Molecular Characterization of the Microsomal Tamoxifen Binding Site

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RUNNING TITLE: Tamoxifen binds to cholesterogenic enzymes and produces the accumulation of new sterols in tumor cells.

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Summary

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 paper, 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-dependant 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: 5α-cholest-8-en-3β-ol for tamoxifen treatment; 5α-cholest-8-en-3β-ol and cholesta-5,7-dien-3β-ol, for PBPE treatment suggesting that these AEBS ligands affect at least two enzymatic steps: the 3β-hydroxysterol-Δ^8-Δ^7-isomerase and the 3β-hydroxysterol-Δ^7-reductase. Steroidal antiestrogens such as ICI 182,780 and RU 58,668 did not affect these enzymatic steps, since they do not bind to the AEBS. Transient co-expression of human 3β-hydroxysterol-Δ^8-Δ^7-isomerase and 3β-hydroxysterol-Δ^7-reductase and immuno-precipitation 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 3β-hydroxysterol-Δ^8-Δ^7-isomerase and the 3β-hydroxysterol-Δ^7-reductase as sub-units 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’s group 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) 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]-ethamine.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 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 (5,10-14). These compounds represent not only specific tools to study AEBS function but are also anticancer drug candidates since the selective AEBS ligand DPPE (Tesmilifene) 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 which contained 20 to 30 times 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 mammary 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 multi-protein complex that contained sub-units 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’s group. They have proposed that the AEBS/HIC might be a cytochrome p450 CYP 3A4 (24). Recently, two different groups have reported that an enzyme involved in the biosynthesis of cholesterol, the 3ß-hydroxysterol-Δ^8-Δ^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 [³H]-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 weight of 40 kDa that we have previously determined as being the size of the binding sub-unit of tamoxifen on the AEBS using photo-affinity labeling (27).

In this paper, 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 paper that tamoxifen and the selective AEBS ligand PBPE produced a massive accumulation of 5α-cholest-8-en-3β-ol and/or 5α-cholesta-5,7-dien-3β-ol that suggests an inhibition of the 3β-hydroxysterol-Δ^8-Δ^7-isomerase (D8D7I) and the 3β-hydroxysterol-Δ^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. Immuno-precipitation followed by tamoxifen binding to the immuno-precipitate complex showed that D8D7I and DHCR7 are associated proteins necessary for the reconstitution of the AEBS.
Experimental Procedures

Chemicals—N-Pyrrolidino-2-[4-(Benzy1)-Phenoxy]-ethanamine.HCl (PBPE), N,N-diethyl-2-[4-(phenylmethyl)phenoxy]-ethamine.HCl (DPPE), N-Morpholino-2-[4-(benzyl)-phenoxy]-ethanamine.HCl (MBPE) and N,N-diethyl-2-[4-(tertiobutyl)-phenoxy]-ethamine 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 (NIH, MD), BM 15,766 was from Roche (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, USA). ICI 182,780 was from Tocris Cookson Ltd. (UK). 7-ketocholestanol was from Steraloïd (Wilton, MH). Tamoxifen, 4OH-tamoxifen, clomiphene, cholesterol-oleate, lanosterol, cholesterol, lathosterol, desmosterol, 6-ketocholestanol, 7-ketocholesterol and 7-dehydrocholesterol were from Sigma-Aldrich (St. Louis, MO). All solvents were from Prolabo (Paris, France). N,O-bis(trimethylsilyl)trifluoro acetamide 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 Fondation, Detroit). These cells were adapted to grow in a chemically defined medium (28): in 5% CO₂ 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-transferin and were called
MCF-7\textsuperscript{WS}. 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 % foetal calf serum (FCS) in 5% CO\textsubscript{2}.

