Identification and pharmacological characterization of cholesterol-5,6-epoxide hydrolase as a target for tamoxifen and AEBS ligands

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Edited by David J. Mangelsdorf, University of Texas Southwestern Medical Center, Dallas, TX, and approved June 9, 2010 (received for review March 5, 2010)

The microsomal antiestrogen binding site (AEBS) is a high-affinity target for the antitumor drug tamoxifen and its cognate ligands that mediate breast cancer cell differentiation and apoptosis. The AEBS, a hetero-oligomeric complex composed of 3β-hydroxysterol-Δ^8-Δ^7-isomerase (D8D7I) and 3β-hydroxysterol-Δ^7-reductase (DHCR7), binds different structural classes of ligands, including ring B oxysterols. These oxysterols are inhibitors of cholesterol-5,6-epoxide hydrolase (ChEH), a microsomal epoxide hydrolase that has yet to be molecularly identified. We hypothesized that the AEBS and ChEH might be related entities. We show that the substrates of ChEH, cholestan-5α,6α-epoxy-3β-ol (α-CE) and cholestan-5β,6β-epoxy-3β-ol (β-CE), and its product, cholestane-3β,5α,6β-triol (CT), are competitive ligands of tamoxifen binding to the AEBS. Conversely, we show that each AEBS ligand is an inhibitor of ChEH activity, and that there is a positive correlation between these ligands' affinity for the AEBS and their potency to inhibit ChEH ($r^2 = 0.95$; $n = 39$; $P < 0.0001$). The single expression of D8D7I or DHCR7 in COS-7 cells slightly increased ChEH activity (1.8- and 2.6-fold), whereas their coexpression fully reconstituted ChEH, suggesting that the formation of a dimer is required for ChEH activity. Similarly, the single knockdown of D8D7I or DHCR7 using siRNA partially inhibited ChEH in MCF-7 cells, whereas the knockdown of both D8D7I and DHCR7 abolished ChEH activity by 92%. Taken together, our findings strongly suggest that the AEBS carries out ChEH activity and establish that ChEH is a new target for drugs of clinical interest, polyunsaturated fatty acids and ring B oxysterols.

breast cancer | chemoprevention | cholesterol metabolism | oxysterol | docosahexaenoic acid

Tamoxifen (Tam) is one of the most commonly used drugs worldwide for hormonal treatment and chemoprevention of estrogen receptor (ER)-positive breast cancers (1). In this application, Tam activity is mediated through the modulation of gene expression under the control of ERs. Tam's pharmacology is complex, however; it exerts nongenomic effects through other targets at therapeutic doses (2). After the ER, the microsomal antiestrogen binding site (AEBS) is the target of highest affinity for Tam. The AEBS has no affinity for estrogens, but binds selective ER modulators (SERMs); these ligands contain a hydrophobic core that mimics the steroid backbone of estrogens, grafted to a dialkylaminoalkyl side chain. Thus, in addition to Tam, SERMs, such as raloxifene, 4-OH-Tam, and RU-39411, are ligands of the AEBS (3–5). The AEBS selectively binds diphenylmethane derivatives of Tam, including (4-benzyl-phenoxy)-ethyl-N-pyrrolidine (PBPE) and tesmilifene (6) and it also binds σ-receptor ligands and inhibitors of cholesterol biosynthesis, such as triparanol and AY-9944 (5, 7). All of these classes of synthetic AEBS ligands have a protonable dialkylaminoalkyl chain in common that is necessary for high-affinity binding to the AEBS (6). In the quest for natural AEBS ligands, several unsaturated fatty acids and ring B oxysterols have been identified (8, 9). Oxysterols that bind to the AEBS are cholesterol-oxidized on ring B of the cholesterol backbone and include 5-cholesten-3β-ol-7-one (7-ketocholesterol), 5α-cholestan-3β-ol-7-one (7-ketocholestanol), and 5α-cholestan-3β-ol-6-one (6-ketocholestanol) (8).

In previous work, we established that the coexpression of 3β-hydroxysterol-Δ^8-Δ^7-isomerase (D8D7I) and 3β-hydroxysterol-Δ^7-reductase (DHCR7) in mammalian cells is necessary and sufficient to reconstitute the high-affinity binding site for [^{3}H]Tam (i.e., the AEBS) (5). D8D7I and DHCR7 are two enzymes involved in specific catalytic steps in the postlanosterol biosynthesis of cholesterol. Consistent with the fact that the AEBS binds σ-receptor ligands (7), D8D7I (an AEBS subunit) carries a binding site for σ-receptor ligands (10). AEBS expression is ubiquitous, and AEBS is highly expressed in proliferative cells, such as tumor cells, and in cholesterogenic tissues, such as the liver and brain, in accordance with its relationship with cholesterol metabolism (5, 10, 11). We recently established that AEBS ligands induce breast cancer cell differentiation and apoptosis through a mechanism involving the production of sterol autoxidation products (3, 4, 12). Cholestan-5α,6α-epoxy-3β-ol (α-CE), cholestan-5β,6β-epoxy-3β-ol (β-CE), and 7-ketocholesterol are among the major autoxidation products of cholesterol (13). CEs (α-CE and β-CE) are the only known substrates of cholesterol epoxide hydrolase (ChEH), and 7-ketocholesterol is an inhibitor of ChEH (14, 15). ChEH (EC 3.3.2.11) catalyzes the hydrolysis of α-CE and β-CE into a unique geminal trans-diol, cholestane-3β,5α,6β-triol (CT) (16) (*SI Appendix*, Fig. S1).

