Supporting Information:

Identification and pharmacological characterization of cholesterol-5,6-epoxide hydrolase: a target for tamoxifen.

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SI Material and Methods

Chemicals: [^{14}C]cholesterol (58 Ci/mol; 1 Ci = 37 GBq) and [^{3}H]Tam (81 Ci/mmol) were purchased from General Electric. Sterols, fatty acids, oxysterols and drugs were from Steraloids or Sigma-Aldrich. Nitromiphene was from Park Davis; raloxifene, RU 39,411 and SR-31747A from Sanofi-Aventis and ICI 182,780 was from Tocris Cookson (UK). BD1008 was a kind gift from Prof. W. Bowen (Brown University, Providence, RI, USA). Other

compounds were synthesized in our laboratory according to published procedures (1, 2). TLC plates (LK-6-DF) were from Whatman. [^{14}C]α- and [^{14}C]β-CE (10 Ci/mol) were synthesized as described (3). The solution was diluted in 10 vol of dichloromethane, and the organic layer washed with 10% aqueous sodium sulfite, 5% aqueous sodium hydrogenocarbonate and then water. The reaction mixture was passed through a Sep-Pack cartridge (Vac C18, 1 cc; Waters) equilibrated with MeOH. CE epimers were then separated isocratically by reversed-phase HPLC (Ultrasep ES 100 RP 18, 6.0 µm**;** Bischoff, Leonberg, Germany) with MeOH/H_2O (95:5) as eluant at a flow rate of 0.7 ml/min. The reaction gave 75% α-CE and 25% β-CE, with a >98% radiochemical purity.

AEBS ligand binding assays. For competition and Scatchard assays, AEBS was labeled as described using rat liver microsomes or COS-7 cell microsomal extracts and [^{3}H]Tam in the presence of 1 µM 17β-estradiol to mask residual estrogen receptors (4). 5% dimethylformamide was used to solubilized compounds.

Chemical synthesis of 5,6-epoxy-cholestan-3β-stearate. Cholesteryl stearate (204 mg; 0.31 mmol; 1 eq) was dissolved in dichloromethane (5 ml). A solution of meta chloro perbenzoate (102 mg; 0.41 mmol; 1.3 eq) dissolved in dichloromethane (2 ml) was added to the solution and stirred at room temperature for 3 hr. The solution was diluted in 10 vol of dichloromethane, and the organic layer washed with 10% aqueous sodium sulfite, 5% aqueous sodium hydrogenocarbonate and then water and subsequently dried under $MgSO_4$. The solution was evaporated, and the white solid was dissolved in 500 µl hexane/diethylether (1:3) and then purified on a silica column. The column was washed with hexane and the synthesis products were eluted with hexane/ethylacetate (8:2) to give a mixture of 5,6α-

epoxy-cholestan-3β-stearate (70%) and 5,6β-epoxy-cholestan-3β-stearate (30%). Yield: 65%; R_F (hexane 8/EtOAc 2): 0.56 (α isomer), 0.58 (β isomer); mass spectroscopy (*m*/*z*; DCI/NH_3): 669 (MH^+); 686 ($M\text{-}NH_4^+$); 651 ($MH^+\text{-}H_2O$).

ChEH activity assays.

An initial kinetic analysis was carried out to determine the optimum incubation time for the subsequent inhibition experiments. The final assay volume was 150 µl with 130 µl buffer (50 mM Tris, pH 7.4; 150 mM KCl), 10 µl microsomal proteins (15 mg/ml) and 10 µl acetonitrile (6.7%) containing the test compound/drug and the labeled α-CE. Tubes were incubated at 37°C for increasing periods of time, from 0 to 30 min. The reaction was stopped by immersing the sample in ice-water and adding 1.5 ml chloroform/methanol (2:1) and 500 µl reaction buffer. After shaking, the lower phase was removed and saved, and the aqueous phase was extracted with 1.5 ml chloroform. The two organic layers were mixed, reduced to dryness under a flux of nitrogen and the residue resuspended in 60 µl ethanol. More than 95% of radioactivity was recovered in the organic layers. Samples were applied to silica gel 60, 20 × 20 plates (Fluka, Germany) that had previously been heated for 1 hr at 100°C and were developed using ethylacetate. The regions corresponding to authentic CE and CT standards were visualized by iodine vapor. Radioactive metabolites were visualized using a Storm apparatus (Molecular Dynamics) and quantified by densitometry with the software Imagequant (Molecular Dynamics). Retention factors (R_F) were determined for each spot on the TLC plates as the ratio of the distance of migration of the eluate from the origin and the distance of the solvent from the origin. α-CE had a R_F = 0.67 (top of the TLC, Fig 1), and CT had a R_F = 0.25. As a result of the initial kinetics, a 9-min incubation and 20 µM [^{14}C]α-CE was chosen for subsequent ChEH inhibition experiments.

Expression of D8D7I and DHCR7 in COS-7 cells. Plasmid constructs, COS-7 cell transfection and the measurement of AEBS binding were done as described in (4). For ChEH activity, microsomes were incubated with 20 μM [^{14}C]α-CE for 9 min and were then treated as described in the above paragraph.

