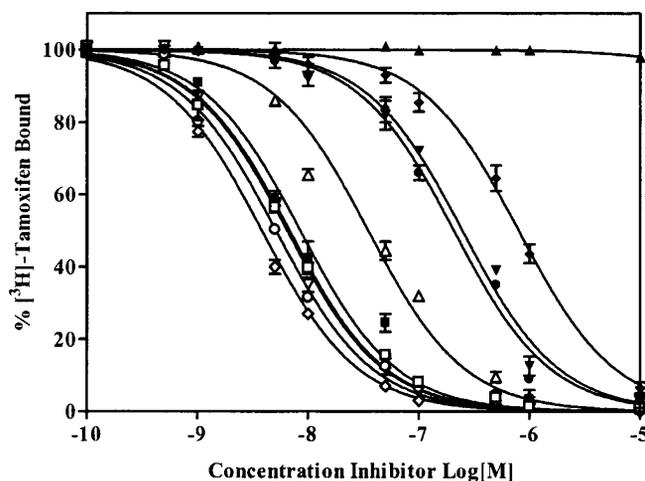
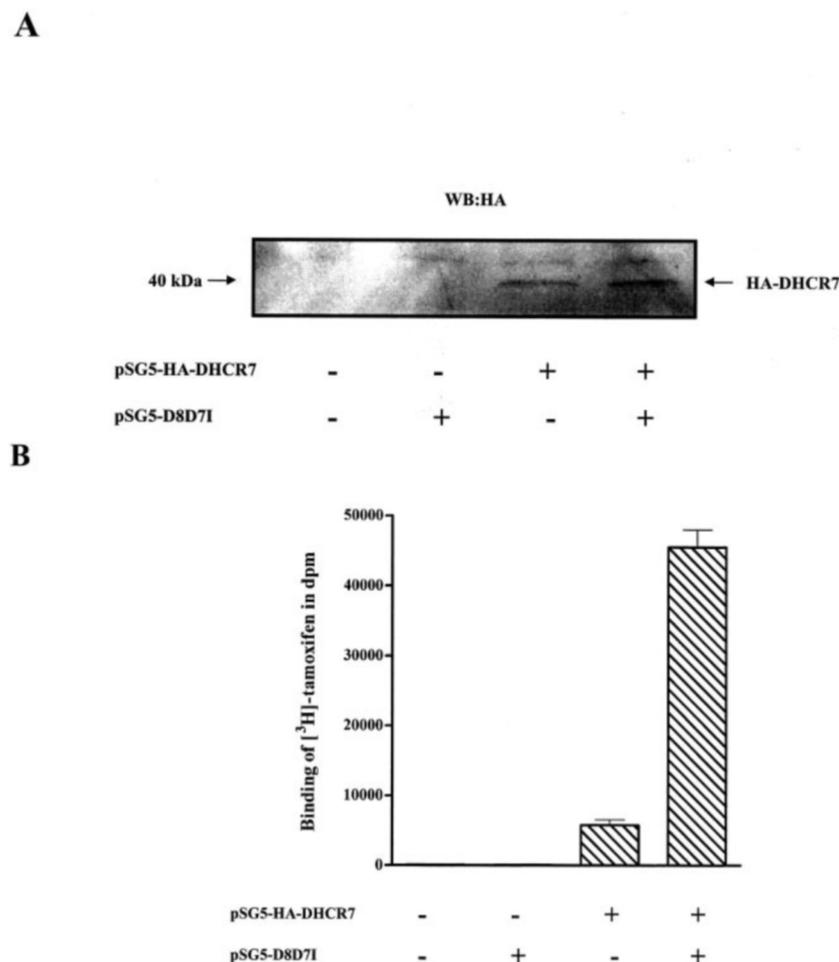