We have observed that the AEBS and ChEH share similar characteristics and pharmacological properties. Indeed, ring B oxysterols, such as 6-ketocholestanol, 7-ketocholestanol, and 7-ketocholesterol, inhibit ChEH (14) and bind to the AEBS (8) with the same order of potency. In addition, ChEH is inhibited by an autoxidation product of 7-dehydrocholesterol (17), and 7-dehydrocholesterol is the substrate of DHCR7, an AEBS subunit (5). Moreover, as with the AEBS, ChEH is located in the endoplasmic reticulum of cells and is found in most mammalian tissues, with the liver being the richest source of both the AEBS and ChEH (18, 19). ChEH is the last member of the epoxide hydrolase family with an unidentified coding gene. The results from these studies, along with the relationship that we established between sterol autoxidation products and the functions of the AEBS (3, 4, 12), suggested to us a pharmacological and structural link between the AEBS and ChEH, which we investigated in the present study. Our results indicate that the enzymes that form the AEBS are involved in the catalytic activity of ChEH.

Author contributions: P.d.M., M.R.P., G.S., and M.P. performed research; P.d.M., M.P., and S.S.-P. analyzed data; M.P. and S.S.-P. designed research; and M.P. and S.S.-P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1002922107/-/DCSupplemental.

Results

α-CE, β-CE, and CT Are Competitive Ligands of the AEBS. We began by investigating whether the substrates of ChEH (α-CE and β-CE) and CT, the product of ChEH activity, are ligands of the AEBS. We performed assays on rat liver microsomes, the richest source of the AEBS (6). Competitive binding assays showed that increasing concentrations of unlabeled Tam, α-CE, β-CE, or CT inhibited the binding of 2.5 nM [^{3}H]Tam to the AEBS in a concentration-dependent manner (Fig. 1*A*). The IC_{50} was 5.9 ± 0.5 nM for Tam, 153 ± 0.5 nM for α-CE, 394 ± 1 nM for β-CE, and 16.3 ± 0.6 μM for CT. To study the modality of the inhibition by α-CE, β-CE, and CT, we performed Scatchard analyses of [^{3}H]Tam binding in the absence or presence of two concentrations of α-CE (Fig. 1*B*), β-CE (Fig. 1*C*), and CT (Fig. 1*D*). For each experiment, a diminished slope of the lines indicated decreasing [^{3}H]Tam affinity, whereas the B_{max} remained unchanged. These data demonstrate that the substrates (α-CE and β-CE) and the product (CT) of ChEH are competitive ligands of the AEBS, with apparent K_i values of 66.5 ± 0.2 nM for α-CE, 171.3 ± 1.4 nM for β-CE, and 7.1 ± 0.6 μM for CT.

The Prototypical AEBS Ligands Tam and PBPE Are Inhibitors of ChEH. To test the effects of AEBS ligands on ChEH, we first measured ChEH activity by incubating rat liver microsomes with 20 μM [^{14}C]α-CE and separated it from CT by TLC. The migration of CE and CT was validated by reference to commercially available standards. As shown in the TLC autoradiogram (*SI Appendix*, Fig. S2*A*, *Left*), the conversion of α-CE to CT was time-dependent. Steady-state kinetics were apparent for the first 10 min of incubation (*SI Appendix*, Fig. S2*A*, *Right*). Consequently, all subsequent experiments were performed using a 9-min incubation period. The K_m was 7.4 ± 0.5 μM, and the V_{max} was 0.62 ± 0.01 nmol CT/mg protein/min (*SI Appendix*, Fig. S2*B*), consistent with data reported in the literature (14, 15). We then assessed ChEH activity, measured using [^{14}C]α-CE or [^{14}C]β-CE as the substrate, in the presence of known inhibitors (6-ketocholestanol and 7-ketocholestanol) (14), as well as in the presence of prototypical AEBS ligands (Tam and PBPE). The conversion of α-CE or β-CE to CT was inhibited by 10 μM 6-ketocholestanol or 7-ketocholestanol and by 1 μM Tam or PBPE (*SI Appendix*, Fig. S2*C*). Taken together, these results indicate that Tam and PBPE inhibit ChEH activity.

