Effect of Guggulsterone and Cembranoids of *Commiphora mukul* on Pancreatic Phospholipase A_2: Role in Hypocholesterolemia

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Guggulsterone (**7**) and cembranoids (**8**−**12**) from *Commiphora mukul* stem bark resin guggul were shown to be specific modulators of two independent sites that are also modulated by bile salts (**1**−**6**) to control cholesterol absorption and catabolism. Guggulsterone (**7**) antagonized the chenodeoxycholic acid (**3**)-activated nuclear farnesoid X receptor (FXR), which regulates cholesterol metabolism in the liver. The cembranoids did not show a noticeable effect on FXR, but lowered the cholate (**1**)-activated rate of human pancreatic IB phospholipase A2 (hPLA2), which controls gastrointestinal absorption of fat and cholesterol. Analysis of the data using a kinetic model has suggested an allosteric mechanism for the rate increase of hPLA2 by cholate and also for the rate-lowering effect by certain bile salts or cembranoids on the cholate-activated hPLA2 hydrolysis of phosphatidylcholine vesicles. The allosteric inhibition of PLA2 by certain bile salts and cembranoids showed some structural specificity. Biophysical studies also showed specific interaction of the bile salts with the interface-bound cholate-activated PLA2. Since cholesterol homeostasis in mammals is regulated by FXR in the liver for metabolism and by PLA2 in the intestine for absorption, modulation of PLA2 and FXR by bile acids and selected guggul components suggests novel possibilities for hypolipidemic and hypocholesterolemic therapies.

Cholesterol homeostasis involves metabolism as well as absorption and excretion. Among the various systemic signal targets of bile salts is the nuclear farnesoid X receptor (FXR), which regulates cholesterol catabolism and bile salt recycling in the liver.[1] Bile salts also show extrasystemic effects. For example, significant structural specificity of bile salts (**1**−**6**) on pig pancreatic IB phospholipase A_2 (pPLA2) activity[2] parallels the effect on gastrointestinal absorption of cholesterol. Thus, cholate (**1**) enhances PLA2 activity and the cholesterol absorption, whereas chenodeoxycholate (**3**) inhibits both.[3−5] Moreover, inhibitors of pPLA2 increase excretion of cholesterol and fat and decrease their gastrointestinal uptake.[6−9] The pancreatic PLA2 rate-lowering effect of **3** depends on the 62−66 loop that is found only in the pancreatic IB PLA2, which possibly evolved for bile salt-mediated processing and uptake of dietary fat emulsion and absorption of cholesterol.[2]

Guggul, the bark resin of *Commiphora mukul* (Arn.) Bhandari (Burseraceae), is known for its hypolipidemic and hypocholesteremic effects with collateral benefits against inflammation and obesity.[10−12] In this paper, we characterize the effect of guggulsterone (**7**) and cembranoids (**8**−**12**) from guggul and other sources (**13**−**16**) on bile salt-regulated PLA2 and FXR activities. This investigation used a kinetic model to study the rate-lowering effects of guggul (**8**−**12**), marine (**13**, **14**), and tobacco (**15**, **16**) cembranoids on the cholate-activated hPLA2 hydrolysis of phosphatidylcholine vesicles and to compare their effects with that of bile salts (**2**−**6**). Cembranoids are natural diterpenes possessing a 14-membered macrocyclic ring substituted by an isopropyl residue at C-1 and by three symmetrically disposed methyl groups at positions C-4, C-8, and C-12. Cembren-1-ol (**8**) and certain other cembranoids (**9**−**13**) were found to lower with significant structural specificity the cholate-activated rate of hydrolysis by human pancreatic IB phospholipase A2 (hPLA2). Also guggulsterone is shown to be an antagonist of the **3**-activated nuclear farnesoid X receptor (FXR), which regulates cholesterol catabolism. Since guggul is believed to lower cholesterol and fat uptake,[10,12,13] the distinct effects of the guggul constituents on hPLA2 and FXR observed in this study raise the possibility that such modulation may be beneficial via the bile salt-modulated hypolipidemic and hypocholesterolemic effects.

