

Tamoxifen Is a Potent Inhibitor of Cholesterol Esterification and Prevents the Formation of Foam Cells

Philippe de Medina, Bruno L. Payré, José Bernad, Isabelle Bosser, Bernard Pipy, Sandrine Silvente-Poirot, Gilles Favre, Jean-Charles Faye, and Marc Poirot

Département Innovation Thérapeutique et Oncologie Moléculaire, Centre de Physiopathologie de Toulouse Purpan, Institut National de la Santé et de la Recherche Médicale Unité 563, Institut Claudius Regaud, Toulouse Cedex, France (P.D.M., B.L.P., S.S.P., G.F., J.C.F., M.P.); Laboratoire de la Signalisation et de la Différenciation des Macrophages, Institut Louis Bugnard, CHU Rangueil, Toulouse Cedex, France (J.B., B.P.); Centre de Microscopie Électronique Appliquée à la Biologie, Faculté de Médecine de Rangueil, Toulouse Cedex, France (B.L.P.); and Affichem, Ramonville-Saint-Agne, France (I.B.).

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ABSTRACT

Tamoxifen is a selective estrogen receptor modulator (SERM) used for the treatment and prevention of breast cancer. Tamoxifen has been reported to protect against the progression of coronary artery diseases in human and different atherosclerosis animal models by blocking the appearance of the atheromatous plaque. However, the molecular mechanism of this effect remains unknown. Acyl-CoA:cholesterol acyl transferase (ACAT) catalyzes the biosynthesis of cholesteryl esters, which are the major lipids found in the atheromatous plaque. In this paper we have tested whether ACAT might be inhibited by tamoxifen. We show, using molecular modeling, that tamoxifen displays three-dimensional structural homology with Sah 58-035 (3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl]-propanamide), a prototypical in-

hibitor of ACAT. We report that tamoxifen inhibits ACAT in a concentration-dependent manner on rat liver microsomal extract. We show that the presence on estrogen receptor ligands of a backbone isosteric to the diphenyl ethane backbone of Sah 58-035 constitutes a pharmacophore for ACAT inhibition. More importantly, tamoxifen was able to inhibit ACAT on intact macrophages stimulated with acetylated low-density lipoproteins and blocked the formation of foam cells, a step that precedes the formation of the atheromatous plaque. This work constitutes the first evidence that tamoxifen is an inhibitor of ACAT and foam cell formation at therapeutic doses and that this may account for its atheroprotective action.

The development of atherosclerotic lesions is associated with an over-accumulation of cholesteryl esters in arteries (Peng et al., 2000). The formation of cholesteryl esters is catalyzed by acyl-CoA:cholesterol acyltransferase (ACAT; EC 2.3.1.26) using both cholesterol and long-chain fatty acyl-coenzyme A as substrate (Chang et al., 1997). ACAT activity is present in a variety of tissues including the intestinal mucosa, liver, adrenals, testes, and macrophages (Chang et

al., 2001). Two isoforms have been described: ACAT-1 and ACAT-2. ACAT-1 is the major isoform in macrophages and in the liver in humans (Chang et al., 2001). ACAT activity is stimulated in macrophages during atheromatous plaque formation, and cholesteryl ester synthesis is greatly enhanced during the foam cell formation (Brown et al., 1980). Foam cell formation is associated with the development of the atheromatous plaque. ACAT inhibitors block the formation of foam cells, preventing in this way the appearance of the atheromatous lesions and lipid deposition (Matsuda, 1994). Foam cell formation can be produced in vitro by treating macrophages with acetylated low-density lipoproteins (acLDLs) (Brown and Goldstein, 1983). This constitutes a simple pathophysiological model for an early-stage development of atherosclerosis and allows the evaluation of the efficiency of

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ABBREVIATIONS: ACAT, acyl-CoA:cholesterol acyl transferase; ER, estrogen receptor; DPE, diphenylethane; DPM, diphenylmethane; RPM, rat peritoneal macrophage; LDL, low-density lipoprotein; AcLDL, acetylated low-density lipoprotein; Sah 58-035, 3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl]-propanamide; RU 39,411, 11 β -[4-N,N-(diethylaminoethoxy)phenyl]-estra-1,3,5(10)-triene-3,17 β -diol; RU 58,668, 11 β -[[[(4,4,5,5,5,-pentafluoropentyl)sulfonyl]pentyloxy]phenyl]-estra-1,3,5(10)-triene-3,17 β -diol; PBPE, 1-[2-(4-benzyl-phenoxy)-ethyl]-N-pyrrolidine hydrochloride; DMBPE, [2-(4-benzyl-phenoxy)-ethyl]-N,N-dimethyl-amine hydrochloride; TLC, thin-layer chromatography; ICI 182,780, Faslodex (fulvestrant); DMEM, Dulbecco's modified Eagle's medium; CI-628, nitromiphen citrate [α -(4-N-pyrrolidinethoxy)phenyl-4-methoxy- α -nitrostilbene]; ICI 164,384, (N-n-butyl-N-methyl-11-[3,17 β -di-hydroxyestra-1,3,5(10)-trien-7 α -yl]-undecanamide).

atheroprotective drug candidates. Apolipoprotein E knockout mice spontaneously developed atherosclerosis and constitute a model of atherosclerosis. On this model, it has been reported that a partial inhibition of ACAT activity is sufficient to induce a regression of the atheromatous lesion without producing side effects (Delsing et al., 2001; Kusunoki et al., 2001; Heinonen, 2002). These data suggest that a partial inhibition of ACAT might be sufficient for atheroprotection in humans.

Tamoxifen is widely used in the treatment and in the prevention of breast cancer (Jordan, 2003). In addition to its antitumoral properties, clinical studies reported that tamoxifen displays a favorable change in some cardiovascular risk factors such as lowering circulating cholesterol, decreasing low-density lipoproteins (LDLs), and modulating high-density lipoproteins. These changes might be relevant to the protection of patients against the development of coronary artery disease (McDonald and Stewart, 1991; Rutqvist and Mattsson, 1993; McDonald et al., 1995; Clarke et al., 2001). Interestingly, tamoxifen has been demonstrated to protect against the development of atheromatous plaques in different atherosclerosis models in mammals such as male C57BL/6 mice (Grainger et al., 1995), transgenic apolipoprotein(a) mice (Lawn et al., 1996), apolipoprotein E-deficient mice (Reckless et al., 1997), and postmenopausal female monkeys fed with an atherogenic diet (Williams et al., 1997). However, the mechanism of action of tamoxifen on this effect has not been clearly elucidated.

Using molecular modeling, we showed that tamoxifen and estrogen receptor ligands displayed three-dimensional structural homologies with Sah 58-035 (3-[decyldimethylsilyl]-*N*-[2-(4-methylphenyl)-1-phenylethyl]-propanamide), a prototypical inhibitor of ACAT (Ross et al., 1984). We demonstrate, for the first time, that tamoxifen inhibits ACAT activity *in vitro* in a concentration-dependent manner and that this effect does not involve the estrogen receptor. More importantly, we show that tamoxifen blocks at therapeutic doses the formation of the foaming cell, a step that precedes the appearance of the atheromatous plaque.