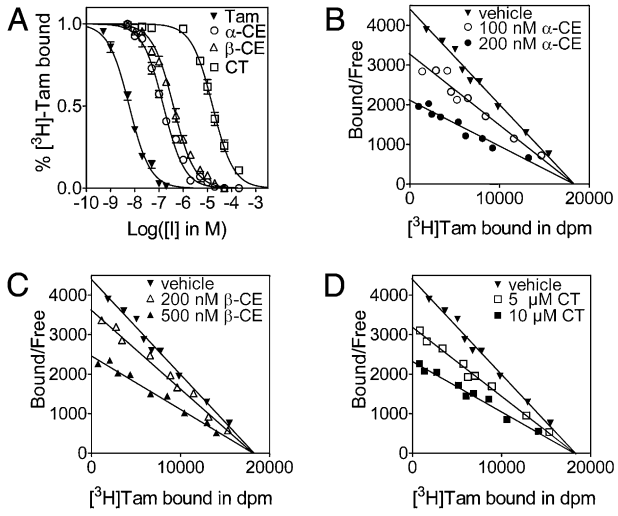

Fig. 1. Competition for [^{3}H]Tam binding to the microsomal AEBS by Tam, α-CE, β-CE, and CT. (*A*) Competition assays with increasing concentrations of unlabeled Tam (▼), α-CE (●), β-CE (▲), and CT (■) were performed on rat liver microsomes using 2.5 nM [^{3}H]Tam. (*B*) Scatchard plots of [^{3}H]Tam binding to the microsomal AEBS in the absence (▼) or presence of 100 nM α-CE (○) or 200 nM α-CE (●). (*C*) Scatchard plots of [^{3}H]Tam binding to the microsomal AEBS in the absence (▼) or presence of 200 nM β-CE (△) or 500 nM β-CE (▲). (*D*) Scatchard plots of [^{3}H]Tam binding to the microsomal AEBS in the absence (▼) or presence of 5 μM CT (□) or 10 μM CT (■). The lines intercept on the *x*-axis, indicating that α-CE, β-CE, and CT are competitive ligands of the AEBS with respect to Tam binding. Measurements were made in triplicate for at least three separate experiments. Data are presented as the mean ± SEM.

Tam and PBPE Are Competitive Inhibitors of ChEH. We next carried out experiments to determine the modality of ChEH inhibition by Tam and PBPE. Tam inhibited ChEH in a concentration-dependent manner, as shown by a double-reciprocal plot (Lineweaver-Burk) of the inhibition of [^{14}C]α-CE hydration by Tam in Fig. 2*A*. The *x*-intercept (i.e., the $1/K_m$ value) decreased, whereas the *y*-intercept (i.e., the V_{max} value) was not affected; these changes are characteristic of competitive inhibition. This was confirmed through a Dixon plot of the inverse of the velocity as a function of increasing Tam concentration in the presence of two concentrations of [^{14}C]α-CE (*SI Appendix*, Fig. S3*A*). The *x*-intercept gave a K_i value of 34 ± 8 nM for Tam. The same experiments carried out with PBPE, a selective AEBS ligand, showed competitive inhibition of ChEH, with a K_i value of 27 ± 6 nM (Fig. 2*B* and *SI Appendix*, Fig. S3*B*). These results indicate that Tam and PBPE are potent competitive inhibitors of ChEH activity.

Synthetic AEBS Ligands Are Inhibitors of ChEH. We tested the potency of AEBS ligands of different pharmacological classes to inhibit ChEH. All of the selective AEBS ligands (compounds 1–8; *SI Appendix*, Fig. S4*A*) inhibited ChEH activity (Table 1). The most potent inhibitors were PBPE, 1-{2-[4-(2-phenylpropan-2-yl) phenoxy]ethyl} pyrrolidine (PCPE), tesmilifene, 4-[2-(4-benzylphenoxy)ethyl]morpholine (MBPE), and 4-{2-[4-(2-phenylpropan-2-yl)phenoxy]ethyl} morpholine (MCPE) (5), with potencies in the nanomolar range, whereas 1-[4-(2-morpholino ethoxy)phenyl]-2-phenylethanone (MCOCH2PE) was the least potent, with a K_i value in the micromolar range for ChEH (Table 1). In the subsequent series, we found that SERMs (compounds 9–14; *SI Appendix*, Fig. S4*B*) inhibited ChEH

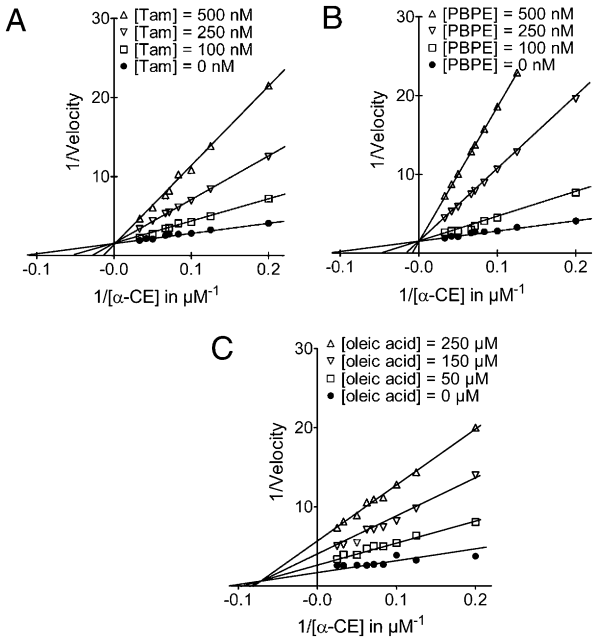

Fig. 2. Inhibition of ChEH by Tam, PBPE, and oleic acid. The relationship between the conversion rates of α-CE to CT and inhibitor concentrations is shown using 10 and 20 μM Tam and PBPE with rat liver microsomal ChEH. Shown are double reciprocal plots of Tam (*A*), PBPE (*B*), and oleic acid (*C*) versus [^{14}C]α-CE.