Results and Discussion

Kinetic Effect of Test Compound on the hPLA2-Catalyzed Rate. The effects of 0.03 and 0.007 mol fraction (MF) test compound on the cholate-activated hPLA2-catalyzed rate of hydrolysis of 1,2-dimyristoyl-*sn*-3-glycerophosphocholine (DMPC) vesicles is summarized in Table 1. Both bile salts (**1**−**6**) and cembranoids (**8**−**16**) showed significant structural specificity. Tauroconjugates of **3** and **4** were the most effective in rate lowering, and among the tauroconjugates, there was a noticeable effect of the position and orientation of the hydroxyl substituent on the steroid nucleus (Table 1). The cembranoids showed significant difference in their efficacy for lowering hPLA2 activity. The rank order **8**, **11**, **12**, **13**, **10**, and **9** for decreasing effectiveness suggested that the hPLA2 rate-lowering activity depends on the position of the hydroxyl substituent, and the polarity of the molecule may also be a contributing factor. The marine and tobacco cembranoids **13**−**16** possess different oxygenation and functional group pattern compared to the guggul cembranoids **8**−**12**. The most active of the guggul cembranoids were nearly as active as 2-*epi*-16-deoxysarcophine (**13**) from the coral *Sarcophyton glaucum.* On the other hand, sarcophine (**14**) and the tobacco cembranoids (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (**15**) and (1*S*,2*E*,4*R*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (**16**) had little effect on hPLA2 activity. It appears that the segment C-2 to C-9 of the cembrene skeleton is important for the hPLA2 rate-lowering effect among these compounds.

The effects of cembren-1-ol (**8**) and tauro-**4** on hPLA2 were further characterized in detail. As modeled in Scheme 1 and shown elsewhere,[2] the cholate-activated rate is sensitive to

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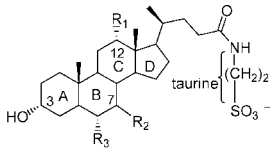
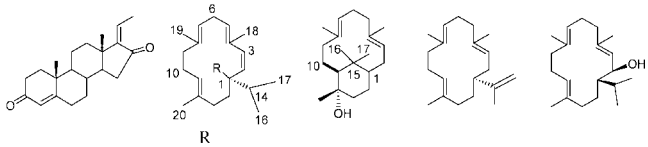
Table 1. Effect of Test Compounds on FXR Agonist and Antagonist Activity[a]

test compound	hPLA2	hPLA2	FXR agonist	FXR antagonist
cholic acid (**1**)	95	95	3.81	103.2
tauro-**1**	80	90		
tauro-**2**	20	46	(8.70)	(91.5)
tauro-**3**	5	25	(17.05)	(100)
tauro-**4**	5	15	(1.53)	(87.2)
tauro-**5**	75	90		
tauro-**6**	40	70	(5.29)	(98.4)
Z-guggulsterone (**7**)	35	75	3.89	166
cembren-1-ol (**8**)	5	40	1.34	81.7
(+)-cembrene (**9**)	30	75		
verticilla-3,7-diene-12-ol (**10**)	24	60		
cembrene A (**11**)	5	45		
mukulol (**12**)	5	30		
2-*epi*-16-deoxysarcophine (**13**)	8	25	1.38	79.5
sarcophine (**14**)	95	95	1.18	86
15	90	95		
16	95	95		
ezetimibe	33	82	1.1	94

[a] Uncertainty in these values is up to 10%. Relative **1**-activated hPLA2 activities (100%) were measured with 0.03 MF (in column 2) or 0.007 MF (in column 3) test compound. FXR activities were measured in the presence of 10 μM concentration of the test compound. Agonist activities are expressed relative to the carrier control (=1), and antagonist activity is relative (=100) to **3**. Activities given in parentheses are for the unconjugated bile salts. Tauro-conjugates of bile acids are abbreviated with the tauro- prefix.