Materials and Methods

Chemicals. [¹⁴C]Oleyl-coenzyme A (34 mCi/mmol), [³H]tamoxifen (84 Ci/mmol), and [³H]oleic acid (7 Ci/mmol) were purchased from Amersham Biosciences Inc. (Piscataway, NJ). Sah 58-035 was kindly provided by Anna Suter from Novartis (Basel, Switzerland). 7 α -Steroidal antiestrogen ICI 164,384 [(*N*-*n*-butyl-*N*-methyl-11-[3,17 β -di-hydroxyestra-1,3,5(10)-trien-7 α -yl]-undecanamide] was provided by Alan Wakeling from AstraZeneca Pharmaceuticals LP (Wilmington, DE) and ICI 182,780 (Faslodex; fulvestrant) was obtained from Tocris Cookson Inc. (Bristol, UK). 11 β -Steroidal antiestrogens RU 39,411 (11 β -[4-*N,N*-[diethylaminoethoxy]phenyl]-estra-1,3,5(10)-triene-3,17 β -diol) and RU 58,668 (11 β -[[[(4,4,5,5,5-pentafluoropentyl)sulfonyl]pentyloxy]phenyl]-estra-1,3,5(10)-triene-3,17 β -diol) were kindly provided by P. van de Velde from Aventis (Romainville, France). Nonphenolic triphenyl ethylenic antiestrogen CI-628 (nitromiphen citrate [α -(4-*N*-pyrrolidinethoxy)phenyl-4-methoxy- α -nitrostilbene]) was obtained from Pfizer (Groton, CT) and tamoxifen citrate [(*z*)-2-[4-(1,2-diphenyl-1-butenyl)-phenoxy]-*N,N*-dimethylethanamine] was obtained from Sigma-Aldrich (St. Louis, MO). Phenolic antiestrogen raloxifene ([6-hydroxy-2-(4-hydroxyphenyl)-benzo[*b*]thiophen-3-yl][4-[2-piperidinoethoxy]phenyl]ketone hydrochloride) was obtained from Aventis and 4-hydroxy-tamoxifen [(*z*)-4-[1-[4-(2-dimethylamino-ethoxy)-phenyl]-2-phenyl-but-1-enyl]-

phenol] was from Sigma-Aldrich. 1-[2-(4-Benzyl-phenoxy)-ethyl]-*N*-pyrrolidine hydrochloride (PBPE) and [2-(4-benzyl-phenoxy)-ethyl]-*N,N*-dimethyl-amine hydrochloride (DMBPE) were synthesized as described before (Poirot et al., 2000). All other compounds and chemicals were obtained from Sigma-Aldrich. All solvents were obtained from Prolabo (Paris, France). Thin-layer chromatography (TLC) plates (LK-6-DF) were obtained from Whatman (Clifton, NJ). Radiochemical purity of the compounds was checked by TLC and was greater than 98%.

Molecular Structure Analysis. Computational chemical calculations were performed on a Silicon Graphics Indigo workstation (Silicon Graphics, Mountain View, CA) using Insight II version 2000 (Accelrys Inc., San Diego, CA). Minimum energy conformations were calculated using the Discover module (2.9.7/95.0/3.0.0) with consistent valence force field. Van der Waals volumes and van der Waals volume intersections were determined using the Search-Compare module version 95.0 (Accelrys Inc.). We first compared the structure of Sah 58-035 and tamoxifen. The structure of Sah 58-035 is extended and the orientation of the diphenyl ethane moiety corresponds to the active conformation of the inhibitor (Dugar et al., 1993). Superimposition of energy-minimized structures was done by superimposing the *trans*-stilbene part of tamoxifen and the diphenyl ethane part of Sah 58-035.

Second, we have compared the van der Waals volume of the diphenyl ethane part of Sah 58-035 to that of the stilbene, steroidal, and benzothiophene backbone of other estrogen receptor ligands and to the diphenylmethane of tamoxifen derivatives that does not bind to the estrogen receptor. Superimpositions were conducted using the diphenyl ethane part of Sah 58-035 that was superimposed carbon to carbon on the stilbene or benzothiophene. For superimposition with steroids, the carbon 3 of the phenyl was adjusted to the carbon 4 of the diphenyl ethane, and the benzylic carbon linked to the carbon 6 of the phenyl of steroid was superimposed on the benzylic carbon of the phenyl of the diphenyl ethane of Sah 58-035. The benzyl of diphenyl methane was superimposed carbon to carbon with the benzyl of the diphenyl ethane of Sah 58-035. The percentage of superimposition was calculated by measuring the ratio of the intersection of the van der Waals volume of compounds with the van der Waals volume of the diphenyl ethane of Sah 58-035.

Quantification of Endogenous Cholesterol. Neutral lipids were extracted according to the method of Bligh and Dyer (1959). Lipids were then separated by TLC using cyclohexane/ethyl acetate (1:1) as eluent. In these conditions, the retention factor for cholesterol was 0.53 and was conformed to published values (Stahl, 1969). Endogenous free cholesterol in the microsomal fraction was estimated to be 26 μ M after titration by gas chromatography coupled to mass spectrometry.

Assays for ACAT Activity. Male Sprague-Dawley rats (purchased from Iffa-Credo, L'Arbresle, France) were killed by decapitation and their livers removed to ice-cold 10 mM Tris-HCl, pH 7.4, containing 0.9% saline or 0.32 M sucrose. The livers were homogenized (after mincing with scissors) using a Potter-Elvehjem Teflon-glass homogenizer in 10 ml/g tissue wet weight of ice-cold 10 mM Tris-HCl/0.32 M sucrose, pH 7.4. The crude homogenate was centrifuged for 10 min at 1000g and the pellet was discarded. The resulting supernatant was centrifuged at 31,000g for 15 min. The pellet was resuspended in 3 ml/g 10 mM Tris-HCl, at pH 7.4 by vortexing, and the suspension was allowed to incubate at 25°C for 15 min. The pellet was then resuspended to 1.53 ml/g in 10 mM Tris-HCl, pH 7.4, by gentle Potter-Elvehjem homogenization, and aliquots were stored at -80°C until use. Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as standard. Rat liver microsomes were prepared as described before (Chailleux et al., 1994), and the 105,000g microsomal pellet was resuspended in 0.1 M phosphate buffer (pH 7.4), 1 mM EDTA, and 2 mM dithiothreitol at a protein concentration of 5 mg/ml. Assays for ACAT activity were performed by measuring the formation of cholesteryl [¹⁴C]oleate from the endogenous cholesterol in the microsomal frac-

tion and exogenous [^{14}C]oleyl-coA as the substrate, following the procedure of Heider et al. (1983). The incubation mixture for microsomal ACAT consisted of 0.25 ml of 0.1 M potassium phosphate buffer (pH 7.4), containing 9 nmol of bovine serum albumin (fatty acid-free) and 60 μg of microsomal proteins. Internal free cholesterol concentration was estimated (using a calibration curve by comparison with authentic cholesterol) to be 52 nmol/mg of protein.