Treatment of cells with drugs—MCF-7\textsuperscript{WS} were grown to 70 % confluence. At day 0, tamoxifen, PBPE, ICI 182,780, RU 56,668 or 17β-estradiol at concentrations of 0.5 µM, 0.5 µM, 0.5 µM, 0.5 µM 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 hours incubation, the cells were washed with Ca\textsuperscript{++} and Mg\textsuperscript{++}-free phosphate-buffered saline (PBS), harvested and counted. 0.1 µCi of [\textsuperscript{14}C]-cholesterol (Isotopchim (France), specific activity = 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 µl of methanol. For each condition 8.10\textsuperscript{7} to 10\textsuperscript{8} cells were used for analysis. Kinetics were performed by treatment of MCF-7\textsuperscript{WS} with tamoxifen and PBPE for 0, 6, 12, 24, 48, 72 and 96 hours. Dose response studies were done with increasing concentrations of tamoxifen and PBPE for 48 hours of incubation with MCF-7\textsuperscript{WS} cells. Tests on other cell lines were performed with 5 µM tamoxifen or 10 µM PBPE for 48 hour. Comparative tests with other drugs were performed by a 48 hours treatment of MCF-7 cells using 5 µM antiestrogens or 10 µM of the other drugs. Sterols were extracted as described above. The percentage recovery of sterols during their purification was 80 % to 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%
silver nitrate in acetonitrile in the absence of light, and then wrapped in aluminium foil before drying in an oven under reduced pressure at 110°C during 1 hour. 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 (Rf) 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 Perkin Elmer 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 µm (25 cm x 4 mm), fitted with a Lichrosorb C18 5 µm (0.5 cm x 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=1). Quantification of sterols were carried out using a calibration curve established with authentic corresponding sterols except for zymostenol 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 ethers derivatives (TMS) were dissolved in hexane. GC-MS analyses were carried out using a HP 5935 instrument housing a fused silica column DB5 (25m x 0.32 mm) coated with a 0.25 µm layer of SE-30, DB-1 ending in the ion source. The oven temperature was about 60°C during the injection and, after 3 minutes 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 (Gibco-BRL) with random hexamers for the reverse transcription step. For PCR, oligonucleotides with the EcoR1 restriction site were used, matching the first 15 and last 16 bases respectively of the open reading frame (ORF) 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 of the influenza virus (HA) was fused to the amino-terminus of the D8D7I (HA-D8D7I) and DHCR7 (HA-DHCR7), using the polymerase chain reaction. The amplification product was cloned into the EcoR1 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 polyethylenenimine methodology (37). Cos-7 cells were seeded in 100 mm plates at a density of 5.10^5 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. β-galactosidase activity was measured by the luminescence derived from 10 µl of each sample incubated in 200 µl of 1 mg/ml O-nitrophenyl-β-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 PBS, pH 7.4, in the presence of a cocktail of protease inhibitors (Sigma) and was homogenized by sonication. The homogenate was centrifuged for 10 min at 13,000 g at 4°C. The protein content was determined by the Bradford method (38). The proteins were incubated in one volume of 2 x Laemmli gel loading buffer and incubated at 60°C for 20 minutes 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 polyvinylene difluoride membranes. The membranes were then saturated with saline buffer (10 mM Tris, 140 mM NaCl (pH 7.4) containing 5% (w/v) non-fat 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% non-fat dried milk and then incubated with a goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech). Visualization was achieved with an ECL plus kit (Amersham Pharmacia Biotech), and fluorescence measured either by autoradiography or using a Phosphor-Imager (Storm 840, Amersham Pharmacia).

**Binding Assay** - 48 hours after transfection, cells were scraped and suspended in 100 µl of cold PBS, pH 7.4, 0.15-0.3 TIU/ml aprotinin (Sigma), 1 mM benzamidine, 1 µg/ml pepstatin and 2 µg/ml leupeptin. The 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 [³H]-tamoxifen (specific activity: 84.0 ci/mmol; Amersham-Pharmacia) from 0.1 nM to 25 nM for 18 hours 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, 4OH-tamoxifen, ICI 164,384, 7-ketocholestanol, t-BuPE, BD 1008, U-18,666A, cholestanol, cholesterol, lathosterol, zymostenol (5α-cholesta-8-en-3β-ol), desmosterol, 7-dehydrocholesterol and 17β-estradiol on whole cell lysates of transfected Cos-7 cells were performed using 8 concentrations of unlabeled test ligand ranging from 0.1 to 10,000 nM with
a single concentration of [³H]-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. Non-specific binding was determined in the presence of 1 µM of tamoxifen and was always less than 20% of total binding. Binding data were determined using the Graphpad Prism program (version 3). 5α-cholest-8-en-3β-ol (zymostenol) was purified by HPLC following the procedure described above after MCF-7 cells treatment with 1 µM of 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 5α-cholest-8-en-3β-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 [³H]-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. IC₅₀ values were determined using the iterative curve fitting program GraphPad Prism. IC₅₀ values were converted into the apparent Kᵢ using the Cheng-Prusoff equation and the Kₐ 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 6 x Histidine tagged recombinant D8D7I (6His-D8D7I). 6His-D8D7I was expressed in E. 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. IPTG 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 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). The cells were broken by 3 consecutive freeze/thaw cycles. The lysate was then diluted with 1 ml of NP40 5% (Roche Molecular Biochemicals) and 8 ml of dilution buffer (10 mM Tris-HCl pH 7.8, 300mM NaCl) and centrifuged (10,000 g, 20 min, 4°C). The protein concentration was measured by the method of Bradford (38). Recombinant proteins were purified on NiNTA agarose beads (Qiagen). The lysate was incubated in batches with the NiNTA 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 washing buffer supplemented with CHAPS (30 mM). 6H-protein was then eluted with: a) 2 x 100 µl of a elution solution of 300 mM NaCl, 50 mM NaH2PO4, 30 mM CHAPS, 50 mM imidazole; b) 2 x 100 µl of the same buffer with 250 mM imidazole and finally 2 x 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 µg 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 Pharmacia) and the fluorescence was measured on a Storm 840 apparatus (Amersham Pharmacia Biotech).