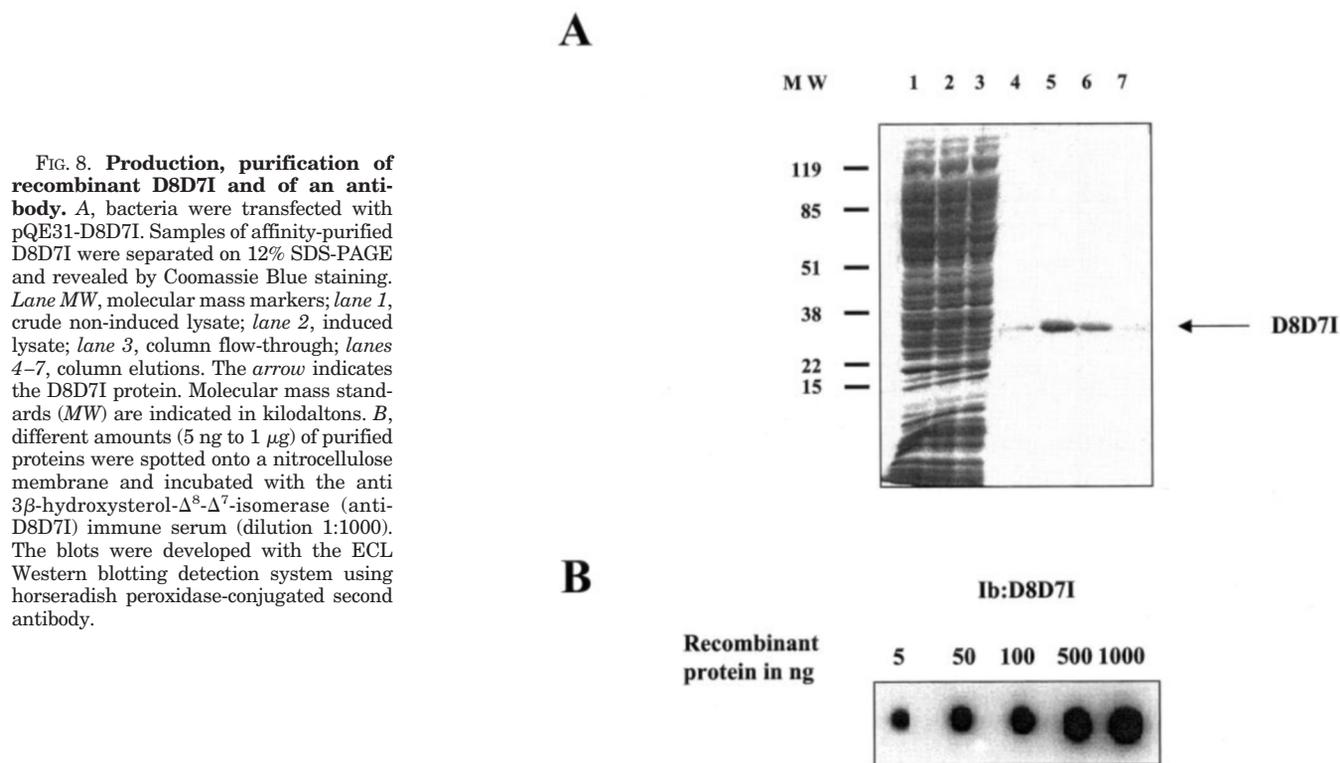