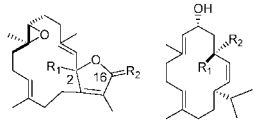


Bile acid	R_1	R_2	R_3
Cholate (**1**)	OH	α-OH	H
Deoxycholate (**2**)	OH	H	H
Chenodeoxycholic acid (**3**)	H	α-OH	H
Ursodeoxycholic acid (**4**)	H	β-OH	H
Hyodeoxycholic acid (**5**)	OH	H	OH
Lithocholic acid (**6**)	H	H	H



R

7 **8** OH **9** H **10** **11** **12**



	R_1	R_2		R_1	R_2
13	α-H	H_2	**15**	OH	CH_3
14	β-H	O	**16**	CH_3	OH

changes in the state of the interface-bound pPLA2 induced by the test compound without binding to the catalytic site. The results in Figure 1A showed an 8-fold increase in the hPLA2-catalyzed rate that saturated at 0.08 MF cholate (**1**). By comparison, the peak rate was only 1.4 -fold higher with tauro-**4**, which decreased to near zero at 0.08 MF. The activation of hPLA2 with **1** was a little weaker and with a smaller Hill number, $n_a = 3-4$, compared with pPLA2 (Scheme 1).[2] The inhibition in the tauro-**4** case is fitted with Hill number $n = 1.5$, which is comparable to the value for the pPLA2 with tauro-**3**.

Cembren-1-ol (**8**) did not show a noticeable rate increase with hPLA2 in the absence of **1**. However, as shown in Figure 1B, tauro-**4** and **8** exhibited a concentration-dependent decrease of the **1**-activated rate. The fits in terms of the model in Scheme 1 suggested that the rate-lowering effect occurred by interference with the interfacial Michaelis complex E_a*S activated by **1**. Thus, E* would bind n_a (=3−4) **1** amphiphiles to form the activated complex E_a*, and then E_a* would bind $n = 1-2$ molecules of the rate-lowering test compound I (such as cembrenol) to form an inactive complex E_a*I_n. The rate in the presence of I was

$$v_I = \frac{k^*_{cat} X^*_S}{K^*_m[1 + (X^*_I/K^*_I)^n] + X^*_S} \quad (1)$$

And in the absence of I, the rate was

$$v_0 = \frac{k^*_{cat}}{K^*_m + 1} \quad (2)$$

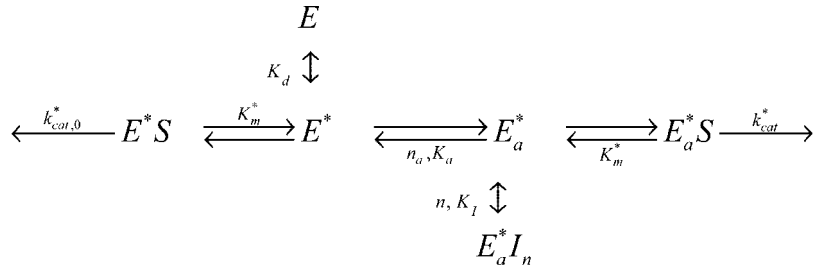
This was the simplest possible kinetic model for the rate-lowering effect monitored with **1**-containing zwitterionic vesicles where the enzyme was bound to the interface and where the partitioning of **1** and other test compounds is ideal.[2] Several other models were also considered. For example, the evidence presented above and the results described below ruled out the rate-lowering effect being due to the desorption of PLA2 from the interface. Binding of **1**, tauro-**3**, and **8** to the active site of PLA2 was also ruled out by the fact that these rate-lowering compounds did not protect catalytic histidine from alkylation.