Knockdown of D8D7I and DHCR7 expression in MCF-7 cells. MCF-7 cells were plated in 10 cm dishes at 5 x 10^5 cells in RPMI medium containing 5% FBS. 24 hours after seeding, the cells were transfected in Opti-MEM with 100 nM Control siRNA (sc-37007, Santa Cruz Biotechnology), 100 nM D8D7I (EBP) siRNA (sc-77218, Santa Cruz Biotechnology) or 50 nM DHCR7 siRNA (sc-60533, Santa Cruz Biotechnology) for 4 h using Oligofectamine (Invitrogen) as recommended by the manufacturer. Cells were harvested at 3 days for enzymatic analyses. To validate the knock-down efficacy, protein levels were analysed on samples under the same conditions. 72 hours after transfection with siRNAs, MCF-7 cells were harvested in RIPA lysis buffer supplemented with protease inhibitors (Roche). Immunoblotting was carried out as described earlier (4). Proteins were separated on a 10% SDS-PAGE gels, electro-transferred onto PVDF membranes and incubated overnight at 4°C with antibodies against human D8D7I (ARP46742, Aviva Systems Biology), human DHCR7 (Ab67664, Abcam) or Actin (C4 Clone, MAB1501, Millipore). Bands were visualized using an ECL plus kit (Amersham Biosciences).

1. Song C, Hiipakka RA, & Liao S (2001) Auto-oxidized cholesterol sulfates are antagonistic ligands of liver X receptors: implications for the development and treatment of atherosclerosis. *Steroids* 66(6):473-479.

2. Poirot M, *et al.* (2000) Synthesis, binding and structure-affinity studies of new ligands for the microsomal anti-estrogen binding site (AEBS). *Bioorg Med Chem* 8(8):2007-2016.

3. Sevanian A & McLeod LL (1986) Catalytic properties and inhibition of hepatic cholesterol-epoxide hydrolase. *J Biol Chem* 261(1):54-59.

4. Kedjouar B, *et al.* (2004) Molecular characterization of the microsomal tamoxifen binding site. *J Biol Chem* 279(32):34048-34061.

SI figure legend

Fig.S1. Scheme describing the reaction of hydration of cholesterol epoxides catalyzed by the Cholesterol-5,6-epoxide hydrolase (ChEH).

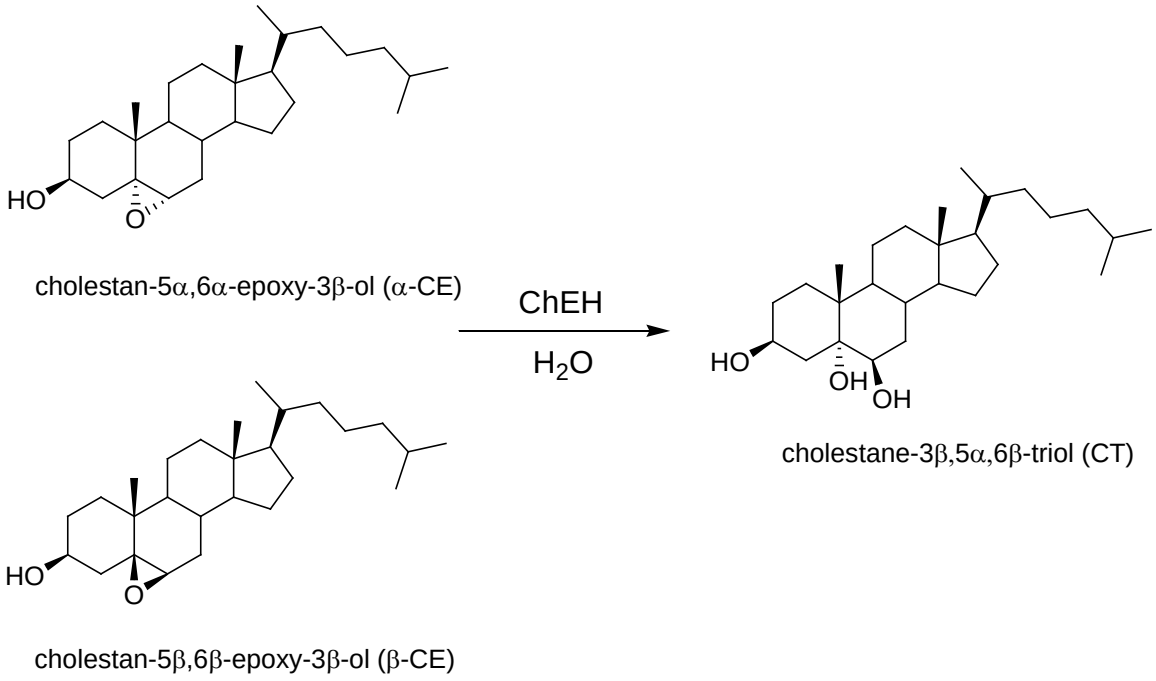

Fig.S2. Effect of the prototypical AEBS ligands Tam and PBPE on ChEH activity. A) A time course of ChEH activity. Rat liver microsomes were incubated with 20 µM [^{14}C]α-CE for the indicated time. ChEH activity was assayed by measuring the conversion of α-CE to CT by TLC. A representative autoradiogram of a TLC from two independent experiments is shown with a plot showing the time course of CT formation by α-CE hydration. The least-squares regression line was computed over the first 10 min. B) Michaelis-Menten plot of ChEH activity. C) Measurement of ChEH activity in rat liver microsomes using 20 µM [^{14}C]α-CE, solvent vehicle (control), 10 µM 6-ketocholestanol (6-KC), 10 µM 7-ketocholestanol (7-KC), 1 µM Tam or 1 µM PBPE. A representative autoradiogram of a TLC from three independent experiments is shown. A plot showing the inhibition of ChEH activity on the hydration of α-CE and β-CE by the test compounds is indicated. The hydration of 20 µM [^{14}C]α-CE or [^{14}C]β-CE into CT was inhibited by 10 µM 6-ketocholestanol, 10 µM 7-ketocholestanol, 1 µM Tam or 1 µM PBPE.