FIG. 7. Inhibition of [ $^3$ H]tamoxifen binding to a microsomal extract of COS-7 cells that co-expressed human recombinant D8D7I and DHCR7. Competition assays with clomiphene (◇), PBPE (○), tamoxifen (□), CI-628 (▽), 7-ketocholestanol (■), 4-OH-tamoxifen (△), BD 1008 (●), U-18,666A (▼), *t*-BuPE (◆), ICI 164,384 (▲) on whole cell lysates of transfected COS-7 cells were performed using eight concentrations of unlabeled ligand ranging from 0.1 to 10,000 nM with a single concentration of [ $^3$ H]tamoxifen of 3 nM. Incubation and separation of bound and free radioligand was performed as described above. Binding and competition assays were performed in duplicate in at least three separate experiments. The competition assay was performed as described under "Experimental Procedures."

#### DISCUSSION

In this study, we first showed that when tumor cells were exposed to tamoxifen and PBPE at concentrations that induced growth control over 48 h, then sterols accumulated that would not normally be found in these cells. By using a combination of analytical purification techniques we have performed a structural analysis of the sterol metabolites that appeared during the treatment of tumor cells with drugs. The metabolites identified were 5 $\alpha$ -cholest-8-en-3 $\beta$ -ol (zymostenol), cholesta-5,7-dien-3 $\beta$ -ol (7-dehydrocholesterol), 5 $\alpha$ -cholesta-8,24-dien-3 $\beta$ -ol (zymosterol); 5 $\alpha$ -cholest-7-en-3 $\beta$ -ol (lathosterol), cholesta-5,24-dien-3 $\beta$ -ol (desmosterol), and cholesta-5,7,24-trien-3 $\beta$ -ol (7-dehydrodesmosterol). Our results showed that AEBS ligands, depending on their chemical structure, inhibited different steps involved in the biosynthesis of cholesterol (Fig. 10): the D8D7I step, the DHCR7 step, and to a lesser extent the DHCR24 and the C5DS steps. These results established for the first time a precise elucidation of the structure of sterol metabolites that accumulated quantitatively in tumor cells treated with a prototypical selective AEBS ligand (PBPE) or with the antitumoral drug tamoxifen. The concentrations that were used corresponded to those at which PBPE and tamoxifen were cytostatic. The accumulation of cholesterol precursors after drug treatment was measurable on MCF-7 cells and on two other tumor cell lines: MDA-MB-231 and SAOS-2, suggesting that tamoxifen and PBPE can block the biosynthesis of cholesterol in various cell lines that express the AEBS.

Diphenylmethane derivatives such as PBPE are competitive inhibitors of tamoxifen binding on the AEBS (27), and diphenylmethane compounds bind with high affinity to one class of binding site (5, 27, 43) showing that diphenylmethane and tamoxifen bound to the same binding site. In the cholesterologenesis pathway, D8D7I occurs before DHCR7. One would have expected that co-treatment with tamoxifen and PBPE might only inhibit D8D7I. However, co-treatment of cells with PBPE and tamoxifen induced a sterol profile that was identical to that obtained with PBPE alone, and PBPE displayed a 5 $\times$  higher affinity than tamoxifen for the AEBS in MCF-7 cells



**FIG. 9. Interaction between D8D7I and DHCR7 and [<sup>3</sup>H]tamoxifen binding to the D8D7I-DHCR7 complex.** *A*, Western blots of immunoprecipitated proteins from cells transfected with empty vector pSG5 (*lane 1*), pSG5-D8D7I (*lane 2*), pSG5-HA-DHCR7 (*lane 3*), or pSG5-D8D7I and pSG5-HA-DHCR7 (*lane 4*). In all experiments, COS-7 cells were also transfected with 50 ng of pCMV-lacZ to control the efficiency of transfection as described under "Experimental Procedures." Immunoprecipitations of the proteins from transfected cells were carried out with anti-D8D7I as described under "Experimental Procedures." DHCR7 was revealed with anti-HA. *B*, binding experiments on immunoprecipitated proteins performed as described under "Experimental Procedures." Immunoprecipitates were resuspended and incubated with 20 nM of [<sup>3</sup>H]tamoxifen in the presence or in the absence of 1  $\mu$ M cold tamoxifen and incubated for 18 h at 4 °C. Specific binding was measured using the Sephadex<sup>TM</sup>-LH20 methodology as described under "Experimental Procedures."

(43). These data showed that the PBPE occupation of the AEBS produced a dual inhibition of D8D7I and DHCR7.

Despite a 5-fold higher affinity than tamoxifen for the AEBS,

PBPE was less efficient at inhibiting D8D7I on intact MCF-7 cells than expected. We propose two explanations. 1) We have shown previously that the uptake of tamoxifen or diphenyl-

TABLE IV

Determination and quantification of sterols accumulated in MCF-7 after 48 h of incubation with antiestrogens of various affinity for the AEBS

Analyses were performed as described in Table II. MCF-7 microsomes were incubated with 3 nM [<sup>3</sup>H]tamoxifen and 12 concentrations of unlabeled test ligands ranging from 0.1 to 100 nM or 1 to 10,000 μM. Assays included 1 μM 17β-estradiol. IC<sub>50</sub> values were determined using the curve fitting program GraphPad Prism. IC<sub>50</sub> values were converted into the apparent  $K_i$  using the Cheng-Prusoff equation and the  $K_i$  values of tamoxifen (39). Metabolism experiments were conducted in triplicate, and the values presented in the table were taken from one representative of three independent experiments

Drug	Affinity for AEBS – $K_i$	Sterol amount/total sterol					
		Δ8,24	Δ8	Δ5,7,24	Δ5,7	Δ7	Δ5,24
	nM	%					
Control							0.4
Tamoxifen	5.3 ± 1.5 <sup>a</sup>	0.4	31.5			0.1	0.6
Clomiphene	0.5 ± 0.8	0.2	36.2			0.1	0.4
CI-628	2.4 ± 1.1	0.4	32.8			0.3	0.4
Raloxifene	5.8 ± 0.9	38.4	1.8		0.08		0.8
4-OH-Tamoxifen	7.2 ± 1.4		1.9				42.1
RU 39,411	38.4 ± 1.6						48.9
ICI 182,780	>10,000						0.4
RU 58,668	>10,000						0.4

<sup>a</sup>  $K_i$  for tamoxifen.