**Immunoprecipitation of D8D7I**— Immuno-precipitation of the recombinant enzymes was performed as described previously for seven transmembrane receptor-G protein complexes (40). 250 µg of proteins extracted from the D8D7I transfected cells with lysis buffer (50 mM Hepes, 150 mM NaCl, 10 mM EDTA, 10 mM Na$_4$P$_2$O$_7$, 100 mM NaF, 1.5% CHAPS, 0.15-0.3 TIU/ml aprotinin (Sigma), 1 mM benzamidin, 1 µg/ml pepstatin and 2 µg/ml leupeptin) were incubated in the presence or not of 50 µl of immune-serum (anti-D8D7I) overnight at 4°C in lysis buffer in which the CHAPS concentration was brought to 1%. Then 20 µl of sheep anti-rabbit IgG coupled to magnetic beads Dyna-beads M-280 (Dynal, Great Neck, NY) was added. After 4 hours incubation at 4 °C, the beads were washed 3 times with 1 ml of washing buffer (30 mM Hepes, 30 mM NaCl, 0.1% Triton X100). The pellet was then resuspended in 2 x 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 immuno-precipitated proteins were performed as follows: proteins were suspended in 200 µl of a buffer containing 0.1% BSA, 20 mM Tris, HCl pH 7.4, CHAPS 2.5 mM, KCl 0.4 M. 100 µl of this suspension was incubated with 20 nM [$^3$H]-tamoxifen, with or without 1 µM cold tamoxifen and incubated for 18 hours at 4°C. Specific binding was measured using the Sephadex™-LH20 methodology as described above.
Results

*Tamoxifen and PBPE are modulators of the biosynthesis of cholesterol in MCF-7<sup>ws</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. 5α-cholest-8-en-3β-ol (zymostenol), and 5α-cholesta-8,24-dien-3β-ol (zymosterol) were not commercially available standard but their presence and their retention factor ($R_F$) 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>ws</sup> cells treated for 48 hours with the solvent vehicle (lane 1), 0.5 µM tamoxifen (lane 2), 0.5 µM PBPE (lane 3) and 10 nM 17β-estradiol (lane 4). In the control lane, the major spot was cholest-5-en-3β-ol (cholesterol) (retention factor ($R_F$) = 0.25). Among the sterols from the C-27 series that were of interest, 2 spots co-migrated with 5α-cholest-7-en-3β-ol (lathosterol) ($R_F = 0.33$) and cholesta-5,24-dien-3β-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 5α-cholest-8-en-3β-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 the 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 5α-cholest-8-en-3β-ol. The spot that co-migrated with desmosterol was more dense 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-3β-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 the cells with 10 nM 17β-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 μM tamoxifen or 0.5 μM PBPE and 10 nM 17β-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**