The mixture was preincubated with the inhibitors added to *N,N*-dimethylformamide as the solvent vehicle (5% of the final volume) for 5 min at 37°C before adding 2 μl of [^{14}C]oleyl-coA (final concentration 40 μM); the mixture was then incubated at 37°C for 5 min (this time was chosen after performing a time-course study and corresponds to a time of linear esterification, allowing reproducible tests). We first verified that 5% dimethylformamide had no effect on the ACAT assay. The reaction was terminated by adding 3 ml of chloroform/methanol (2:1) and 500 μl of 0.04 N HCl. After shaking, the lower phase was taken and reduced to dryness under nitrogen. The residue was resuspended in 30 μl of hexane containing cholesterol oleate (1 mg/ml) at 4°C. Samples were spotted on LK-6-DF Whatman 20 \times 20-cm silica gel plates previously heated for 1 h at 100°C and developed using *n*-hexane/diethyl ether/acetic acid (70:30:1). After drying the plate, the cholesteryl oleate was visualized with iodine vapor. In these conditions, the retention factor for cholesteryl oleate was 0.77. The radioactive metabolites were identified and quantified on TLC plates either by a Berthold TLC linear detector [LB 2821; the peak integration was obtained by using the program CHROMA 1D (PerkinElmer Life and Analytical Sciences, Boston, MA)] or by liquid scintillation counting of the cholesteryl oleate region. The ACAT activity was expressed as the percentage of the ACAT activity measured in the absence of inhibitors (control assay realized with solvent vehicle). ACAT control was 48.3 ± 2.3 pmol of cholesteryl [^{14}C]oleate \cdot mg microsomal protein $^{-1}$ \cdot min $^{-1}$. Background represents less than 1% of the specific signal. Competitiveness of ACAT inhibition by tamoxifen was evaluated using 4 μM , 8 μM , 16 μM , and 40 μM [^{14}C]oleyl-CoA in the presence or absence of 8 μM tamoxifen. The concentration of compound required to inhibit ACAT activity by 50% (IC $_{50}$) was calculated using GraphPad Prism software, version 3.0 (GraphPad Software Inc., San Diego, CA). Calculation of IC $_{50}$ values was performed with data from triplicate assay tubes at each drug concentration. Ten different concentrations were tested for each inhibitor.

Cell Culture. Resident peritoneal macrophages were harvested from male Sprague-Dawley rats (5–6 weeks old, 200–250 g, purchased from Iffa-Credo) as described by Pollaud-Cherion et al. (1998). Briefly, the peritoneal cavity was washed with 100 ml of sterile Medium 199 with Hanks' salts containing 20 IU of heparin per ml. The cells collected were centrifuged at 800g for 10 min and resuspended in DMEM with 44 mM NaHCO $_3$ buffer (pH 7.2) containing 1% fetal calf serum. The cells were plated out in 24-well tissue culture plates at 10 6 cells in a total volume of 0.3 ml per well. About 60% of the cells in the peritoneal perfusate were nonspecific esterase-positive and had the morphological appearance of macrophages when examined by May-Grünwald and Giemsa staining. Cell viability was assessed by the trypan blue exclusion assay.

Preparation and Modification of LDLs. LDLs were isolated from the serum of normal volunteers in the density range 1.006 to 1.063 g/ml by ultracentrifugation as previously described (Poumay and Ronveaux-Dupal, 1985). Isolated LDLs were dialyzed against 150 mM NaCl containing 5 mM Tris and 0.3 mM EDTA (pH 7.4) at 4°C for 48 h. The lipoproteins were filtered through a 0.45- μm filter (Sartorius Corp., Edgewood, NY), and the apolipoprotein concentrations were determined by kinetic turbidimetry (Behring Turbitimer; Behring Diagnostics, Somerville, NJ). Fresh LDLs were acetylated using an excess of acetic anhydride (Basu et al., 1976). The acLDLs were passed through a Sephadex G-25 PD-10 column (Amersham Biosciences Inc.) and were filtered over a 0.45- μm filter (Sartorius Corp.). The acLDLs were stored sterile under nitrogen at 4°C in the dark and used within 3 weeks after their preparation.

Treatment of Macrophages with acLDLs and Measurement of [^3H]Oleate Incorporation into Macrophage Lipids. The macrophages were allowed to adhere to the culture plate by incubating for 2 h at 37°C under 5% CO $_2$ and 95% air. Nonadherent cells were removed by washing the dishes with DMEM. To investigate the effect of ACAT inhibitors and antiestrogens on cholesteryl ester synthesis induced by acLDLs, macrophages were incubated for 15 h with [^3H]oleate during the treatment with acLDLs. Briefly, the adherent cells were preincubated 15 min with 5 or 10 μM ACAT inhibitor and antiestrogens (see Table 3) and then incubated without or with 100 $\mu\text{g}/\text{ml}$ acLDL in DMEM containing 5 $\mu\text{Ci}/\text{ml}$ [^3H]oleate and 1% FCS in the CO $_2$ incubator for 15 h.

Extraction and Assay of Cholesteryl-oleate. The [^3H]oleate incorporation process was studied, as already described (Pollaud-Cherion et al., 1998), at the end of the incorporation course by analyzing cellular lipids into which [^3H]oleate had been incorporated. The extraction of membrane phospholipids and neutral lipids used a technique initially described by Bligh and Dyer (1959). Briefly, after removing the supernatant, the ^3H -labeled macrophages were scraped off into 800 μl of distilled water using a rubber policeman, and the cell lipids were extracted after adding a mixture of chloroform and methanol (water/chloroform/methanol; 1:1:1, v/v). The chloroform phase containing the lipids was evaporated to dryness under nitrogen, and the residues were dissolved in 80 μl of chloroform and separated by TLC as described above.

Staining Procedures for Foam Cells. Cells were fixed with 10% formaldehyde in PBS for 10 min and were washed twice with PBS. The cells were pretreated with 60% 2-propanol for 2 min and then stained with 0.2% oil red O (Sigma-Aldrich) in 60% 2-propanol for 10 min. The slides were washed with 2-propanol and then with PBS. Finally, the cells were treated with hematoxylin (Sigma-Aldrich) for 5 min to stain nuclei.

Transmission Electron Microscopy. Cells were fixed on the culture plates with 2% glutaraldehyde in cacodylate buffer (0.1 M sodium cacodylate, 0.012 M calcium chloride, pH = 7.4) for 4 h and washed with 0.2 M sodium cacodylate with 0.006 M calcium chloride for 12 h. Then cells were postfixated with 1% OsO $_4$ in cacodylate buffer with 1.5% K $_3$ Fe(CN) $_6$ for 1 h, washed twice with distilled water, and prestained with an aqueous solution of uranyl acetate at 2% for 12 h. Samples were dehydrated in an ascending ethanol series and embedded in epoxy resin (Epon 812). Ultrathin sections (50 nm) were mounted on 150 mesh collodion-coated copper grids and poststained with 3% uranyl acetate in 50% ethanol and with 8.5% lead citrate before being examined on an H300 Hitachi electron microscope at an accelerating voltage of 75 kV. Approximately 150 cells were surveyed per data point.