TABLE V

Determination and quantification of sterols accumulated in MCF-7 cells during 48 h of incubation with AEBS ligands and inhibitors of cholesterologenesis of various affinity for the AEBS

Analysis was performed as described in Table II.

Drug	Affinity for AEBS – $K_i$	Sterol amount/total sterol					
		Δ8,24	Δ8	Δ5,7,24	Δ5,7	Δ7	Δ5,24
	nM	%					
PBPE	1.2 ± 0.4	1.2	20.8	0.001	0.004		0.8
DPPE	32 ± 1.2	0.4	18.2	0.001	0.004		0.4
PCPE	2.5 ± 0.6	0.2	24.2	0.001	0.005		0.9
MBPE	5.1 ± 0.8	0.4	18.8	0.001	0.004		0.4
MCPE	7 ± 0.6	0.2	18.6	0.001	0.005		0.4
t-BuPE	200 ± 4.5						0.4
7-Ketocholestanol	8.4 ± 0.8		18.3				0.4
7-Ketocholesterol	284 ± 2		6.1				0.4
6-Ketocholestanol	32.5 ± 1.2		11.5				0.4
BD 1008	62.8 ± 3.5	0.4	19.8	0.001	0.0035		1.2
AY-9944	358 ± 6.1		6.6		0.013		48.9
BM 15,667	642 ± 11				0.022		1.2
U-18,666A	62.5 ± 3.5						0.4

methane compounds was rapid and reaches equilibrium after 3 min of incubation with cells (43). Diphenylmethane derivatives such as PBPE are 2 orders of magnitude less lipophilic than tamoxifen (44) and require a 5–10× higher concentration for a comparable uptake by cells. 2) The full occupation of the AEBS does not induce a complete inhibition of the enzymes. This suggests that the drug binding sites were different from the catalytic sites of the enzymes and explains why we observed a shift between the affinity of compounds for the AEBS and their efficiency to block cell proliferation.

Transient expression experiments in COS-7 cells showed that the single expression of D8D7I did not significantly change the binding parameters of tamoxifen when compared with the mock-transfected COS-7 cells. The single expression of DHCR7 produced a slight increase of the  $B_{max}$  for tritiated tamoxifen. This is interesting because tamoxifen did not inhibit the activity of this enzyme in our experiments as much as in the case of DHCR7 expressed in yeast (34). This illustrates that DHCR7 is involved in the binding of tamoxifen. The co-expression of both enzymes potentiated this increase of binding more than additively showing that the binding of [<sup>3</sup>H]tamoxifen required both enzymes. These data might explain the observations of Moebius *et al.* (26). They have showed that the addition of microsomes from mammalian cells into yeast extracts containing recombinant mammalian D8D7I increased the  $B_{max}$  for tritiated emopamil and they suggested that cofactors might be present in such extracts that helped emopamil binding to D8D7I (EBP) expressed in yeast. Co-expression of D8D7I and

DHCR7 gave a [<sup>3</sup>H]tamoxifen displacement profile consistent with the pharmacological profile established for the AEBS from tumor cell lines (5, 11, 19, 45). None of the intermediates of the post-lanosterol cholesterol biosynthesis pathway we have tested display any detectable affinity for the reconstituted AEBS. Moreover, we have shown that compounds that compete with tamoxifen for binding to the AEBS do not systematically inhibit D8D7I and DHCR7. This suggests that the catalytic domains of D8D7I and DHCR7 were different from the binding sites for drugs on the AEBS. This is consistent with reports showing that tamoxifen or AY-9944 and BM 15,766 were non-competitive inhibitors of DHCR7 or D8D7I in rat liver extracts (26, 42, 46).

DHCR7 has a calculated size of 54 kDa. However, its mobility in SDS-PAGE corresponded to a 40 kDa protein when expressed in mammalian cells or yeast (this study and Ref. 34). This apparent mobility corresponds to the mobility of the tamoxifen binding subunit of 40 kDa in the AEBS that we identified by photoaffinity labeling experiments (27). Immunoprecipitation demonstrated that both D8D7I and DHCR7 were associated. The calculated molecular mass of the complex of D8D7I and DHCR7 is 82 kDa, which corresponds to the size that has been measured by inactivation with ionizing radiation for the solubilized AEBS (5, 18) and D8D7I (47).