Table 1. Inhibition of [^{3}H]Tam binding to the AEBS and catalytic activity of ChEH by drugs

Compound		K_i AEBS, nM	K_i ChEH, nM
Selective AEBS ligands			
PBPE	1	9 ± 1	27 ± 6
PCPE	2	10 ± 1	35 ± 8
Tesmilifene	3	56 ± 2	62 ± 3
MBPE	4	18 ± 1	27 ± 6
MCPE	5	48 ± 2	57 ± 8
PCOPE	6	64 ± 4	203 ± 11
MCOPE	7	102 ± 16	241 ± 7
MCOCH2PE	8	850 ± 12	902 ± 13
SERMs			
Tamoxifen	9	2.5 ± 0.2	34 ± 8
4OH-Tamoxifen	10	11 ± 1	145 ± 4
Raloxifene	11	6 ± 1	36 ± 4
Nitromiphene	12	2.4 ± 0.3	18 ± 6
Clomiphene	13	1.5 ± 0.2	9 ± 2
RU 39,411	14	38 ± 1	155 ± 8
σ receptor ligands			
BD-1008	15	83 ± 1	99 ± 9
Haloperidol	16	5,322 ± 9	18,067 ± 14
SR-31747A	17	1.2 ± 0.1	6 ± 2
Ibogaine	18	920 ± 12	2,150 ± 11
AC-915	19	1,120 ± 8	3,527 ± 9
Rimcazole	20	640 ± 5	2,325 ± 8
Amiodarone	21	432 ± 22	733 ± 9
Trifluoroperazine	22	14 ± 2	135 ± 7
Cholesterol biosynthesis inhibitors			
Ro 48–8071	23	110 ± 4	89 ± 5
U-18666A	24	84 ± 2	90 ± 5
AY-9944	25	358 ± 12	649 ± 6
Triparanol	26	17 ± 2	39 ± 3
Terbinafine	27	3,720 ± 16	9,105 ± 33
SKF-525A	28	897 ± 10	1,904 ± 11
Ring B oxysterols			
6-Ketocholestanol	29	1,122 ± 30	2,251 ± 21
7-Ketocholestanol	30	580 ± 12	864 ± 22
7-Ketocholesterol	31	1,223 ± 31	4,212 ± 32
7α-Hydroxycholesterol	32	2,252 ± 32	6,151 ± 22
7β-Hydroxycholesterol	33	4,471 ± 22	6,941 ± 21
6-Keto-5α-hydroxycholestanol	34	5,320 ± 22	8,522 ± 12
Cholestane-3β,5α,6β-triol	35	7,131 ± 28	9,744 ± 11
Fatty acids			
Oleic acid	36	48,144 ± 19	54,235 ± 38
α-Linolenic acid	37	38,232 ± 41	36,341 ± 42
ARA	38	26,171 ± 17	24,094 ± 18
DHA	39	18,284 ± 19	12,111 ± 16

Rat liver microsomes were incubated with a single concentration of 2.5 nM [^{3}H]Tam and increasing concentrations of inhibitors ranging from 0.1 nM to 1,000 μM under the conditions described in *SI Appendix, SI Materials and Methods*. IC_{50} values were determined using the iterative curve-fitting program GraphPad Prism version 4 (GraphPad Software). For the AEBS, the apparent K_i was expressed as $K_i = [IC_{50}]/(1 + ([[^3H]Tam])/K_d)$, using 2.5 nM [^{3}H]Tam and a K_d of 2 nM. The K_i values of drugs for ChEH inhibition were determined using 150 μg of rat liver microsomal protein and 10 and 20 μM of [^{14}C]α-CE with increasing concentrations of inhibitors ranging from 0.01 to 1000 μM, under the conditions described in *SI Appendix, SI Materials and Methods*. K_i was measured as the projection on the *x*-axis of the intersection of the lines obtained from 1/V versus [inhibitor] plots for ChEH. Values are the average of three experiments ± SEM, each carried out in duplicate.

in the following order of potency: clomiphene > nitromiphene > Tam ≥ raloxifene > 4OH-tamoxifen ≥ RU 39,411 (Table 1). ER ligands with no affinity for the AEBS, such as 17β-estradiol, diethylstilbestrol, ICI-164,384, RU-56668, and ICI-182,780 (*SI Appendix*, Fig. S7), did not inhibit the ChEH at concentrations up to 10 μM (*SI Appendix*, Table S1). Of the σ-receptor ligands (*SI Appendix*, Fig. S5*A*), compounds 15–22 were both ligands of the AEBS and inhibitors of ChEH with the following order of potency: SR-31747A > BD-1008 > trifluoroperazine > amiodarone > ibogaine ≥ rimcazole > AC-915 > haloperidol (Table 1). SR-31747A had the highest affinity for the AEBS and was the most potent ChEH inhibitor of the compounds tested, with a K_i value of 1.2 ± 0.1 nM for the inhibition of Tam binding to the AEBS and a K_i value of 6 ± 2 nM for ChEH inhibition. Other σ-receptor ligands (compounds S6–S10; *SI Appendix*, Fig. S7), including ditolyl guanidine (DTG), (+)-pentazocine, (+)-3PPP, PRE-084, and progesterone, failed to bind to the AEBS and inhibit ChEH, even at concentrations up to 1,000 μM (*SI Appendix*, Table S1). In the last series of synthetic compounds, inhibitors of cholesterol biosynthesis already reported to be AEBS ligands (5) (compounds 23–28; *SI Appendix*, Fig. S5*A*), including U-18666A, triparanol, AY-9944, and SKF-525A, and newly identified AEBS ligands, such as terbinafine and Ro 48–8071, were inhibitors of ChEH (Table 1). Together, these results establish that every tested drug that bound to the AEBS was an inhibitor of ChEH.