*Analysis of the structure of cholesterol metabolites that accumulated in MCF-7* during 48 hours treatment with tamoxifen or PBPE—The second set of experiments was done 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 the cells, and were separated by high pressure liquid chromatography (HPLC) according to the methodology of Popjak et al (31). The collected fractions were evaporated to dryness, trimethylsilylated and analyzed by gas chromatography coupled to mass spectrometry (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 that was not separated by HPLC, was achieved by the 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 (Relative Retention Time (RRT) = 1.00) and another peak at 31.6 min (RRT = 0.71) that co-migrated with commercial cholesterol/ lathosterol and desmosterol respectively (Fig 2, A) (41). The subsequent analyses of the RRT = 1.00 peak showed that it contained exclusively cholesterol. This result indicated that the spot of $R_F = 0.33$ 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, the 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 2, B). Two supplementary 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 1) 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 fragmentation profile (Table 1) was consistent with the fragment profile of zymostenol (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 fragments profile characteristic of commercial desmosterol that had been trimethylsilylated (41). The peak at 29.5 min (RRT= 0.67) gave one peak in the GC with a molecular ion of 456 (100%) that corresponded to the base peak. The fragmentation profile
(Table 1) 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 3β-hydroxysterol-Δ8-Δ7-isomerase (D8D7I) step and to a lesser extent at the 3β-hydroxysterol-Δ24-reductase (DHCR24) and the 3β-hydroxysterol-C5-desaturase (C5DS) steps. This illustrates that tamoxifen may affect multiple steps in the cholesterogenesis pathway and this partly confirms the previous observations of Cho et al (42) showing that D8D7I step and DHCR24 step were inhibited by tamoxifen in normal rat liver microsome 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 1) 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 zymostenol, 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 ($\lambda_{\text{max}1} = 272 \mu m \varepsilon_1 = 11,250; \lambda_{\text{max}2} = 282 \mu m \varepsilon_2 = 11,900; \lambda_{\text{max}3} = 293 \mu m \varepsilon_3 = 6,650$). It gave one peak in GC. Analyses of the fragmentation profile (Table 1) 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 1. 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 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 zymostenol and their Δ24 derivatives, and the accumulation of 7-dehydrocholesterol, it is reasonable to suppose that this compound might be the cholesta-5,7,24-trien-3ß-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 the 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 cells— We have performed the structural identification of the sterols that accumulated in MCF-7 cells during treatment with various compounds such as E2, tamoxifen, 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 2). Solvent vehicle and untreated cells produced 91 µg of cholesterol and 0.3 µg of desmosterol per 4.10^7 cells. Treatment with tamoxifen produced 88 µg of cholesterol, 79 µg of zymostenol and 1.3 µg of desmosterol per 4.10^7 cells. As expected, pure antiestrogens such as ICI 182,780 and RU 56,668 did not significantly modify the post-lanosterol profile of the cells; these compounds have no affinity for the AEBS. 80 µg of cholesterol was found in the cells treated with PBPE and the accumulation of 29 µg of zymostenol, 1.3 µg of desmosterol, 0.8 ng of 7-dehydrocholesterol, 0.4 ng of 7-dehydridesmosterol could be measured. Tamoxifen was more potent than PBPE in inhibiting D8D7I. The amount of Δ^{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 µM tamoxifen and a 0.5 µM 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 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-depndant accumulation of $5\alpha$-cholest-8-en-3β-ol (zymostenol) in MCF-7. The accumulation of zymostenol reached a plateau 24 hours after the start of treatment and decreased strongly after 48 hours. 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 (BHT), 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 hours 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 hours with tamoxifen or PBPE. Both drugs induced a concentration-depndant accumulation of zymostenol with an ED$_{50}$ of 163 nM and 630 nM respectively. The accumulation of zymostenol reached a plateau at a mean value of 75 µg for 4.10$^7$ 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$_{50}$ 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 G0-G1 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 hours 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% FCS. The cholesterol profiles are described in Table 3. Table 3 shows that MCF-7 treated with 5 µM tamoxifen or 10 µM PBPE gave a sterol profile that was similar to that obtained with MCF-7ws. The sensitivity of wild type MCF-7 cells to the accumulation of sterols induced by AEBS ligands was 10 times less than for MCF-7ws. The same concentrations of drugs were used to treat MDA-MB-231 (an ER 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-7ws. GC-MS analysis of HPLC purified sterols confirmed the identity of the different sterols that accumulated in the 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 ER status of the 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 weights 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 $[^3H]$-tamoxifen were $7.2 \pm 0.9$ nM and $1.82 \pm 0.6$ pmole/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 $[^3H]$-tamoxifen were $7.47 \pm 0.6$ nM and $5.9 \pm 0.5$ pmol/mg protein. The $B_{max}$ of $[^3H]$-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, done using proteins without the HA tag, gave similar results showing that the fusion with HA had no effects on the binding activity of $[^3H]$-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 $[^3H]$-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) > 4OH-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, 17β-estradiol and pure antiestrogen ICI 164,384 did not compete for $[^3H]$-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 $[^3H]$-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, 17β-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 sub-units. To validate this hypothesis we produced a polyclonal antibody against D8D7I in order to conduct immuno-precipitation studies to show whether D8D7I and DHCR7 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 Figure 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 the 6 x Histidine tagged D8D7I was 28 kDa (Fig. 8A, lane 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 immuno-precipitated with the immune serum directed against the D8D7I. Extracts were analyzed for β-galactosidase activity and the variation of activity was less than 4 % for the different conditions. Immuno-precipitated proteins were analyzed by SDS-PAGE and electro-transfered onto a PVDF membrane. Immuno-blotting with anti-HA
antibodies were then performed. Fig 9A shows that in the absence of HA-DHCR7 no band could be detected (Fig 9A, lane 1, 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, lane 3, 4). In lane 4, the band corresponding to DHCR7 was more intense than in lane 3 due to the expression of the recombinant DHCR7. Fig 9B shows [3H]-tamoxifen binding experiments with immuno-precipitated 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 [3H]-tamoxifen binding experiments described in the above section in which the Bmax 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 to the inhibition of D8D7I or DHCR7 in MCF-7 cells**—Firstly we have shown that two ligands of the AEBS (tamoxifen and PBPE) inhibited the catalytic activities of D8D7I or/and DHCR7, and secondly that the over-expression of D8D7I and DHCR7 resulted in the reconstitution of the [3H]-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 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 4 and 5 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 hours treatment with these compounds. All these compounds were apparent competitive inhibitors of [3H]-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 4. Non-phenolic triphenyl ethylenic compounds such as tamoxifen, CI-628 (nitromiphene) and clomiphene (Clomid) gave a similar sterol profile; the major metabolite that accumulated in the cells was zymostenol. 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 5) and were almost equipotent at 10 µM 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 had a 53.6 time 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.
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 hours, 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 5α-cholest-8-en-3ß-ol (zymostenol), cholesta-5,7-dien-3ß-ol (7-dehydrocholesterol), 5α-cholesta-8,24-dien-3ß-ol (zymosterol); 5α-cholest-7-en-3ß-ol (lathosterol), cholesta-5,24-dien-3ß-ol (desmosterol), and cholesta-5,7,24-trien-3ß-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 3ß-hydroxysterol-Δ^8-Δ^7-isomerase (D8D7I) step, the 3ß-hydroxysterol-Δ^7-reductase (DHCR7) step, and to a lesser extent the 3ß-hydroxysterol-Δ^24-reductase (DHCR24) and the 3ß-hydroxysterol-C^5-desaturase (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 cholesterogenesis 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 (43). These data showed that the PBPE occupation of the AEBS produced a dual inhibition of D8D7I and DHCR7.