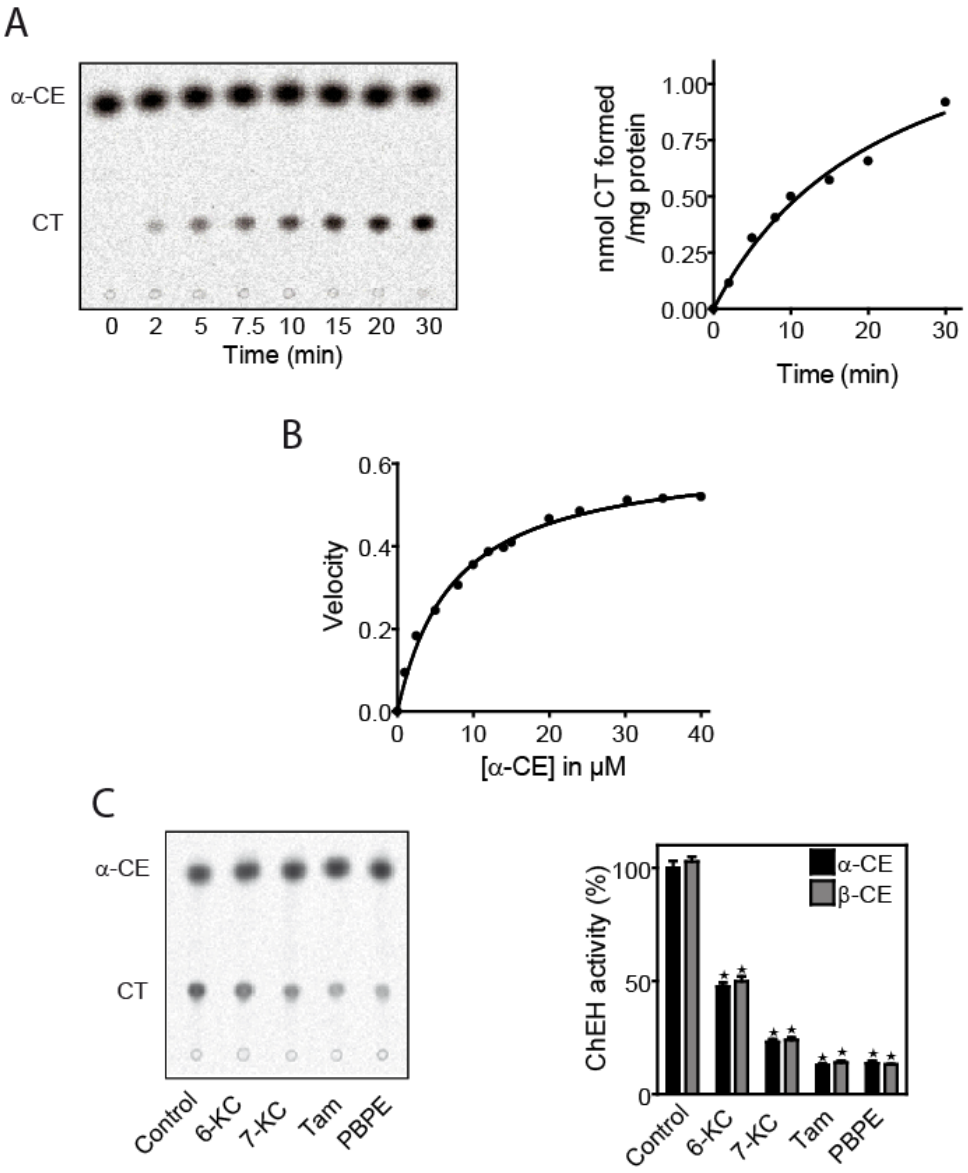

Fig.S3. Dixon Plot of ChEH inhibition by Tam, PBPE and oleic acid. The potency of Tam, PBPE and oleic acid to inhibit the ChEH activity was assayed as described in the legend of Fig.2. Dixon plots of [^{14}C]α-CE versus Tam (A), PBPE (B) or oleic acid (C) with rat liver microsome ChEH. Concentrations in Tam or PBPE used in the assay were: 0, 100, 250, 300, 400, 500, 750 and 1000 nM. Concentrations of oleic acid were: 0, 50, 100, 150, 200 and 250 µM.