Unesterified Ring B Oxysterols Are Inhibitors of ChEH. We next evaluated a set of oxysterols (*SI Appendix*, *SI Appendix*, Figs. S6 and S8). Ring B oxysterols (compounds 29–34; *SI Appendix*, Fig. S6) inhibited ChEH according to the following order of potency: 7-ketocholestanol > 6-ketocholestanol > 7-ketocholesterol > 7α-hydroxycholesterol > 7β-hydroxycholesterol > 6-keto-5α-hydroxycholestanol > CT (Table 1). In contrast, side-chain oxysterols (compounds S13–S16; *SI Appendix*, Fig. S8) did not inhibit ChEH activity or bind to the AEBS (*SI Appendix*, Table S1). Ring B oxysterols were previously shown to be competitive inhibitors of ChEH (14) as well as of Tam binding to the AEBS (8). In addition, the sulfate ester α-CE (S17) and the stearic acid ester of CE (S18) had no affinity for the AEBS and were not inhibitors of ChEH (*SI Appendix*, Table S1). Thus, unlike α-CE, esterified forms of α-CE are not substrates of ChEH. Our data indicate that unesterified ring B oxysterols are both inhibitors of ChEH and ligands of the AEBS, whereas side-chain oxysterols and esterified ring B oxysterols are not.

Unsaturated Fatty Acids That Are AEBS Ligands Are Inhibitors of ChEH. Because oleic acid is a noncompetitive ligand of the AEBS (20), we next studied whether oleic acid can inhibit ChEH activity, and analyzed the modality of its inhibition. Using Lineweaver-Burk analysis (Fig. 2*C*) and Dixon analysis (*SI Appendix*, Fig. S3*C*), we found that oleic acid is a noncompetitive inhibitor of ChEH, with a K_i value of 54 μM (Table 1). We extended this study by testing other fatty acids [compounds 36–39 (*SI Appendix*, Fig. S6) and S19–S21 (*SI Appendix*, Fig. S8)]. Unsaturated fatty acids, such as docosahexaenoic acid (DHA), α-linoleic acid, and arachidonic acid (ARA), are inhibitors of ChEH activity, whereas the saturated fatty acids stearic acid and palmitic acid and the methyl ester of oleic acid are not (*SI Appendix*, Table S1). These data indicate that unsaturated fatty acids are inhibitors of ChEH, and that oleic acid is a noncompetitive inhibitor.

Ligands' Affinity for the AEBS Positively Correlates with Their Inhibition of ChEH. Plotting the pK_i [-log(K_i)] of compounds that bound to the AEBS as a function of their pK_i values for their inhibitory potency of ChEH activity yielded a positive linear correlation between both parameters, with an r^2 value of 0.95 (n = 39; $P < 0.0001$) (Fig. 3). This demonstrates a clear correlation between the affinity for the AEBS and ChEH inhibition for the different classes of molecules.

D8D7I and DHCR7 Coexpression Allows the Reconstitution of ChEH. We previously reported that the coexpression of D8D7I and DHCR7 is necessary for reconstitution of the AEBS in mammalian COS-7 cells (5). We evaluated whether these two enzymes were involved in ChEH activity. As shown in Fig. 4*A*, the basal

activity of ChEH was low in COS-7 cells transfected with the vector control (mock). The single expression of D8D7I (D8) or DHCR7 (D7) in COS-7 cells induced a slight increase in ChEH activity (1.8- and 2.6-fold, respectively) compared with the vector control. In contrast, the coexpression of D8D7I and DHCR7 (D8 + D7) in COS-7 cells potentiated ChEH activity by 8.5-fold (Fig. 4*A*). A V_{max} value of 0.46 ± 0.04 nmol CT/mg protein/min and a K_m value of 4.47 ± 0.05 μM was measured in the D8D7I and DHCR7 coexpression experiments, compared with a V_{max} = 0.05 ± 0.002 nmol CT/mg protein/min and K_m = 7.01 ± 0.05 μM for the vector-only transfected cells (Fig. 4*B*). These data indicate that coexpression of D8D7I and DHCR7 led to the reconstitution of robust ChEH activity in mammalian cells, indicating that ChEH activity requires both enzymes. We tested the inhibition of ChEH by various AEBS ligands on COS-7 cell lysates that coexpressed D8D7I and DHCR7 (Fig. 4*C*). The order of potency of AEBS ligands for inhibiting the reconstituted ChEH was as follows: clomiphene (K_i = 32.3 ± 0.5 nM) > PBPE (K_i = 43.3 ± 0.4 nM) > Tam (K_i = 57.3 ± 0.6 nM) > 4OH-tamoxifen (K_i = 295.8 ± 0.8 nM) > 7-ketocholestanol (K_i = 711.6 ± 0.9 nM). 17β-Estradiol, which does not bind to the AEBS (*SI Appendix*, Table S1), did not inhibit the reconstituted ChEH. These data establish that the pharmacological profile obtained with the ChEH is similar to that of the AEBS (5).