Our results support the hypothesis that the hPLA2 rate-lowering test compound (I) interferes with the allosteric activation of the enzyme by **1** at the interface. The fit parameters in the legend to Figure 1B are based on the formation of an E_a*I_n complex that is catalytically inert. The K_I* values are apparent inhibition constants. Besides a modest difference between the K_I* values for tauro-**4** and cembren-1-ol (**8**), there was a difference in apparent cooperativity. The model includes the binding of I to the enzyme at the interface and also as a diluent in the interface. Binding of the substrate and the inhibitor are assumed to be mutually exclusive even if the inhibitor did not bind in the active site. Also, the model in Scheme 1 includes the effect of I only on the enzyme parameters such as K_d, K_m*, or k_{cat}* for E_a*, but we could not distinguish the model where I also binds to E_a*S and blocked catalysis.

Binding of Bile Salts to PLA2 Is Specific but Not to the Vesicles. The difference in the kinetic effect of bile salts (Table 1) was attributed to their effects on the enzyme bound to the interface (Scheme 1). Such interfacial complexes cannot be characterized by the available methods. However, the results described below show that in the aqueous phase specific binding of bile salt to PLA2 correlates qualitatively with the interfacial kinetic effects. As shown before for pPLA2,[2] quenching of the resonance energy transfer signal from the hPLA2 + trimethyl-ammonium-diphenylhexatriene complex by bile salts has a K_d of 10 μM for tauro-**4** compared to >150 μM for **1** (results not shown), which is consistent with the kinetic results.

The saturation transfer difference (STD) NMR spectra a and b in Figure 2A of tauro-**3** and **1** bound to pPLA2 are different, and similar results were obtained with hPLA2 (not shown). The STD signatures of the ^{1}H NMR spectroscopic intensities provided an averaged measure of the proton−proton interactions of the test compound with the protein in the complex.[14] The differences in the spectra a and b in Figure 2A showed that the affinity of pPLA2 for tauro-**3** is high compared to **1** and that the epitope for the bile salt ring protons in the complex were noticeably different. Such

Scheme 1. Kinetic Model for the Biphasic Activation/Inhibition of PLA2 by Bile Salts[a]



[a] E_a* is the interface-activated form bound with a number (n_a) of bile salt molecules. E_a*S is the activated complex with substrate at the active site, and E_a*I_n is the inhibited complex with n copies of the bound rate-lowering test compound (I). The analysis in this paper assumes that the unactivated rate $k^*_{cat,0}$ is negligible. The model was fully developed before to account for the effects of bile salts on the pPLA2-catalyzed rates.[2]