AEBS ligands produced different sterol profiles in MCF-7 cells. Non-hydroxylated triphenylethylenic antiestrogens, 6- or 7-ketosterols were inhibitors of D8D7I whereas hydroxylated antiestrogens were inhibitors of DHCR24. The presence of one

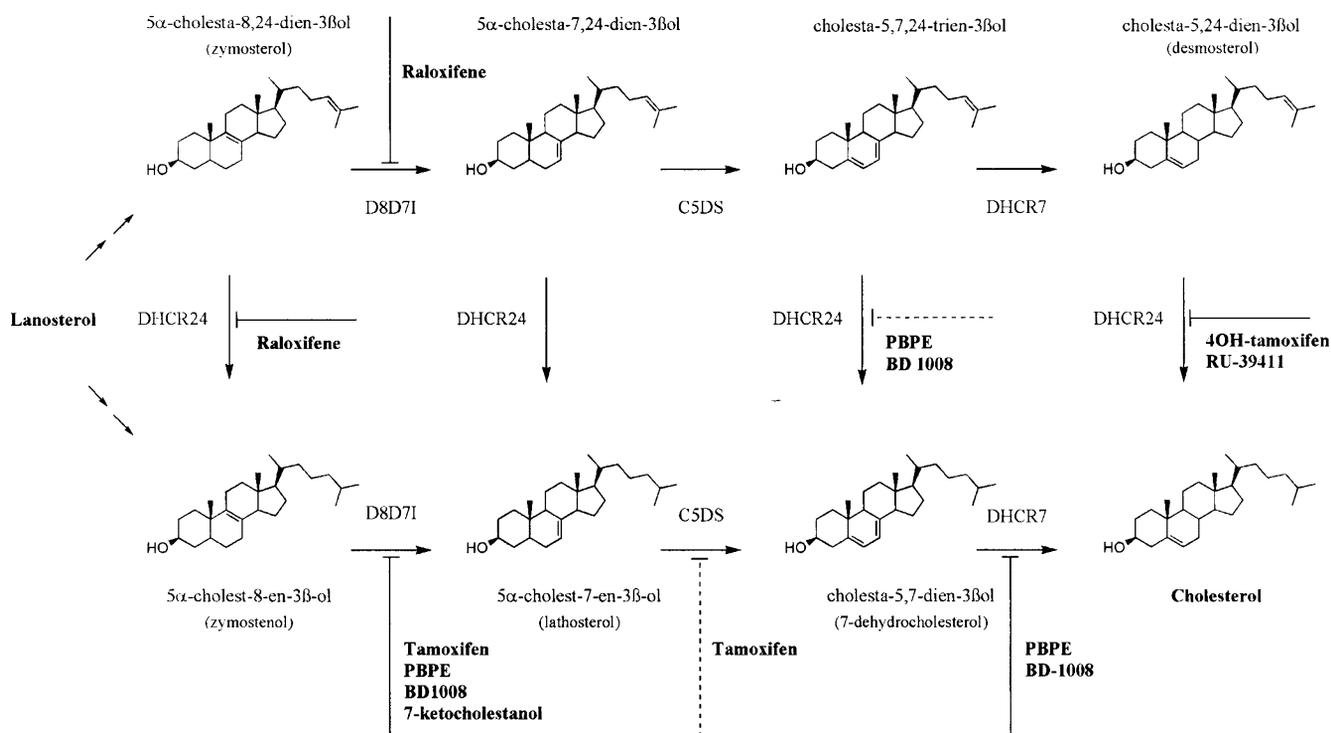


FIG. 10. A proposed mechanism for the action of various AEBS ligands in the post-lanosterol pathway in human tumor cell lines. The signal denotes a strong (solid line) or a weak (dashed line) blocking of the reaction catalyzed by the various enzymes. Tamoxifen is a strong inhibitor of D8D7I at micromolar concentrations and a weak inhibitor of the DHCR24. Derivatives of tamoxifen that are selective AEBS ligands such as PBPE are strong dual inhibitors of D8D7I and DHCR7 and weak inhibitors of DHCR24. Selective AEBS ligands of the oxysterol series are strong inhibitors of the D8D7I. 4-OH-Tamoxifen and RU 39,411 are selective inhibitors of the DHCR24. Raloxifene is a dual inhibitor of D8D7I and DHCR24.