Results

Tamoxifen has been shown to protect against the development of the atheromatous plaque in different animal models of atherosclerosis by a mechanism that has not been clearly elucidated (Grainger et al., 1995; Lawn et al., 1996; Reckless et al., 1997; Williams et al., 1997). The major lipids found in fatty streak lesions on the vascular wall are cholesteryl esters, which are synthesized by acyl-CoA cholesterol:acyl transferase. Since the accumulation of cholesteryl esters can be blocked by the inhibition of ACAT, and tamoxifen displayed some structural similarities with Sah 58-035, a prototypical inhibitor of ACAT, we have investigated whether tamoxifen could be an inhibitor of this enzyme.

Tamoxifen Shares Structural Similarities with Sah 58-035. We first compared the structure of Sah 58-035, a prototypical inhibitor of ACAT, with tamoxifen (Fig. 1A). Three-dimensional structures of these compounds in their minimal energy conformation are depicted in Fig. 1B. The

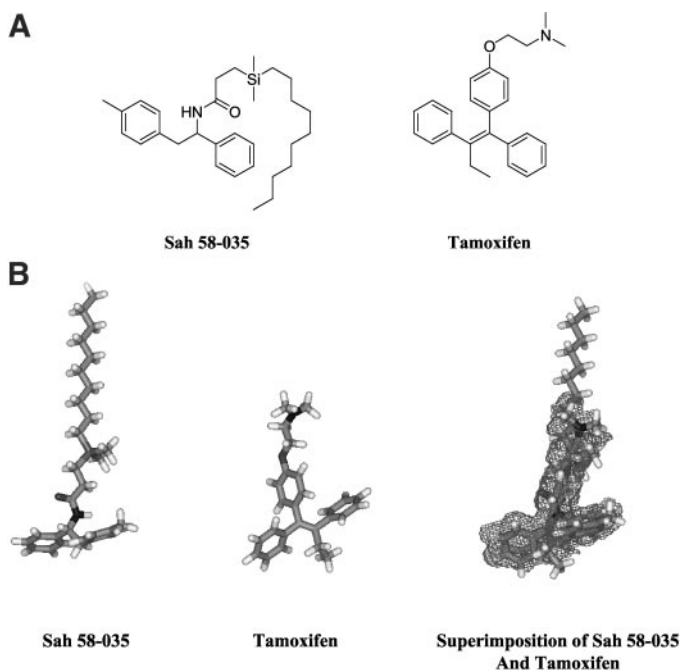


Fig. 1. A, chemical structure of Sah 58-035 and tamoxifen. B, molecular structure of Sah 58-035 and tamoxifen. Three-dimensional structure of the calculated minimum energy conformation of Sah 58-035 (A) and Tamoxifen (B) was carried out using the discover module of Insight II (version 2000) as described under *Materials and Methods*. Overlay of Sah 58-035 and tamoxifen as well as van der Waals volume calculation and intersection measurement were done using the Search-Compare module. The van der Waals volume intersection is depicted as the gray grid and illustrates the structural similarities that exists between Sah 58-035 and tamoxifen. In their minimal energy, the van der Waals volume of Sah 58-035 and of tamoxifen are of 431.7 \AA^3 and 322 \AA^3 , respectively. A total of 48.3% of the van der Waals volume of tamoxifen is in common with the van der Waals volume of Sah 58-035, and 84.5% between the *trans*-stilbene part of tamoxifen and the diphenylethane of Sah 58-035.

van der Waals volumes of Sah 58-035 and tamoxifen are, respectively 431.7 \AA^3 and 322 \AA^3 . The intersection of the van der Waals volume of Sah 58-035 and tamoxifen (Fig. 1B) showed 48.3% homology. Interestingly, 84.5% of this homology was between the diphenylethane (DPE) moiety of Sah 58-035 and the stilbene moiety of tamoxifen (Figs. 4 and Fig. 5, A+B). These results indicated that Sah 58-035 and tamoxifen share a structural homology on the DPE motif of Sah 58-035 that suggests a pharmacological relationship. We then tested whether tamoxifen inhibited ACAT activity.

Tamoxifen Is a Potent Inhibitor of Cholesterol Esterification in Vitro. To determine whether tamoxifen is an inhibitor of acyl-CoA:cholesterol acyltransferase in a cell-free system, we carried out an enzymatic test on a rat liver microsomal homogenate. The formation of cholesteryl [$1\text{-}^{14}\text{C}$]oleate from rat liver microsomes was linear up to 9 min. Therefore, a 5-min incubation with tamoxifen was used in the subsequent experiments. As shown in Fig. 2, Sah 58-035 inhibited ACAT with an IC_{50} of $0.38 \pm 0.12 \text{ \mu M}$, similar to that reported in the literature (Clader et al., 1995). Tamoxifen inhibited in a concentration-dependent manner ACAT with an IC_{50} of $6.74 \pm 0.84 \text{ \mu M}$ (Fig. 2). At 10 \mu M , tamoxifen inhibited ACAT activities up to 63%. Interestingly, the therapeutic concentration of tamoxifen has been measured to be between 1 and 10 \mu M in the blood of mammals and patients (Etienne et al., 1989; Trump et al., 1992), indicating that the blockage of cholesterol esterification by inhib-

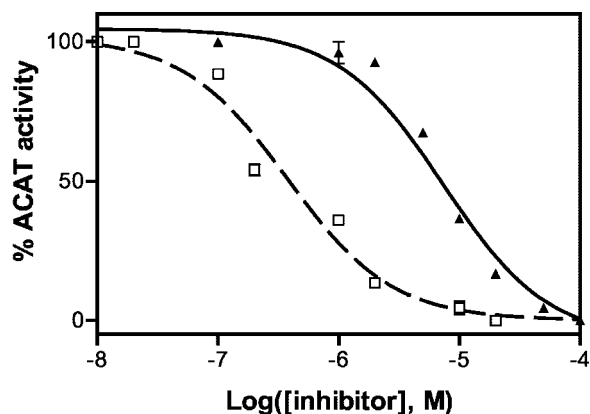


Fig. 2. Effect of the prototypical ACAT inhibitor Sah 58-035 (\square) and tamoxifen (\blacktriangle) on cholesterol esterification (ACAT) activity in rat liver microsomal homogenates. Rat liver microsomal proteins (60 \mu g) were incubated with 10 different concentrations of each drug and 40 \mu M [^{14}C]oleyl-CoA. Cholesteryl esters were quantified under conditions described under *Materials and Methods*. The ACAT activity was expressed as the percentage of the ACAT activity measured in the absence of inhibitors (control assay realized with solvent vehicle). ACAT control was $48.3 \pm 2.3 \text{ pmol of cholesteryl } [^{14}\text{C}] \text{oleate} \cdot \text{mg microsomal protein}^{-1} \cdot \text{min}^{-1}$. Curves were analyzed using the curve-fitting program GraphPad Prism (version 3.0). Values are the average of three experiments \pm S.E.M., each carried out in duplicate. In some cases, S.E.M. values were less than the size of the symbol and the error bars were not visible.

iting the ACAT activity could explain the atheroprotection effect of tamoxifen. We have performed ACAT inhibition experiments with tamoxifen to determine the mode of inhibition. A Lineweaver-Burk plot shown in Fig. 3 suggests that tamoxifen is acting as an apparent competitive inhibitor of ACAT activity.