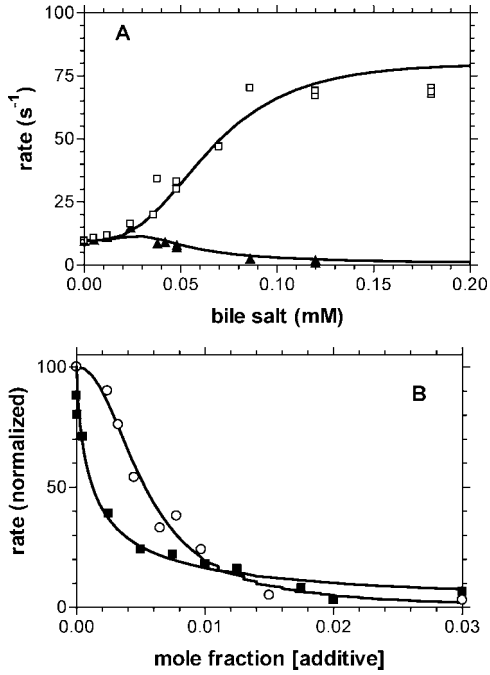


Figure 1. (A) Effect of cholate versus tauro-UDCA. The rate of hydrolysis of 1 mM DMPC vesicles by hPLA2 was monitored in the presence of added (squares) cholate or (triangles) tauro-ursodeoxycholate (tauro-**4**). The fitted curve is obtained with the model in Scheme 1[2] with partition coefficient $K' = 0.1$ MF, $K_d = 3$ mM, k^*_{cat}(from E*S) = 35 s^{-1}, k^*_{cat}(from E_a*S) = 150 s^{-1}, and $K_I^* > 0.5$ MF for cholate and 0.005 MF for tauro-UDCA. (B) Rate lowering effect of tauro-**4** and cembren-1-ol (**8**). The rate of hPLA2-catalyzed hydrolysis of 1 mM DMPC + 0.05 mM cholate in 2 mM $CaCl_2$ at pH 8 was monitored in the presence of (squares) tauro-**4** or (circles) cembren-1-ol (**8**). The fitted line is based on Scheme 1 assuming $K_m^* = 0.65$ MF. The best fit for TUDOC is for Hill number $n = 0.78$ and $K_I^* = 0.000306$ MF, and the best fit for cembrenol is with $n = 2.2$ and $K_I^* = 0.00325$ MF.

differences were not apparent in the crystal structure of pPLA2 complexes with bile salts.[15] Altogether, the kinetic and biophysical behavior of pPLA2 and hPLA2 was qualitatively similar, and the quantitative difference was analytically discernible. Also, biophysical results supported the kinetic measurements and were consistent with Scheme 1, where the effect of the rate-lowering solutes was due to their interaction with the interface-bound enzyme.

The STD signatures of tauro-**3** and **1** in DMPC vesicles were similar (Figure 2A, spectra c and d), which suggested that their partitioning in the bilayer is not noticeably different. These spectra were observed at high bile salt concentrations, where the difference between their kinetic effects on the PLA2-catalyzed reaction is large (Figure 1A). Also, all the proton peaks were observed, which suggests that contacts of **1** and tauro-**3** in DMPC vesicles are comparable. Results (not shown) for other bile salts also showed that the hydrophobicity or the number and positions of hydroxyl groups had little effect on their partitioning epitope in DMPC vesicles. These controls rule out the kinetic specificity of bile salts being due to their interaction with DMPC vesicles.

The low solubility of guggul cembranoids in water precluded determination of STD epitopes. However, the results in Figure 2B (a versus b) showed that **8** changes the STD signatures of tauro-**2** bound to hPLA2. As also suggested by a weaker rate-lowering effect of tauro-**2** (Table 1), its apparent K_d was about 30 μM for the complex with hPLA2. In the presence of 5 μM cembren-1-ol (**8**), the STD signal intensities from tauro-**2** protons were somewhat weaker, and the epitope of the taurine and ring protons was also less prominent. When taken together, the results obtained showed that the STD epitopes of the rate-lowering bile salts bound to hPLA2 or pPLA2 were more pronounced than those of the rate-activating bile salts.

PLA2 and Cholesterol Adsorption in the Intestine. Details of the gastrointestinal fat uptake remain to be settled. Specific interaction of bile salts with the pancreatic PLA2 is likely to influence gastrointestinal function, where about 0.5 g (2 mM) of bile salt is also secreted per day. This amount of bile salt is not sufficient to micellize 60 g (100 mM) of triglyceride and 5 g (6 mM) of phospholipid in a healthy diet. Our estimate is that the mole ratio of bile salts in the phospholipid and cholesterol monolayer on the surface of the dietary emulsion with a core of triacylglycerol and cholesterol esters would be in the same range as in our PLA2 assay mixture (Figure 1). Thus, rate-activating and -lowering effects of different bile salts and conjugates would regulate the PLA2-catalyzed changes in the phospholipid monolayer, which is likely to influence the size of the emulsion particles for absorption and excretion. Also, the PLA2 inhibitors, schniol and masticadienoic acid, from the pink peppercorn,[16] the berries of *Schinus terebinthifolius* used as a spice in butter sauce, may also have a similar effect on fat and cholesterol uptake. It is also intriguing and possibly not unrelated that nonpolar cembranoids are found in the saliva of the crocodile[17] and the frontal gland secretion of termites.[18] When taken together, the results with PLA2 suggest that the pancreatic enzyme could be a viable target for the extrasystemic regulation of fat and cholesterol absorption in intestine and secretion.