(4-OH-tamoxifen) or two hydroxyl groups within the hydrophobic backbone (RU 39,411 and raloxifene) seems to be associated with the inhibition of DHCR24 because triparanol and ethamoxitriphetol are known inhibitors of DHCR24 (31, 48). Raloxifene is the only phenolic antiestrogen compound of our series that is also an inhibitor of D8D7I.

*t*-BuPE is a weak affinity ligand for the AEBS initially designed to characterize the biological properties of the AEBS. *t*-BuPE is not cytotoxic to tumor cell lines (5, 49). We showed that this compound did not inhibit cholesterol synthesis in MCF-7 cells suggesting that the lack of antiproliferative activity of this compound might be related to its absence of inhibition of cholesterol biosynthesis.

Different structural categories of AEBS ligands such as tamoxifen, raloxifene, 4-OH-tamoxifen and PBPE induced the accumulation of different species of sterol, which might have different pharmacological consequences in terms of growth control. In each case, the increase of sterols doubled the total sterol content of cells. This could mean that new sterols may have effects, by themselves, on cell biology or generated secondary metabolites that show antiproliferative properties. Their incorporation into plasma membranes might have direct effects on lipid raft formation (50) and may alter the functionalities of numerous proteins including cell surface receptors or membrane GTPases, which are involved in the control of various proliferation pathways. Their activities have been shown to be dependent upon membrane fluidity, the presence of lipid rafts or the chemical structure of sterols present in the membrane (51). These sterols or their oxidation products have been shown to bind and modulate the transcription activation function of nuclear receptors such as ROR $\alpha$  and LXR $\alpha$  involved in the control of cell growth and differentiation (52–56).

These sterols can manifest numerous oxidation products on different positions of their steroidal backbone (9, 57, 58). Enzymatic or non-enzymatic hydroxylations require the presence of saturated carbons, and no reaction can occur on ethylenic carbons. The pattern of oxidation produced by the precursors of cholesterol that accumulate in tumor cells under AEBS ligand treatment will depend upon the structure of cholesterol precursors that accumulate because they differ by the number and the position of double bonds. Thus tamoxifen, raloxifene, 4-OH-tamoxifen, and PBPE will produce different oxysterol species that might have different physiological properties. Interestingly, tamoxifen treatment of tumor cell lines has been reported to stimulate the production of reactive oxygenated species (59, 60). It will be of particular interest to compare the effect of 4-OH-tamoxifen, raloxifene and PBPE on the stimulation of the production of reactive oxygenated species in tumor cell lines and to identify the structures of the putative oxidation products of the cholesterol precursors that accumulate in cells.

There are autosomal recessive disorders associated with defects in post-squalene sterol metabolism and particularly with both enzymes involved the AEBS reconstitution: DHCR7 is associated with Smith-Lemli-Opitz syndrome (SLOS) and D8D7I with *Chondrodysplasia punctata* type 2 (CDPX2) (61). These syndromes involve enzyme defects resulting in an over accumulation of cholesterol precursors and a diminution of cholesterol content, underlying the importance of these enzymes during embryogenesis. We have shown that treatment of tumor cells with tamoxifen and PBPE induced a massive accumulation of unsaturated sterols in cells before cell growth inhibition was observed. At the tested concentrations of the compounds, cells were first arrested in the G<sub>1</sub> phase of the cell cycle by tamoxifen and PBPE, and then after a lag time, cells

died by apoptosis.<sup>2</sup> We have no evidence for a diminution of the cholesterol content but instead the accumulation of cholesterol precursors. This suggests that the overaccumulation of sterols was associated with the growth control and apoptosis of cells.

In conclusion, we have established that tamoxifen and PBPE, a prototypical and selective AEBS ligand, produce a massive accumulation of precursors of cholesterol in tumor cell lines by high affinity binding to a hetero-oligomeric proteinaceous complex composed of  $3\beta$ -hydroxysterol- $\Delta^8$ - $\Delta^7$ -isomerase and  $3\beta$ -hydroxysterol- $\Delta^7$ -reductase.

The relationship between the presence of certain cholesterol precursors and the antitumoral activity of some classes AEBS ligands opens new insights into the analysis of the molecular mechanisms involved in their cytotoxicity. We are currently investigating how these sterols are metabolized in tumor cell lines.

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