Despite a five 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 diphenylmethane compounds was rapid and reaches equilibrium after 3 minutes incubation with cells (43). Diphenylmethane derivatives such as PBPE are 2 orders of magnitude less lipophilic than tamoxifen (44) and require a 5 to 10 times 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 Bmax 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 [3H]-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 co-factors might be present in such extracts that helped emopamil binding to D8D7I (EBP) expressed in yeast. Co-expression of D8D7I and DHCR7 gave a [3H]-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 MW 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 (34)). This apparent mobility corresponds to the mobility of the tamoxifen binding sub-unit of 40 kDa in the AEBS that we identified by photo-affinity labeling experiments (27). Immunoprecipitation demonstrated that both D8D7I and DHCR7 were associated. The calculated molecular weight of the complex of D8D7I and DHCR7 is 82 kDa, which corresponds to the size that has been measured by inactivation with ionizing radiations 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 (4OH-tamoxifen) or two hydroxyl groups within the hydrophobic backbone (RU 39,411 and raloxifene) seems to be associated with the inhibition of DHCR24 since triparanol and ethamoxytriphetol 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 cholesterogenesis 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, 4OH-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 the cells. This could mean that new sterols may have effects by themselves on the cell’s 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, that 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 RORα and LXRα involved in the control of cell growth and differentiation (52-56).