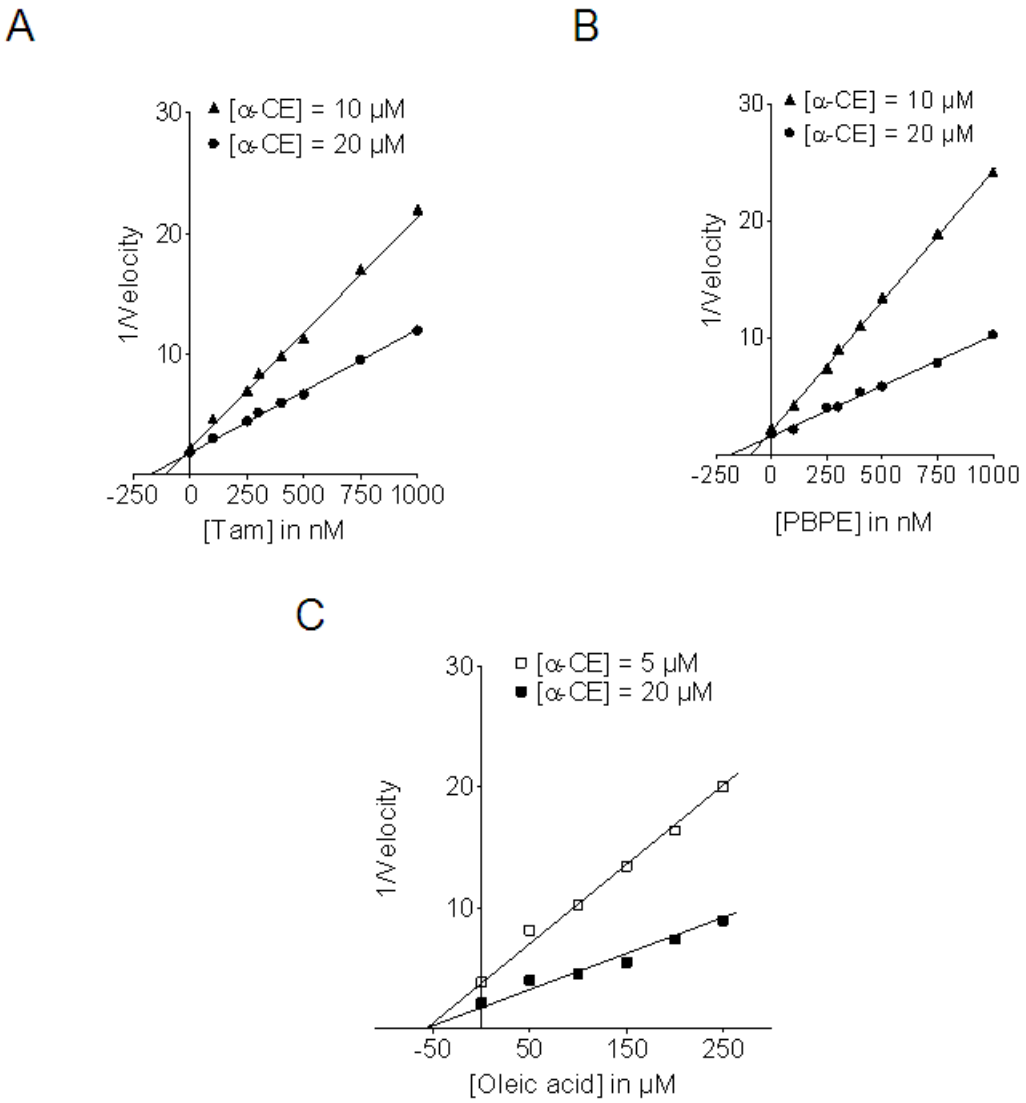

Table S1. Measurement of the inhibition of [^{3}H]Tam binding to the AEBS and catalytic activity of ChEH by drugs. Rat liver microsomes were incubated with a single concentration of 2.5 nM [^{3}H]Tam and increasing concentrations of test compounds ranging from 0.01 to 1000 µM under the condition described in the "Materials and Methods" section. For the ChEH inhibition tests 150 µg of rat liver microsomal proteins and 10 and 20 µM of [^{14}C]α-CE with increasing concentrations of compounds ranging from 0.01 to 1000 µM were used under the conditions described in the "Material and Methods" section. N.M.: no measurable inhibition up to the maximal concentration of inhibitor used.

	Compound		K_i AEBS	K_i ChEH
Estrogen receptor ligands	17β-estradiol	S1	N.M.	N.M.
	DES	S2	N.M.	N.M.
	ICI 164,384	S3	N.M.	N.M.
	ICI 182,780	S4	N.M.	N.M.
	RU 58,668	S5	N.M.	N.M.
Sigma receptor ligands	(+)-pentazocine	S6	N.M.	N.M.
	(+)-3PPP	S7	N.M.	N.M.
	PRE-084	S8	N.M.	N.M.
	DTG	S9	N.M.	N.M.
	progesterone	S10	N.M.	N.M.
Miscalenous	ketoconazole	S11	N.M.	N.M.
	MCH_3PE	S12	N.M.	N.M.
Side chain oxysterols	22(R)-hydroxycholesterol	S13	N.M.	N.M.
	24(S)-hydroxycholesterol	S14	N.M.	N.M.
	25-hydroxycholesterol	S15	N.M.	N.M.
	26-hydroxycholesterol	S16	N.M.	N.M.
Epoxysterol-esters	α-CE-sulfate	S17	N.M.	N.M.
	CE-stearate	S18	N.M.	N.M.
Fatty acids and ester	palmitic acid	S19	N.M.	N.M.
	stearic acid	S20	N.M.	N.M.
	oleic acid methyl ester	S21	N.M.	N.M.