Knockdown of D8D7I and DHCR7 Abolishes ChEH Activity. To confirm that D8D7I and DHCR7 both contribute to ChEH, we conducted knockdown experiments using siRNA against D8D7I and DHCR7 in a human breast adenocarcinoma cell line, MCF-7 cells. The siRNA specificities were evaluated at the mRNA and at the protein levels for the expression of D8D7I and DHCR7. The impact of knockdowns on the kinetic parameters of ChEH and on the binding parameters of [^{3}H]Tam to the AEBS was measured. MCF-7 cells expressed the AEBS (K_d = 5.2 ± 1.4 nM, B_{max} = 1,553 ± 25 fmol/mg proteins) (4), and ChEH activity was found, with a V_{max} value of 0.38 ± 0.07 nmol CT/mg protein/min and a K_m value of 5.91 ± 0.06 μM (Fig. 4 *G* and *H*). Transfection of the cells with D8D7I siRNA, but not with scrambled siRNA, led to decreased D8D7I expression at the mRNA level (72%) (Fig. 4*D*) and protein level (60%) (Fig. 4*E*). Interestingly, it also reduced ChEH activity by 47% (Fig. 4*F*), with V_{max} = 0.18 ± 0.09 nmol CT/mg protein/min, K_m = 3.87 ± 0.07 μM (Fig. 4*G*), and a 42% decrease in the amount of AEBS (K_d = 6.1 ± 0.4 nM, B_{max} = 897 ± 18 fmol/mg proteins) (Fig. 4*H*). Transfection of the cells with DHCR7 siRNA, but not with scrambled siRNA, decreased DHCR7 expression at the mRNA level (73%) (Fig. 4*D*) and protein level (64%) (Fig. 4*E*). Knockdown of DHCR7 increased the K_m value of ChEH by 66% (K_m = 17.22 μM) with no significant changes in the V_{max} value (Fig. 4*G*), and reduced the affinity of Tam for the AEBS with no changes in the B_{max} value (K_d = 7.2 ± 0.6 nM, B_{max} = 1,445 ± 23 fmol/mg proteins) (Fig. 4*H*). Finally, transfection of the cells with D8D7I siRNA and DHCR7 siRNA produced a comparable decrease in D8D7I and DHCR7 expression as in the single knockdown experiments, associated with a drastic reduction (92%) in ChEH activity (Fig. 4*F*) that was more than additive (V_m = 0.023 ± 0.06 nmol CT/mg protein/min, with no change in the K_m value (5.75 ± 0.07 μM) (Fig. 4*G*). A similar effect on the AEBS was observed, with a 93% decrease in the B_{max} value (103.5 ± 29 fmol/mg proteins) with no changes in the K_d value (Fig. 4*H*). These decreases are greater than expected based on the reduction in protein expression and strongly suggest that D8D7I and DHCR7 cooperate in the ChEH activity. These data further demonstrate that D8D7I and DHCR7 are involved in the ChEH activity.

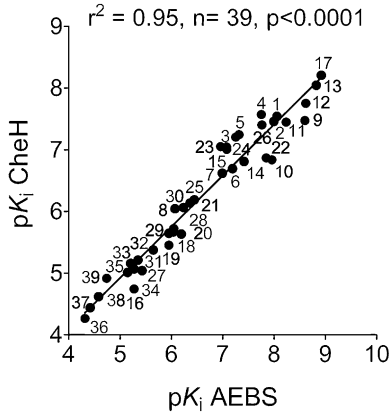

Fig. 3. Correlation between affinity of AEBS ligands for the AEBS and their potency to inhibit ChEH. Graph of the pK_i for 39 compounds tested for the inhibition of [^{3}H]Tam binding as a function of pK_i on ChEH activity. The drug numbers and the corresponding pK_i values [−log(K_i)] are listed in Table 1. Here *r* is the correlation coefficient between pK_i values calculated for the inhibition of Tam binding and ChEH activity. The r^2 value of 0.95 and significance of correlation (P < 0.0001) are given for all structural classes of compounds (n = 39).