Structure-Activity Studies. Since we showed a high homology between the DPE of Sah 58-035 and the stilbene of tamoxifen, we then investigated whether other compounds related to tamoxifen could have a similar homology. In Fig. 4, we have drawn the different classes of compounds tested and

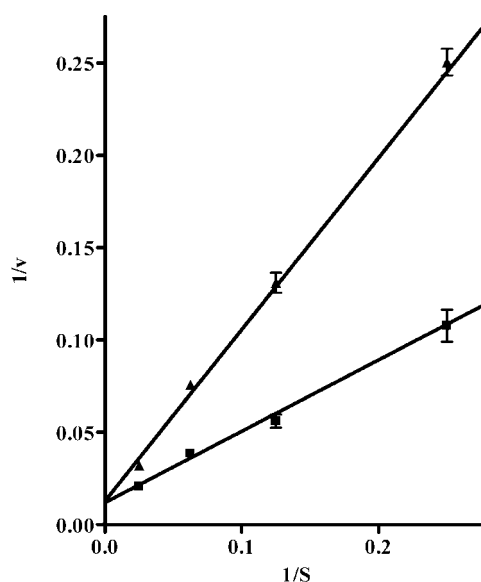


Fig. 3. Lineweaver-Burk plot of the effect of tamoxifen on ACAT activity. \blacksquare , no tamoxifen; \blacktriangle , 8 \mu M tamoxifen. V, pmol of cholesteryl [^{14}C]oleate formed $\cdot \text{mg proteins}^{-1} \cdot \text{min}^{-1}$; S, [^{14}C]oleyl-CoA (\mu M). Values are the average of three experiments \pm S.E.M., each carried out in duplicate. In some cases, S.E.M. values were less than the size of the symbol and the error bars were not visible.

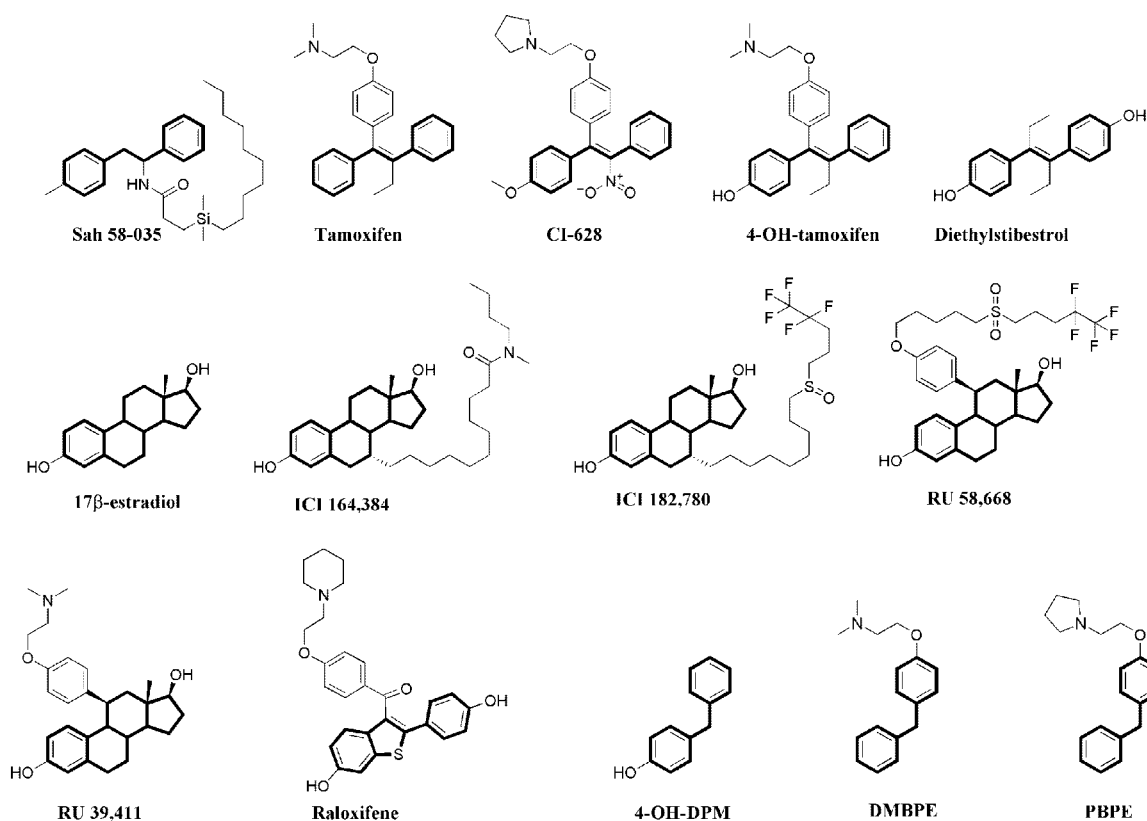


Fig. 4. Chemical structure of the inhibitor of ACAT, Sah 58-035; triphenylethylene compounds tamoxifen, CI-628, and 4-OH-tamoxifen; nonsteroidal estrogen, diethylstilbestrol; steroidal estrogen, 17 β -estradiol; steroidal antiestrogens, ICI 164,384, ICI 182,780, RU 58,668, and RU 39,411; benzothiothiophene; raloxifene; and diphenylmethanes 4-OH-DPM, DMBPE, and PBPE. In these molecules, the groups that may be superimposable with the diphenylethane part of Sah 58-035 are in bold.

shown in bold the putative motifs similar to that of DPE. The conformations of these different groups, extracted from the minimum energy conformations of their parent compounds, are depicted in Fig. 5. Their van der Waals volumes have been measured, superimposed with the DPE, and the intersection of the van der Waals volumes have been reported in Table 1. The substructure that shared the weakest homology with the DPE is diphenylmethane, found in tamoxifen derivatives (OH-DPM, PBPE, DMPE). The diphenylmethane group was used to obtain tamoxifen derivatives that lacked

the capacity to bind the estrogen receptor (ER); consequently, OH-DPM, PBPE, and DMPE were not estrogen receptor ligands (Poirot et al., 2000). The stilbene group can be found in triphenylethylene compounds (Fig. 5B) and in diethylstilbestrol (Fig. 5C). In triphenylethylene compounds (tamoxifen, CI-628, hydroxytamoxifen), the stilbene group adopts a conformation in which the two phenyl groups are not coplanar with the ethylene (Fig. 5B). In diethylstilbestrol, the minimal energy conformation of the stilbene group, the phenyl groups are coplanar with the ethylene (Fig. 5C). The steroidal back-