Fig.S4. trivial names, IUPAC names and chemical structure of selective AEBS ligands (A) and estrogen receptor modulators (B) that bind to the AEBS and inhibit ChEH. PBPE: 1-(2-(4-benzylphenoxy)ethyl)-pyrrolidin; PCPE: 1-(2-(4-(2-phenylpropan-2-yl) phenoxy)ethyl)pyrrolidine; tesmilifene: 2-(4-benzylphenoxy)-N,N-diethylethanamine, MBPE: 4-(2-(4-benzylphenoxy)ethyl)morpholine; MCPE: 4-(2-(4-(2-phenylpropan-2-yl)phenoxy) ethyl)morpholine; PCOPE: phenyl(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)methanone; MCOPE: (4-(2-morpholinoethoxy)phenyl)(phenyl)methanone; MCOCH2PE: 1-(4-(2-morpholino ethoxy)phenyl)-2-phenylethanone; tamoxifen: 2-[4-[(Z)-1,2-di(phenyl)but-1-enyl]phenoxy]-N,N-dimethylethanamine; 4OH-tam: 4-[(Z)-1-[4-(2-dimethylaminoethyloxy)phenyl]-2-phenyl but-1-enyl]phenol; raloxifene: [6-hydroxy-2-(4-hydroxyphenyl)-1-benzothiophen-3-yl]-[4-(2-piperidin-1-ylethoxy)phenyl]methanone; nitromiphene:1-[2-[4-[(Z)-1-(4-methoxyphenyl)-2-nitro-2-phenylethenyl]phenoxy]ethyl]pyrrolidine; clomiphene: 2-[4-[(Z)-2-chloro-1,2-di (phenyl)ethenyl]phenoxy]-N,N-diethylethanamine; RU 39,411: 11-[4-*N*,*N*-[diethylamino ethoxy]phenyl]-estra-1,3,5(10)triene-3,17-diol.