These sterols can manifest numerous oxidation products on different position 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, 4OH-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 4OH-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 the 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 the cells before cell growth inhibition was observed. At the tested concentrations of the compounds, cells were first arrested in the G1 phase of the cell cycle by tamoxifen and PBPE, and then after a lag time, cells died by apoptosis (Kedjouar et coll, manuscript in preparation). We have no evidence for a diminution of the cholesterol content but instead the accumulation of cholesterol precursors. This suggests that the over accumulation of sterols was associated with the growth control and apoptosis of the 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 3ß-hydroxysterol-\(\Delta^8-\Delta^7\)-isomerase and 3ß-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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Abbreviations: AEBS, Antiestrogen binding site; tamoxifen, trans-2-[4-(1,2-diphenyl-1-buteryl)phenoxy]-N,N-dimethylethylamine; PBPE, N,N-pyrrolidino-[(4-benzyl)-phenoxy]-ethanamine; mEH, microsomal epoxide hydrolase; ORF, open reading frame; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; D8D7I, 3ß-hydroxysterol-∆8-∆7-isomerase; DHCR7, 3ß-hydroxysterol-∆7-reductase; DHCR24, 3ß-hydroxysterol-∆24-reductase; C5DS, 3ß-hydroxysterol-C5-desaturase; HA-D8D7I, 3ß-hydroxysterol-∆8-∆7-isomerase fused with a hemaglutinin tag; HA-DHCR7, 3ß-hydroxysterol-∆7-reductase fused with a hemaglutinin tag; zymosterol, 5α-cholesta-8,24-dien-3ß-ol; zymostenol, 5α-cholest-8-en-3ß-ol; lathosterol, 5α-cholest-7-en-3ß-ol; lanosterol, 4,4’14α-trimethyl-5α-cholesta-8,24-dien-3β-ol; 7-dehydrocholesterol, cholesta-5,7-dien-3ß-ol; desmosterol, cholesta-5,24-dien-3ß-ol; cholesterol, cholest-5-en-3ß-ol; cholestanol, 5α-cholestan-3β-ol
Table 1. Gas chromatography-mass spectrometry analysis of trimethylsilyl ether 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 Rt in min | 19.4 | 20.1 | 19.6 | 19.9 | 20.08 | 20.02 |
| m/z | m/z | m/z | m/z | m/z | m/z | m/z |
| 458 | 51 | 95 | 100 | 456 | 15 | 100 | 19 |
| 443 | 10 | 18 | 26 | 441 | 11 | 26 | 2 |
| 368 | 97 | 10 | 9 | 372 | 20 | 5 | 0 |
| 353 | 35 | 22 | 26 | 366 | 19 | 28 | 34 |
| 345 | 2 | 10 | 9 | 351 | 23 | 36 | 100 |
| 329 | 79 | 2 | 2 | 343 | 65 | 10 | 2 |
| 275 | 6 | 2 | 4 | 327 | 25 | 1 | 5 |
| 260 | 4 | 6 | 8 | 325 | 4 | 2 | 82 |
| 255 | 27 | 100 | 20 | 253 | 58 | 7 | 20 |
| 247 | 23 | 5 | 5 | 245 | 14 | 3 | 1 |
| 229 | 5 | 30 | 30 | 229 | 4 | 18 | 1 |
| 213 | 16 | 40 | 34 | 227 | 6 | 6 | 7 |
| 143 | 18 | 11 | 12 | 213 | 16 | 22 | 9 |
| 142 | 2 | 3 | 6 | 211 | 9 | 9 | 24 |
| 129 | 100 | 12 | 14 | 129 | 100 | 17 | 23 |

Position insaturation: $\Delta 5$, $\Delta 7$, $\Delta 8$, $\Delta 5,24$, $\Delta 8,24$, $\Delta 5,7$
Table 2. Determination and quantification of sterols accumulated in MCF-7"w" during 48 hours treatment with tamoxifen and PBPE. Analyses were performed by HPLC as described in Fig 2. The identification of sterols was done as reported in Table 1. 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 in % |
|-------------|---------------------------------|
|             | \(\Delta 8,24\) | \(\Delta 8\) | \(\Delta 5,7,24\) | \(\Delta 5,7\) | \(\Delta 7\) | \(\Delta 5,24\) |
| 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 |
Table 3. Determination and quantification of sterols accumulated in MCF-7, SAOS-2 and MDA-MB-231 during 48 hours treatment with tamoxifen or PBPE. Analysis were performed as described in Table 2.