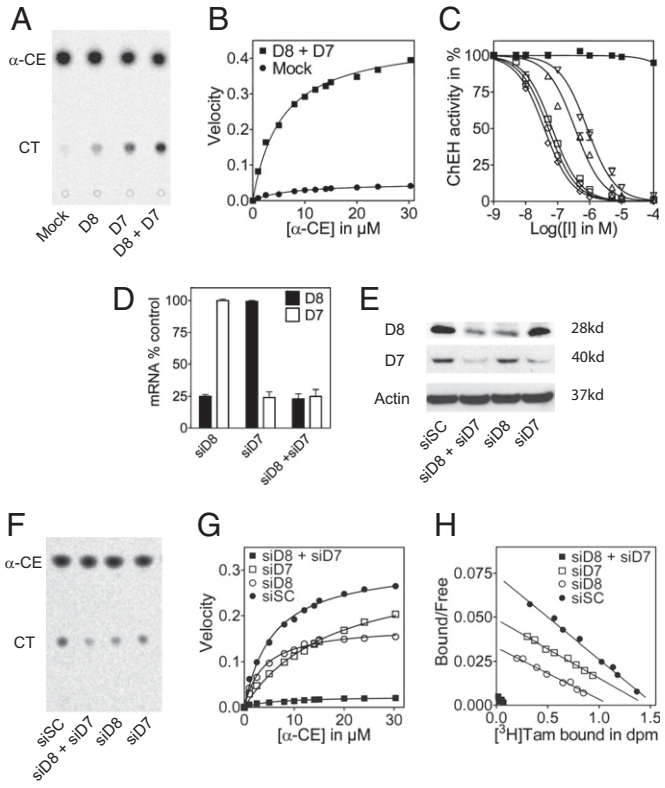

Fig. 4. Expression and knockdown of D8D7I and DHCR7 in mammalian cells: Impact on ChEH and AEBS activities. (*A*) ChEH activity of microsomal extracts from COS-7 cells transfected with control vector (mock), D8, D7, and D8 + D7. (*B*) Michaelis-Menten plot of velocity versus α-CE in ChEH assays from COS-7 cells transfected with mock (●) or D8 + D7 (■). (*C*) Inhibition of ChEH in microsomal extracts from COS-7 cells coexpressing human recombinant D8 and D7 with increasing concentrations of clomiphene (◇), PBPE (○), Tam (□), 4OH-tamoxifen (△), 7-ketocholestanol (▽), or 17β-estradiol (■). (*D*, *E*) Expression of D8 and D7 in MCF-7 cells transfected with siSC scrambled, siD8, siD7, or siD8 + siD7 at the mRNA level (*D*) and at the protein level (*E*). (*F*) Representative TLC autoradiogram showing ChEH activity in MCF-7 cells from three independent experiments. (*G*) Michaelis-Menten plot of velocity versus α-CE in ChEH assays from MCF-7 cells transfected with control scrambled siRNA (siSC; ●), siD8 (○), siD7 (□), or siD8 + siD7 (■). (*I*) Scatchard plots of [^{3}H]Tam binding to microsomal AEBS from MCF-7 cells transfected with siSC (●), siD8 (○), siD7 (□), or siD8 + siD7 (■). Measurements were made in triplicate for at least three separate experiments. Data are presented as mean ± SEM.

Discussion

The present study provides evidence that the AEBS and ChEH are pharmacologically and molecularly related. We have shown that substrates (CEs) and the product (CT) of ChEH are competitive inhibitors of Tam binding to the AEBS, and have established that ChEH is inhibited by all AEBS ligands tested, demonstrating that inhibition of ChEH is a hallmark of AEBS ligands. We found that different structural classes of AEBS ligands inhibit ChEH with the same modality as they inhibit [^{3}H]Tam binding to the AEBS. We established the pharmacological similarities by showing a positive correlation between inhibition of ChEH activity and affinity of compounds for the AEBS. Importantly, compounds belonging to different pharmacological or biochemical classes that had no affinity for the AEBS did not inhibit ChEH. With the series of synthetic compounds, the arylaminoalkyl structure was not sufficient for ChEH inhibition; such compounds as (+)-3-PPP, (+)-pentazocine, PRE-084, and MCH_3PE did not inhibit ChEH or compete with Tam for binding to the AEBS.

As shown previously for reconstitution of the AEBS (5), transient coexpression of D8D7I and DHCR7 in COS-7 cells led to the reconstitution of ChEH. We confirmed the involvement of D8D7I and DHCR7 in ChEH through knockdown experiments using siRNA directed against D8D7I and DHCR7. These data show that both D8D7I and DHCR7 are required for the dual reconstitution of ChEH and the AEBS and provide more evidence of the molecular nature of the ChEH. The fact that our single knockdown experiments showed that D8D7I and DHCR7 affect the kinetic parameters of ChEH differently suggests that D8D7I carries the catalytic activity, and that DHCR7 cooperates in the binding of substrates to ChEH (Fig. 5). We find it interesting that three epoxide hydrolases in three unrelated structural classes have such high structural complexity. The leukotriene A4 hydrolase/aminopeptidase (21) and the soluble epoxide hydrolase/phosphatase (22) are both bifunctional enzymes. Our data suggest even greater complexity with ChEH in being bifunctional and composed of two independent gene products that unite in the microsomes to create a functional protein.

Here we have shown that anticancer drugs of clinical interest are inhibitors of ChEH at pharmacologically and therapeutically active concentrations. In vitro studies on breast cancer cells have previously used 1- to 40-μM concentrations of selective AEBS ligands, SERMS, or σ-receptor ligands (3, 4, 7) and 10–100 μM polyunsaturated fatty acids (23). The present study has demonstrated the existence of ChEH activity in MCF-7 cells, and thus ChEH inhibition and CE accumulation are likely to play a role in the mechanism of induction of breast cancer cell differentiation and apoptosis by AEBS ligands that require sterol autoxidation products. The therapeutic plasma concentration of Tam is 1–10 μM (24), and that of tesmilifene is 5 μM (25), whereas their respective K_i values for ChEH are 33 nM and 26 nM, indicating total inhibition of ChEH at therapeutic concentrations. The plasma concentration of nonesterified DHA is 2–10 μM in humans receiving a diet supplemented with 1.5 g DHA/d (26), whereas the DHA K_i value for the AEBS is 12.1 μM, indicating possible inhibition of ChEH. Thus, the accumulation of CEs is likely to be part of the pharmacologic action of these compounds and might be involved in the anticancer and chemopreventive actions of SERMs and DHA; this merits further investigation.

CEs are autoxidation products of cholesterol, and their production can be blocked by lipophilic antioxidants such as vitamin E (27). We recently established that AEBS ligands induce differentiation and apoptosis in breast cancer cells through a mechanism involving the production of sterol autoxidation products, and that vitamin E inhibits these effects (3, 4, 12). Consistent with these data, previous reports have shown a limited clinical outcome with Tam treatment when cholesterol metabolism enzymes (28) or antioxidant enzymes (29) are overexpressed in breast tumors. These data, along with our present findings, suggest that the modulation of CE metabolism resulting from ChEH inhibition by AEBS ligands might be involved in these effects. Further investigation is warranted. In particular, it will be interesting to study the relationship that may exist between CE metabolism and the sensitivity of and resistance to Tam of breast cancer cell lines and to define how accumulation of CEs or the products of their transformation might be involved in the effects of AEBS ligands.