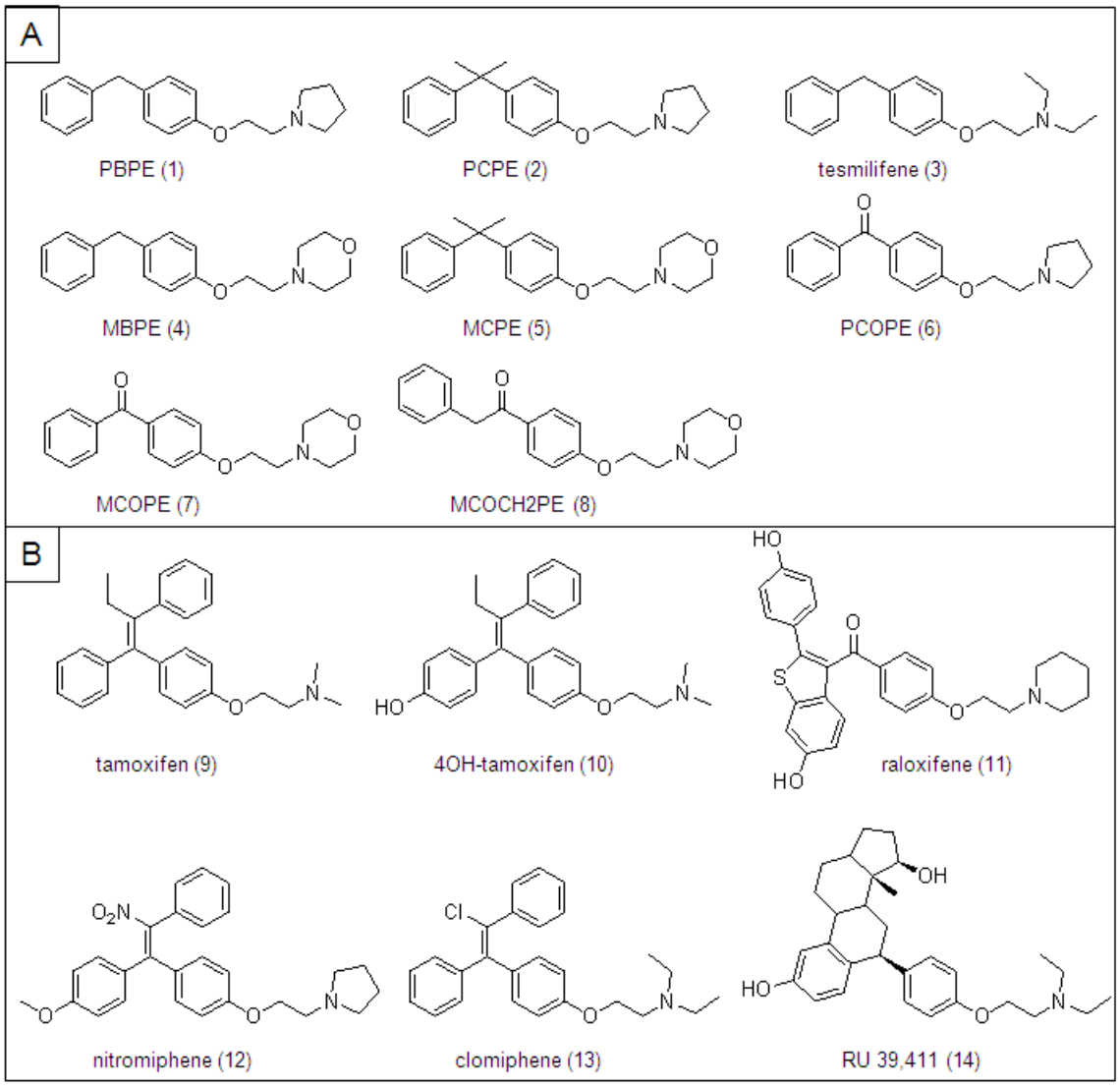

Fig.S5. name and chemical structure of Sigma-receptor ligands (A) and cholesterol biosynthesis inhibitors (B) that bind to the AEBS and inhibit ChEH. BD-1008: N-(3,4-dichlorophenethyl)-N-methyl-2-(pyrrolidin-1-yl)ethanemine; Haloperidol: 4-(4-(4-chloro phenyl)-4-hydroxypiperidin-1-yl)-1-(4-fluorophenyl)butan-1-one; SR-31747A: (E)-N-(4-(3-chloro-4-cyclohexylphenyl)but-3-enyl)-N-ethylcyclohexanamine; RO 48-8071: (4-(6-(allyl (methyl)amino)hexyloxy)phenyl)(4-bromophenyl)methanone; U-18666A: 3-beta-(2-(diethyl amino)ethoxy)androst-5-en-17-one; AY-9944: trans-1,4-Bis(2-chlorobenzaminomethyl) cyclohexane; Triparanol: 2-(4-chlorophenyl)-1-(4-(2-(diethylamino)ethoxy)phenyl)-1-p-tolyl ethanol; terbinafine: (E)-N,3,6,6-tetra methyl-N-(naphthalene-1-ylmethyl)hept-2-en-4-yn-1-amine; SKF-525A: 2-diethylaminoethyl2,2-diphenylpentanoate.

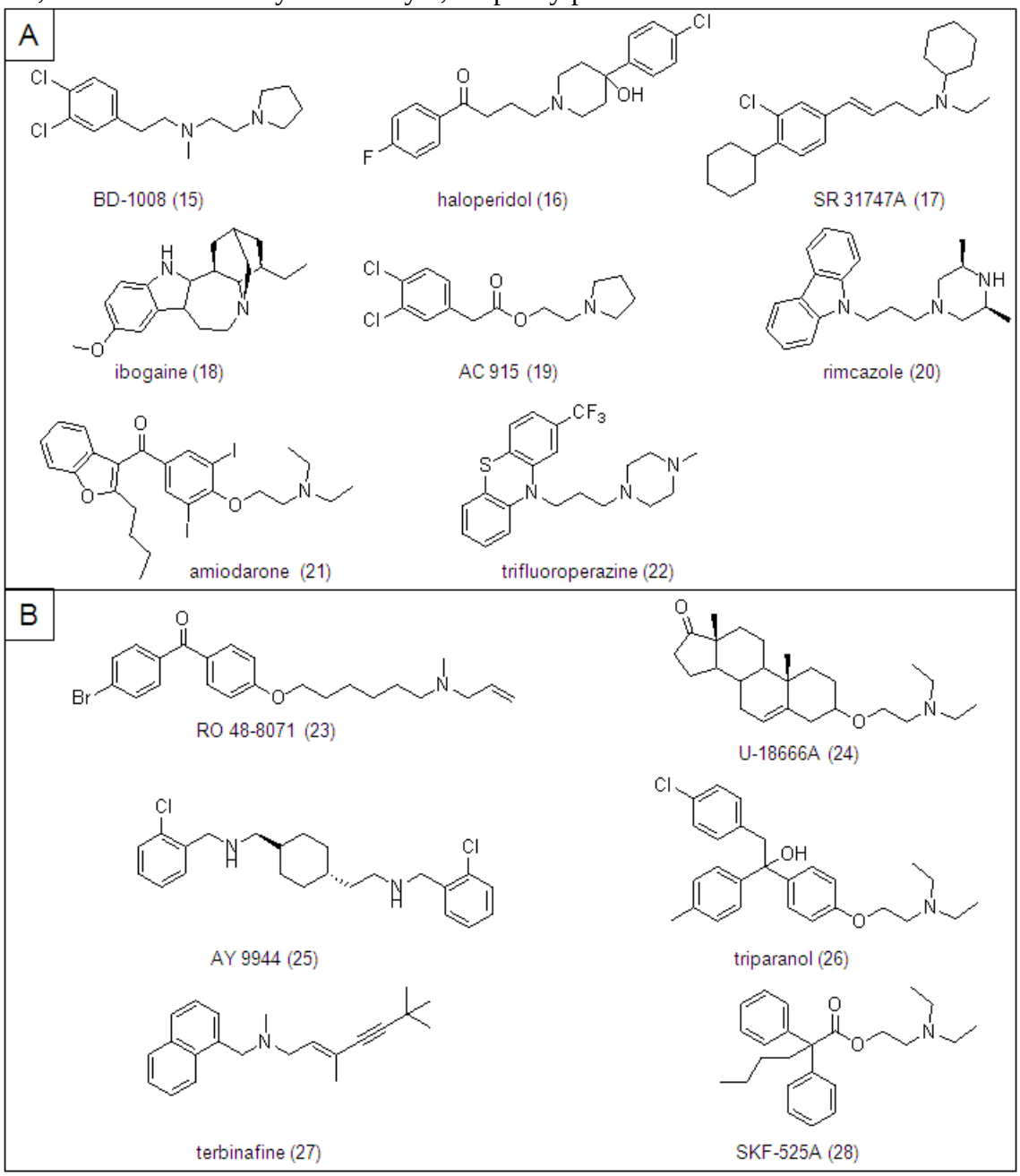

Fig.S6. Trivial name, chemical name and chemical structure of ring B oxysterols and fatty acids that bind to the AEBS and that inhibit ChEH.