| Cells          | Treatment | Sterol Amount/Total Sterol in % |
|----------------|-----------|---------------------------------|
|                | ∆8,24     | ∆8                              | ∆5,7,24 | ∆5,7 | ∆7 | ∆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   |
Table 4. Determination and quantification of sterols accumulated in MCF-7 after 48 hours incubation with antiestrogens of various affinity for the AEBS. Analyses were performed as described in Table 2.

| Drug         | Affinity for AEBS | Sterol amount/Total Sterol in % |
|--------------|-------------------|---------------------------------|
|              | $K_i$ (nM)        | $\Delta8,24$ $\Delta8$ $\Delta5,7,24$ $\Delta5,7$ $\Delta7$ $\Delta5,24$ |
| control      | -                 | -                              |
| tamoxifen    | 5.3 ± 1.5*        | 0.4                            |
| clomiphene   | 0.5 ± 0.8         | 0.2                            |
| CI-628       | 2.4 ± 1.1         | 0.4                            |
| raloxifene   | 5.8 ± 0.9         | 38.4                           |
| 4OH-tamoxifen| 7.2 ± 1.4         | -                              |
| RU 39,411    | 38.4 ± 1.6        | -                              |
| ICI 182,780  | > 10,000          | -                              |
| RU 58,668    | > 10,000          | -                              |

* $K_d$ for tamoxifen. MCF-7 microsomes were incubated with 3 nM [³H]-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. IC₅₀ values were determined using the curve fitting program GraphPad Prism. IC₅₀ 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 table were taken from one representative of three independent experiments.
Table 5. Determination and quantification of sterols accumulated in MCF-7 cells during 48 hours incubation with AEBS ligands and inhibitors of cholesterogenesis of various affinity for the AEBS. Analysis were performed as described in Table 2.

| Drug         | $K_i$ (nM) | $\Delta 8,24$ | $\Delta 8$ | $\Delta 5,7,24$ | $\Delta 5,7$ | $\Delta 7$ | $\Delta 5,24$ |
|--------------|------------|---------------|------------|-----------------|-------------|----------|---------------|
| 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           |
FIGURE LEGENDS

Fig.1. **AEBS ligands induce accumulation of putative cholesterol precursors in MCF-7ws cells.** Cells were grown for 2 days in the presence (lane 1) or absence of 0.5 µM tamoxifen (lane 2), 0.5 µM PBPE or 10 nM 17β-estradiol. The lipids were extracted and separated by silver nitrate impregnated TLC plates as described in the Experimental Procedures. The TLC were developed with a H2SO4/methanol mixture and compared with commercial standards. The positions of identified sterols are indicated.

Fig.2. **HPLC profiles of sterols from MCF-7ws cells treated with AEBS ligands.** The treatment of cells and the extraction of lipids were performed as described in the Experimental Procedures section. Sterols were analysed 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/H2O (96/4, v/v). MCF-7ws cells treated for 48 hours with solvent vehicle (A), 0.5 µM tamoxifen (B), or 0.5 µM PBPE (C). Numbers indicate the relative retention times (RRT). Arrows indicate peaks corresponding to commercial standard sterols: desmosterol; 7-dehydrocholesterol; cholesterol; lanosterol.

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 minutes) (─) and at RRT 0.64 (28.2 minutes) (--) from the HPLC chromatogram shown in Fig 2.C

Fig.4. **Kinetics and dose response study of the production of 5α-cholest-8-en-3β-ol by MCF-7 treated with tamoxifen or PBPE.** A) Kinetics of the accumulation of 5α-cholesta-8-en-3β–ol (zymostenol) in MCF-7ws after treatment with tamoxifen (□) and PBPE (O) for 0,
6, 12, 24, 48, 72 and 96 hours. B) Dose response studies were done with increasing concentrations of tamoxifen (□) and PBPE (O) for 48 hours of incubation of MCF-7 cells. 8.10^7-to 10^8 cells were used per condition. Analysis of 5α-cholesta-8-en-3β−ol was done by HPLC as described in Fig 2.

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. β-galactosidase activity was measured as described in the “Experimental Procedures” section and used to control the transfection efficiency. 40µg of total proteins were subjected to SDS-PAGE and Western blotting. Expressed proteins were detected with an anti-HA antibody (16B10 clone).