The physiological function of ChEH has been proposed to be involved in the control of lipid metabolism (16) based on the biological properties of CT, not for detoxification as initially proposed (14). As opposed to toxic aliphatic and aromatic epoxides, which can spontaneously alkylate proteins and nucleic acids in association with their cytotoxicity or carcinogenicity, CEs are stable (30) and are less toxic than CT (13, 31) and nontumorigenic substances (32). CT's greater toxicity and the mutagenic nature of its oxidation products (33, 34) suggest that the inhibition of ChEH and the inhibition of CT production protects cells against cytotoxic insults.

Several other lines of evidence point to the existence of a dynamic metabolism centered on CEs. α-CE is the only epimer of CE found in the adrenal cortex, where it is produced by an as-yet-unidentified cytochrome p450 (35). α-CE can be esterified by Sult2B1b (36) to give a 3β-sulfated product that antagonizes liver X-receptor sig-

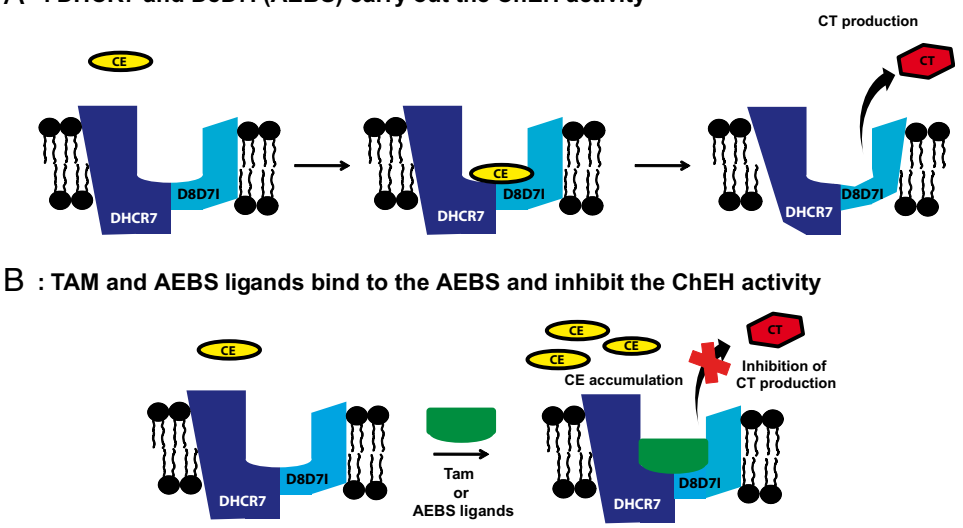

Fig. 5. Functional relationship between the AEBS and ChEH. (*A*) The subunits of the AEBS that bind Tam (D8D7I and DHCR7) carry out the ChEH activity. (*B*) Ligands of the AEBS, such as cationic SERMs (Tam and raloxifene), diphenylmethane compounds (tesmilifene and PBPE), ring B oxysterols (7-ketocholesterol and 7-hydroxycholesterol) and polyunsaturated fatty acids (DHA), are inhibitors of ChEH, leading to the blockage of CT production and CE accumulation.

naling (37, 38), or it can be transformed by glutathione transferase into 3β,5α-dihydroxycholestan-6β-yl-S-glutathione (39, 40). In addition, we have reported that the aminolysis of α-CE by biogenic amines under catalytic conditions is possible and generates powerful cell-differentiating alkylaminooxysterols (30). In contrast, β-CE is nonreactive even under catalytic conditions (30) and has been reported to accumulate in breast fluids (41) and in the plasma of endometrial cancer patients (42). Thus, it would be interesting to study whether β-CE can deregulate CE metabolism at the level of the epoxidation step or of CE metabolism, including ChEH, leading to the appearance of toxic CT.

In conclusion, these data shed light on the molecular nature and potential functions of ChEH and open up the existence of an active metabolic pathway at the level of the CEs.

Materials and Methods

Descriptions of chemical synthesis, AEBS binding, cell culture, and transfections are provided in *SI Appendix, SI Materials and Methods.*

ChEH Activity Assays. Rat liver microsomes were prepared as described previously (43), and ChEH activity was assayed as described previously (14). Drugs and [^{14}C]α-CE were dissolved in acetonitrile for the biological tests. The concentration of [^{14}C]α-CE in the test tubes was 10 or 20 μM for the Dixon analyses and 5, 10, 20, 30, or 40 μM for the Lineweaver-Burk analyses. The maximal velocity (V_{max}) and Michaelis constant (K_m) were determined by nonlinear regression analysis using GraphPad Prism version 4.01 for Windows (GraphPad Software). One-way ANOVA with Dunnett's multiple-comparison posttest was performed with vector-only cells as the control using GraphPad Prism 4.01.

ACKNOWLEDGMENTS. We thank Dr. Michel Record for his critical reading of the manuscript. This work was funded by Institut National de la Santé et de la Recherche Médicale Conseil Regional Midi-Pyrénées, the Institut National du Cancer through the ResisTH network, the Ministère Français de la Recherche et de l'Enseignement Supérieur through the GenHomme project and a predoctoral fellowship (G.S.), Affichem, and European Commission FP6 Integrated Project EuroHear (LSHG CT-2004-512063A). S.S.-P. is in charge of research at the Centre National de la Recherche Scientifique.

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