6-ketocholestanol: 5α-cholestan-3β-ol-6-one**;** 7-ketocholestanol: 5α-cholestan-3β-ol-7-one; 7-ketocholesterol: 5-cholesten-3β-ol-7-one; 7α-hydroxycholesterol: 5-choles tene-3β,7α-diol; 7β-hydroxycholesterol: 5-cholestene-3β,7β-diol; 6-keto-5α-hydroxy cholestanol: cholestane-3β,5α-diol-6-one; oleic acid: (Z)-octadec-9-enoic acid; α-linolenic acid: (9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid; arachidonic acid: (5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic acid; docosahexaenoic acid: (4Z,7Z,10Z, 13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenoic acid.

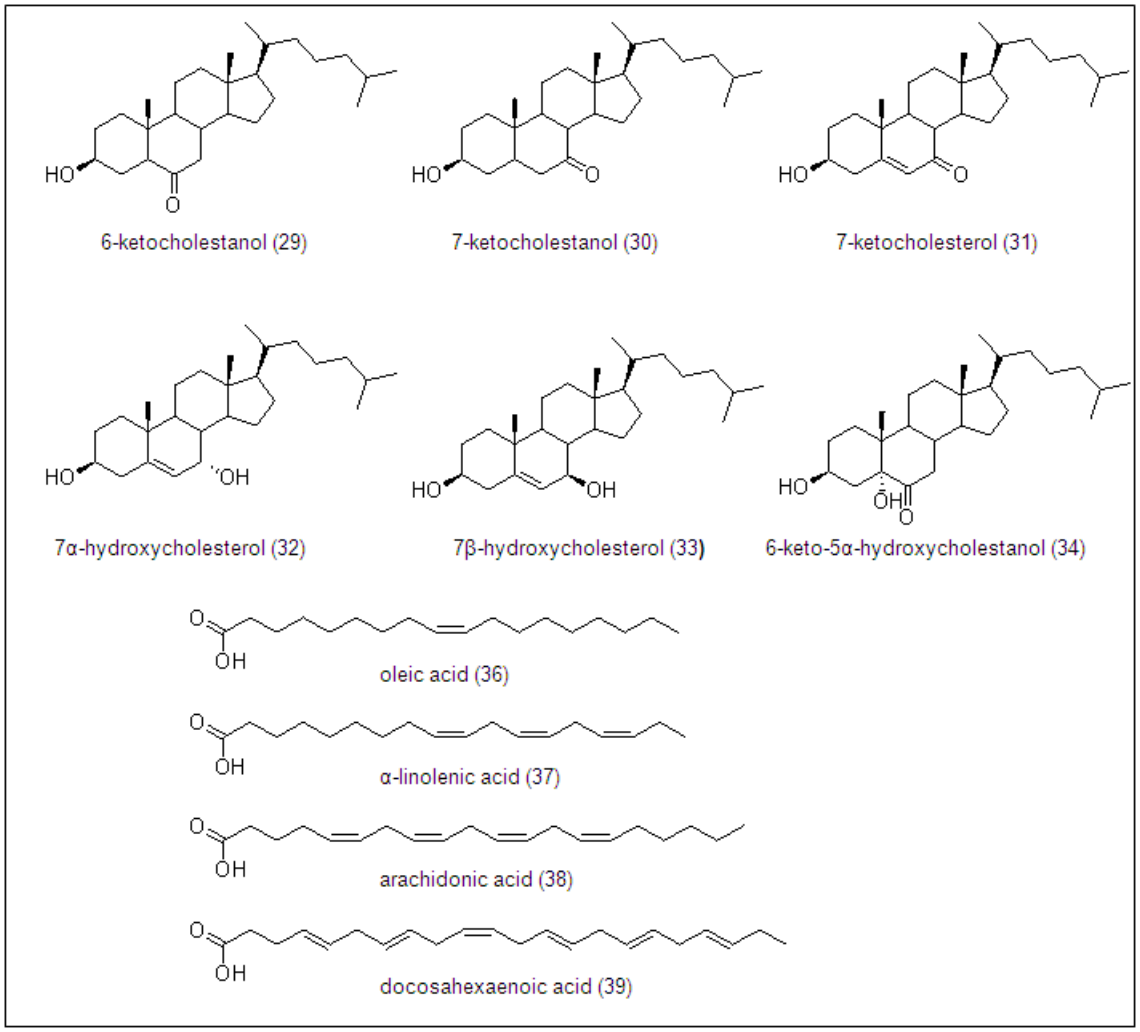

Fig.S7.Trivial name, chemical name and chemical structure of compounds that do not bind to the AEBS and do not inhibit ChEH up to 10 µM (compounds S2-S5) or 1000 µM (S6-S12). ICI 164,384: [(*N*-*n*-butyl-*N*-methyl-11-[3,17-di-hydroxyestra-1,3,5(10)-trien-7-yl]-undecanamide]; ICI 182,780: Faslodex, fulvestrant, 7-alpha-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]estra-1,3,5(10)-triene-3,17-beta-diol; RU 58668: 11-[[[(4,4,5,5,5,-pentafluoropentyl)sulfonyl]pentyloxy]phenyl]-estra-1,3,5(10)-triene-3,17-diol; (+)-3PPP: (R)-3-(1-propylpiperidin-3-yl)phenol; PRE-084: 2-morpholinoethyl-1-phenyl cyclohexanecarboxy late; DTG: 1,3-dio-tolylguanidine; progesterone; ketoconazole: 1-(4-(4-(((2R,4S)-2-((1H-imidazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl)methoxy) phenyl)piperazin-1-yl) ethanone; MCH3PE: 4-(2-(p-tolyloxy)ethyl)morpholine.