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 A) control vector pSG5 (▼), pSG5-D8D7I (▲), pSG5-DHCR7 (♦), or B) pSG5-D8D7I and pSG5-DHCR7 (●). For all experiments, Cos-7 cells were also transfected with 50 ng of pCMV-lacZ to measure the efficiency of transfection. β-galactosidase activity was measured as described in the “Experimental Procedures” section and used to control the transfection efficiency. Microsomes from transfected cells were prepared as described in the “Experimental Procedures” section. 10 µg of microsomal proteins were incubated in a binding buffer (20 mM tris-HCl, 2.5 mM EDTA, pH 7.4, 2.5 mM thioglycerol) with various concentration of [^3H]-tamoxifen (84.0 Ci/mmol; Amersham-Pharmacia) from 0.1 nM to 25 nM ± 1µM of cold tamoxifen for 18 hours at 4°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.

Fig.7. Inhibition of [3H]-tamoxifen binding to a microsomal extract of Cos-7 cells that co-expressed human recombinant D8D7I and DHCR7. Competition assay with clomiphene, O) PBPE, □) tamoxifen, ▽) CI-628, ■) 7-ketocholestanol, △) 4OH-tamoxifen, ●) BD 1008, ▼) U-18,666A, ◆) t-BuPE, ▲) ICI 164,384 on whole cell lysates of transfected Cos-7 cells was performed using 8 concentrations of unlabeled ligand ranging from 0.1 to 10,000 nM with a single concentration of [3H]-tamoxifen of 3 nM. Incubation and separation of bound and free radio-ligand 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".

Fig.8. Production, purification of recombinant D8D7I and of an antibody. A) Bacteria were transfected with pQE31-D8D7I. Samples of affinity purified D8D7I were separated on 12 % SDS-PAGE and revealed by Coomassie blue staining. Lane MW, molecular weight markers; lane 1, Crude non-induced lysate; lane 2, induced lysate; lane 3, column flow-through; lanes 4-7, column elutions. The arrow indicates the D8D7I protein. Molecular weight standards (MW) are indicated in kilodaltons. B) Different amounts (5 ng to 1 µg) of purified proteins were spotted onto a nitrocellulose membrane and incubated with the anti 3ß-hydroxysterol-Δ8-Δ7-isomerase (anti-D8D7I) immune serum (dilution 1:1000). The blots were developed with the ECL Western blotting detection system using horseradish peroxidase-conjugated second antibody.
Fig.9. Interaction between D8D7I and DHCR7 and [3H]-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 in "Experimental Procedures". Immuno-precipitations 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 immuno-precipitated proteins performed as described in "Experimental Procedures": Immunoprecipitates were resuspended and incubated with 20 nM of [3H]-tamoxifen in the presence or in the absence of 1 µM of cold tamoxifen and incubated for 18 hours at 4°C. Specific binding was measured using the Sephadex™-LH20 methodology as described in the "Experimental Procedures" section.

Fig.10. A proposed mechanism for the action of various AEBS ligands in the post-lanosterol pathway in human tumor cell lines. D8D7I, 3ß-hydroxy-sterol-Δ⁸-Δ⁷-isomerase; C5DS, 3ß-hydroxy-sterol-C⁵-desaturase; DHCR24, 3ß-hydroxy-sterol-Δ²⁴-reductase; DHCR7, 3ß-hydroxy-sterol-Δ⁷-reductase. The mark |—— denotes a strong or |----- a weak blocking of the reaction catalysed by the various enzymes. Tamoxifen is a strong inhibitor of D8D7I at µM 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. 4OH-tamoxifen and RU 39,411 are selective inhibitors of the DHCR24. Raloxifene is a dual inhibitor of D8D7I and DHCR24.
Figure 1 Kedjouar et coll
Figure 2 Kedjouar et coll
Figure 3  Kedjouar et coll
Figure 4 A Kedjouar et coll
Figure 4 B Kedjouar et coll
Figure 5 Kedjouar et coll
Figure 6 A Kedjouar et coll
Figure 6 B Kedjouar et coll
Figure 7: Kedjouar et coll
Figure 8 Kedjouar et coll
A

Figure 9A Kedjouar et al
Figure 9B Kedjouar et al
Figure 10 Kedjouar et coll
Molecular characterization of the microsomal tamoxifen binding site
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