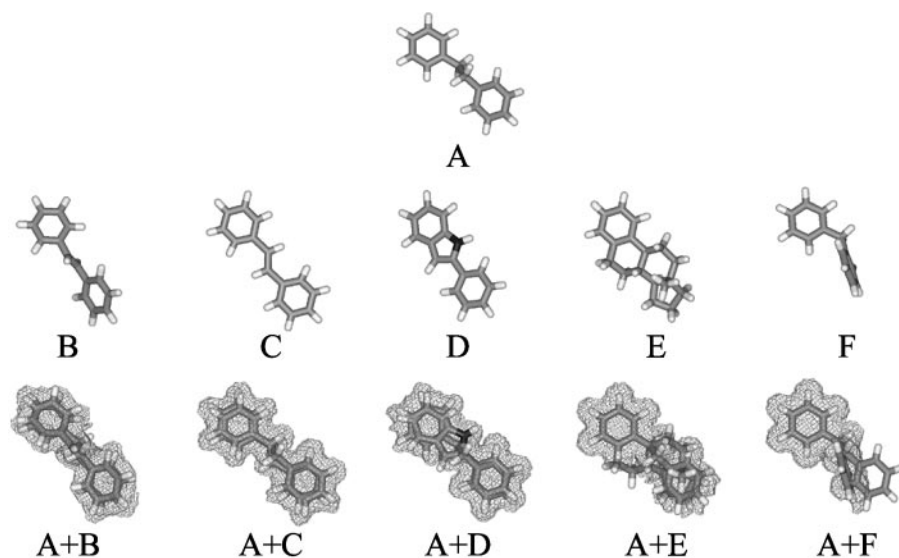


Fig. 5. Molecular structure comparison between the diphenyl ethane (DPE) moiety of Sah 58-035 (A), the stilbene core of triphenylethylene (B), the stilbene core of diethylstilbestrol (C), the steroidal core estradiol and steroidal antiestrogens (D), the benzothiothiophene of raloxifene (E), and the benzyl group of 4-OH-DPM, DMBPE, and PBPE (F). Overlay, van der Waals volume calculation and intersection measurement were done as described in the legend of Fig. 1 and reported in Table 1.

TABLE 1

Van der Waals volumes and van der Waals volume intersections between Sah 58-035 and antiestrogens

Van der Waals volumes and van der Waals volume intersections were calculated using the Search-Compare module of Insight II (version 2000) as described under *Materials and Methods*.

Compound	Van der Waals Volume	Volume Intersection	Percentage in Common
	\AA^3		
Diphenylethane	160.51		
Stilbene diethylstilbestrol ^a	156.52	140.35	87.44
Stilbene triphenylethylene ^b	150.74	135.70	84.54
Estra-1,3,5(10)-triene	220.27	133.21	82.99
Benzothiophene	161.27	128.79	80.24
Diphenylmethane	146.02	109.24	68.06

^a Stilbene in the conformation of diethylstilbestrol.

^b Stilbene in the conformation of triphenylethylenes.

bone found in 17 β -estradiol and in steroidal antiestrogens (ICI 164,384; ICI, 182,780; RU 58,668; RU 39,411) (Fig. 5D) and the benzothiophene motif from raloxifene (Fig. 5E) displayed more than 80% homology in common with the DPE group. These results showed that the molecular volumes of these motifs are highly similar to that of the DPE of Sah 58-035. Moreover, these results suggested that these estrogen receptor ligands might be inhibitors of ACAT activity and that these structural similarities could drive the inhibition of ACAT activity. To test this hypothesis, we next tested whether ER ligands and diphenylmethane compounds could inhibit ACAT activity on the microsomal assay.

Estrogen Receptor Ligands Are Inhibitors of ACAT Activity "in Vitro". The dose-response curves for each compound tested are reported in Fig. 6 and the corresponding IC₅₀ values in Table 2. The results indicated that all compounds that contained a hydrophobic motif highly similar (more than 80%) to the DPE motif were inhibitors of ACAT. In contrast, compounds that contained a diphenylmethane motif (4-OH-DPM, PBPE, DMBPE), which displayed weaker similarities with the DPE motif of Sah 58-035, were not able to inhibit ACAT activity. The consequences of the presence of different substituents on this motif have been evaluated. ICI

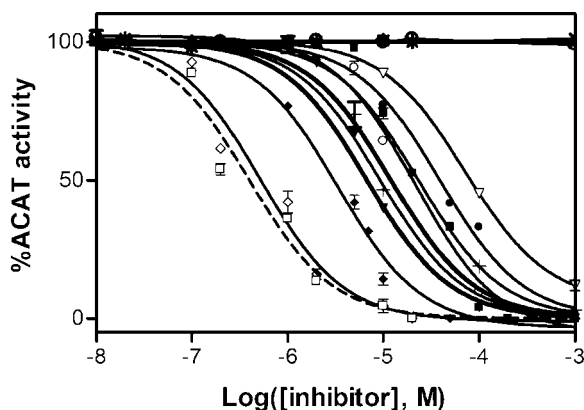


Fig. 6. Effect of Sah 58-035 and estrogen receptor ligands on rat liver microsomal cholesterol esterification (ACAT) activity. \square , Sah 58-035; \triangle , ICI 164,384; \diamond , ICI 182,780; \blacklozenge , RU 58,668; \blacktriangle , tamoxifen; $+$, diethylstilbestrol; \blacktriangledown , CI-628; \blacksquare , 4-OH-tamoxifen; \bullet , raloxifene; ∇ , RU 39,411; \circ , 17 β -estradiol; $*$, DMBPE; \times , PBPE; $=$, 4-OH-DPM. Assays were conducted as described in Fig. 2. ACAT control, 48.3 ± 2.3 pmol of cholesteryl [¹⁴C]oleate \cdot mg microsomal protein⁻¹ \cdot min⁻¹. Values are the average of three experiments \pm S.E.M., each carried out in duplicate. In some cases S.E.M. values were less than the size of the symbol and the error bars were not visible.

TABLE 2

IC₅₀ of Sah 58-035, estrogen receptor ligands, and tamoxifen derivatives on rat liver microsomal ACAT assay

Rat liver microsomes were incubated with 10 concentrations ranging from 0.01 to 1000 μ M under the conditions described under *Materials and Methods*. IC₅₀ values were determined using the iterative curve-fitting program GraphPad Prism (version 3.0). Values are the average of three experiments \pm S.E.M., each carried out in duplicate. cLogP (the log of the calculated partition coefficient for compounds between water and an organic solvent such as *n*-octanol) values were calculated using ChemDraw Ultra (version 7.01).