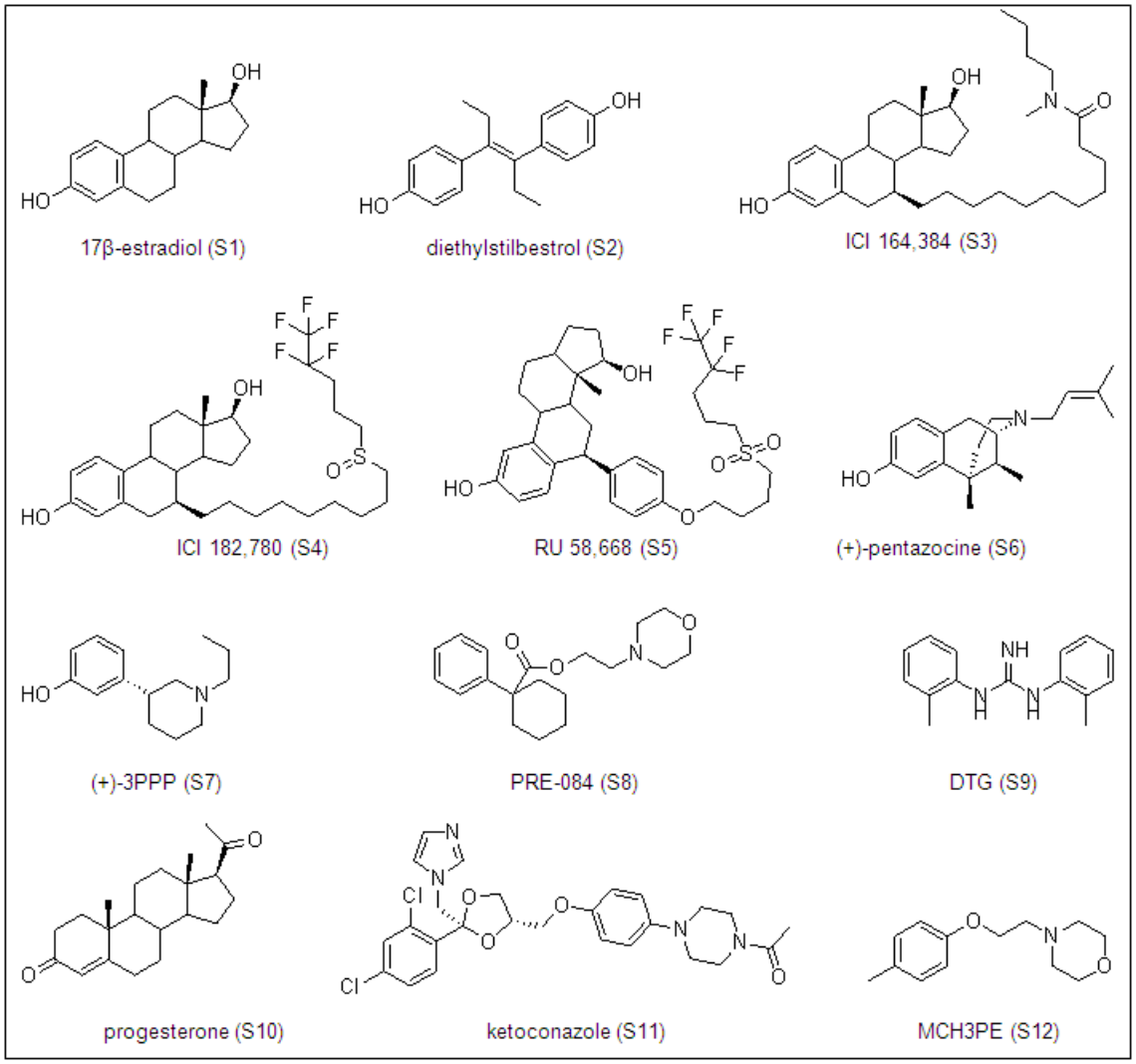

Fig.S8. Trivial name, chemical mane and chemical structure of side chain oxysterols and fatty acids that do not bind to the AEBS and do not inhibit ChEH up to 50 µM.
22(R)-hydroxycholesterol: 5-cholestene-3β,22R-diol; 24(S)-hydroxycholesterol: 5-cholestene-3β,24(S)-diol; 25-hydroxycholesterol: 5-cholestene-3β,25-diol; 26-hydroxy cholesterol: 5-cholestene-3β,26-diol; α-CE-sulfate: cholestan-5α,6α-epoxy-ol-3β-sulfate; CE-stearate: cholestan-5,6-epoxy-3β-ol-stearate; palmitic acid: hexadecanoic acid; stearic acid: octadecanoic acid;