Compound	IC ₅₀	cLogP
	μ M	
Sah 58-035	0.38 ± 0.12	10.24
Tamoxifen	6.74 ± 0.84	6.82
17 β -Estradiol	21.49 ± 1.92	3.78
Diethylstilbestrol	11.99 ± 1.01	4.96
CI-628	9.20 ± 1.08	5.62
ICI 182,780	0.52 ± 0.28	7.35
ICI 164,384	0.61 ± 0.22	8.43
RU 58,668	3.36 ± 1.12	6.12
4-OH-Tamoxifen	23.10 ± 1.23	6.15
Raloxifene	38.51 ± 2.32	6.86
RU 39,411	85.58 ± 1.51	5.21
4-OH-DPM	N.M.	3.54
PBPE	N.M.	4.77
DMBPE	N.M.	4.23

N.M., no measurable inhibition.

164,384, ICI 182,780, and RU 58,668 displayed an IC₅₀ of 0.61 ± 0.22 μ M, 0.52 ± 0.28 μ M, and 3.36 ± 1.12 μ M. These results indicate that the presence of a long hydrophobic side chain gave compounds almost equipotent to Sah 58-035 to inhibit ACAT activity. Moreover, these results indicated that the presence of two hydroxyl functions has no impact on the inhibition of ACAT activity. The absence of the long aliphatic side chain (17 β -estradiol or diethylstilbestrol) induced a diminution of the ACAT inhibition compared with Sah 58-035. CI-628 and tamoxifen displayed an IC₅₀ of 9.20 ± 1.08 μ M and 6.74 ± 0.84 μ M. This showed that the presence of the aminoethoxyphenyl side chain protonable at physiologic pH on the DPE motif is favorable for ACAT inhibition. However, compounds such as 4-OH-tamoxifen, raloxifene, and RU 39,411 are weaker inhibitors of ACAT compared with Sah 58-035. This indicated that the simultaneous presence of one or two phenol groups and a cationic aminoethoxyphenyl or aminoethoxyphenylketone side chain on the DPE-like backbone is not favorable for ACAT inhibition. Altogether, these data showed that estrogen receptor ligands containing a backbone isosteric with the DPE moiety of Sah 58-035 are inhibitors of ACAT activity and that their potency depends on the nature of the side chain and the presence of hydroxyl phenolic groups. We have estimated the hydrophobicity of the drugs tested by calculating their cLogP, which is an estimation of their partition coefficient between water and a hydrophobic organic solvent (Table 2), to determine whether this parameter was important for ACAT inhibition. The results showed that there is no correlation between the IC₅₀ and cLogP and, consequently, that ACAT inhibition is not dependent on the hydrophobicity of the compounds.

Tamoxifen Inhibits ACAT Activity on Intact Rat Peritoneal Macrophages Treated with acLDLs. To estimate the physiological relevance of the ACAT inhibition by antiestrogens, we tested the effect of several of the most representative antiestrogens on ACAT activity measured on rat peritoneal macrophages (RPMs) activated with acLDLs. This treatment allows the transformation of macrophages

into foam cells that contain a high level of cholesterol esters because of a strong increase of ACAT activity (Brown et al., 1980). This models an early event that precedes the development of the atheromatous plaque. We obtained an 8-fold elevation of cholesterol esterification when the RPMs were treated with acLDLs. The increase of ACAT activity induces a strong accumulation of cholesterol esters that are colorable with oil red O, as shown in Fig. 7B. The results of ACAT activity measurement in these conditions of treatment are presented in Table 3. Two concentrations were chosen (5 and 10 μM). Treatment of macrophages with Sah 58-035 induced 31.5% (5 μM) and 64.1% (10 μM) inhibition of ACAT in macrophage. Interestingly, tamoxifen produced a more robust effect with 42.6% (5 μM) and 92.3% (10 μM) inhibition, which is consistent with the IC_{50} measured in microsomal extract. A similar observation was made with CI 628, with 48.3% (5 μM) and 84.8% (10 μM) inhibition. ICI 164,384 produced a weaker effect than expected, with 21.3% (5 μM) and 34.2% (10 μM) inhibition. Phenolic nonsteroidal antiestrogens showed a modest but significant effect consistent with their IC_{50} , determined on microsomes, with 6.4% (5 μM) and 22.2% (10 μM) inhibition for 4-OH-tamoxifen and 8.4% (5 μM) and 26.8% (10 μM) inhibition for raloxifene. It is important to note that the toxicity was monitored during the time of the experiments, and cells were viable at more than 95%. These results showed that for all the compounds tested, the inhibition of ACAT is as efficient *in vitro* on rat liver microsomal suspension than on intact macrophages stimulated with acLDLs, except for ICI 164,384 and Sah 58-035. Since tamoxifen was determined the most potent inhibitor of ACAT activity in intact rat peritoneal macrophages treated with acLDLs, we next measured its activity to inhibit the formation of foam cells.

Tamoxifen Inhibits the Formation of Foam Cells.

Normal macrophages do not contain high levels of neutral lipids and are not colored with Oil red O, a dye specific for neutral lipids (Fig. 7A). Normal macrophages do not contain a high level of vacuoles in their cytoplasm (Fig. 7D). When macrophages were treated with acLDLs, they became highly colorable with oil red O (Fig. 7B) and were full of lipid droplets and whorls (Fig. 7E), which is characteristic of foam cells (Tabas, 2002). When the macrophages were treated by acLDLs and 10 μM tamoxifen, they were not colored red (Fig. 7C) and did not contain lipid droplets and whorls (Fig. 7F), indicating that they were not transformed into foam cells.

TABLE 3

Effect of ER ligands on ACAT activity measured in rat peritoneal macrophages stimulated with acLDLs

Rat peritoneal macrophages were preincubated with compounds and then allowed to convert into foam cells by 15-h incubation with 100 $\mu\text{g}/\text{ml}$ acLDL. Cellular lipids and cholesteryl ester amounts were determined by following the incorporation of ^{14}C -labeled oleic acid into cholesteryl esters as described under *Materials and Methods*. Values are the average of three experiments \pm S.E.M., each carried out in duplicate.

Compound	Percentage of ACAT Inhibition in Rat Peritoneal Macrophage Stimulated with acLDLs (at the Indicated Concentration of Compound)	
	5 μM	10 μM
Sah 58-035	31.5 \pm 2.7	64.1 \pm 4.6
Tamoxifen	42.6 \pm 3.5	92.3 \pm 6.4
CI-628	48.3 \pm 2.7	84.8 \pm 4.1
ICI 164,384	21.3 \pm 4.8	34.2 \pm 7.8
OH-Tamoxifen	6.4 \pm 4.8	22.2 \pm 5.4
Raloxifene	8.4 \pm 7.5	26.8 \pm 4.3

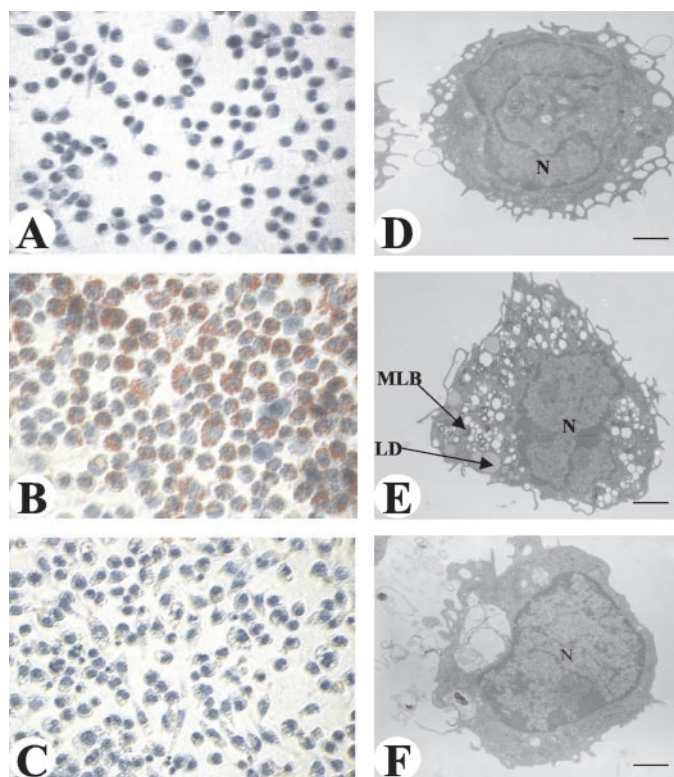


Fig. 7. A–C, staining of neutral lipids with oil red O of rat peritoneal macrophages treated 15 h with solvent vehicle (panel A), 100 $\mu\text{g}/\text{ml}$ acLDL (panel B), or 100 $\mu\text{g}/\text{ml}$ acLDL plus 10 μM tamoxifen (panel C). After incubation, the cells were fixed and stained with oil red O and hematoxylin. Stimulation of macrophages by acLDLs induced a massive appearance of oil red O coloration (panel B). Data are representative of three repeated experiments. D–F, electron micrographs of rat peritoneal macrophages. Macrophages were treated with solvent vehicle (panel D), acLDLs (panel E), and acLDLs with 10 μM tamoxifen (panel F). After fixation of the cells and embedding into Epon 812, ultrathin sections of the cells were prepared, stained with uranyl acetate and lead citrate, and examined on an H300 Hitachi electron microscope. Numerous lipid droplets were detected in the cytoplasm of RPMs treated with acLDLs (panel E). In contrast, these lipid droplets were not found in the cytoplasm of cells treated with acLDLs plus tamoxifen (panel F). N, nucleus; MVB, multivesicular body; LD, lipid droplet. Bars: 1 μm for panels D and F; 1.4 μm for panel E.

These results showed that tamoxifen blocks the transformation of the macrophages into foam cells.

Discussion

In the present study, we have tested the hypothesis that tamoxifen, used for the treatment and the prevention of breast cancer, displays an atheroprotective effect by inhibiting ACAT activity. We first demonstrated that the ACAT inhibitor Sah 58-035 presents a high structural homology on its diphenylethane moiety with tamoxifen. This is not peculiar to tamoxifen, since a similar homology was found between Sah 58-035 and different estrogen receptor ligands that contained a structural motif isosteric to the diphenylethane moiety of Sah 58-035. Thus, this motif may constitute a pharmacophore for the inhibition of cholesterol esterification. In accordance with this structural homology, tamoxifen and estrogen receptor ligands were able to inhibit ACAT activity, in a concentration-dependent manner, both on rat liver microsomes and on intact foaming macrophages. Triphenylethyl compounds such as tamoxifen and CI-628

were found to be the most potent inhibitors of ACAT activity on foaming macrophages, whereas ICI 164,384 was less efficient than expected.

Cytological studies showed that the inhibition of ACAT activity by tamoxifen on macrophages stimulated with acLDLs is associated with a blockage of the accumulation of neutral lipids and prevents their transformation into foam cells. To our knowledge, these data show for the first time that tamoxifen, by inhibiting the formation of foam cells, is able to block a step that precedes the formation of the atheromatous plaque. Thus, the inhibition of ACAT activity by tamoxifen could explain its atheroprotective effect, described in the different atherosclerosis mammal models such as male C57BL/6 mice (Grainger et al., 1995), transgenic apolipoprotein(a) mice (Lawn et al., 1996), apolipoprotein E-deficient mice (Reckless et al., 1997), and postmenopausal female monkeys fed an atherogenic diet (Williams et al., 1997), and its protective effect against the development of coronary artery diseases in patients treated with tamoxifen (McDonald and Stewart, 1991; Rutqvist and Mattsson, 1993; McDonald et al., 1995; Clarke et al., 2001).

The therapeutic concentration of tamoxifen has been determined to be between 1 and 10 μM in the blood of treated patients and animals (Etienne et al., 1989; Trump et al., 1992). In the present study, 10 μM tamoxifen prevents foam cell formation and inhibits 92.3% of ACAT activity. Since partial inhibition of ACAT is sufficient to protect from atheromatous plaque deposition in apo-E knockout mice treated with an ACAT inhibitor (Kusunoki et al., 2001), our results strongly argue that the inhibition of ACAT by tamoxifen plays an important role in its atheroprotective effect.

Sah 58-035 is a selective inhibitor of ACAT (Ross et al., 1984). Its mechanism of action is assumed to be direct and competitive with regard to oleyl-CoA (Sliskovic et al., 2002). We have shown in this study that tamoxifen is a competitive inhibitor of ACAT.

It has been reported that ACAT inhibition induced free cholesterol accumulation that might be toxic for cells (Warner et al., 1995). Cholesterol over-accumulation was associated, in this case, with the appearance of cholesterol crystals that were not observed during tamoxifen treatment. This could suggest a stimulation of the efflux of free cholesterol. Tamoxifen has been reported to stimulate the production of transforming growth factor- β in mouse aorta (Grainger et al., 1995). Recently, transforming growth factor- β has been shown to enhance the free cholesterol efflux through the up-regulation of ATP-binding cassette transporters in foam cells (Argmann et al., 2001). This mechanism might explain the absence of over-accumulation of free cholesterol and cell toxicity in our experiments. This work constitutes the first evidence that tamoxifen is an inhibitor of ACAT and foam cell formation at therapeutic doses and that this may account for its atheroprotective action. Regarding the structure-activity data on the other compounds that we have tested in the present study, it appears that not only tamoxifen but also other related estrogen receptor ligands inhibit ACAT. These data suggest that these compounds may act like tamoxifen and may display an atheroprotective action.

Thus, the inhibition of ACAT by tamoxifen may give a benefit for patients against the development of cardiovascular diseases and supports its application for long-term treat-

ment and prevention of cancer. Furthermore, these results offer a rationale for the possible use of tamoxifen for the prevention of cardiovascular diseases in males.

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Address correspondence to: Marc Poirot, Département Innovation Thérapeutique et Oncologie Moléculaire, Institut Claudius Regaud, INSERM U 563, CPTP, 20–24 rue du Pont Saint Pierre, 31052 Toulouse Cedex, France. E-mail: poirot@icr.fnclcc.fr