Significance of the Interactive Roles of FXR and PLA2 in Cholesterol Homeostasis. Bile salts exhibit specific effects in the two assays (Table 1) used. In the FXR assay, unconjugated bile salts were used because they readily cross the cell membrane. Chenodeoxycholate (**3**) is the most potent agonist of FXR (column 4), followed by **2** and **1**. This rank ordering is consistent with published results.[19−21] Humans produce half a dozen bile salts, and **1** and **3** are the two major representatives.[22] About 10% of the gastrointestinal bile salts are excreted, and 90% are recycled to the liver. Feeding **1** increases gastrointestinal uptake of cholesterol, whereas feeding **3** or **4** inhibits cholesterol absorption.[3−5] As a potent FXR agonist, **3** represses bile acid synthesis. Also cholesterol

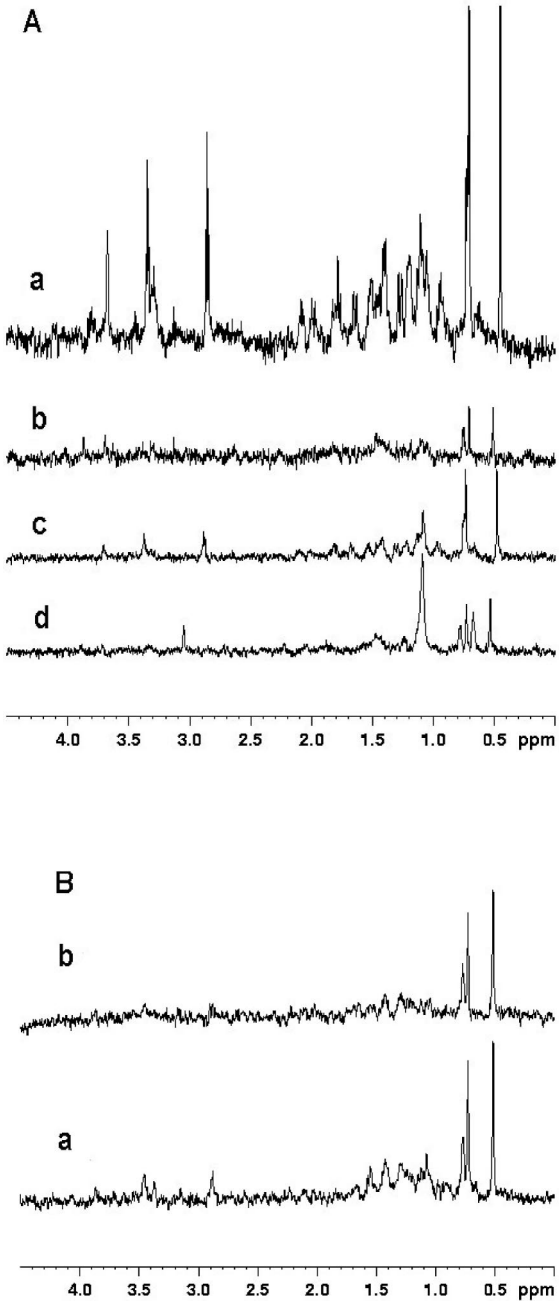


Figure 2. (A) Saturation transfer difference (STD) NMR spectra at 15 °C in 20 mM bicarbonate buffer in D_2O (99%) at pH 7.5 of (a) 100 μM tauro-**3** in the presence of 7 μM pPLA2; (b) 100 μM **1** in the presence of 7 μM pPLA2; (c) 150 μM tauro-**3** in the presence of 10 μM DMPC; (d) 150 μM **1** in the presence of 10 μM DMPC. (a) and (b) have the same scaling factor, and (c) and (d) have the same settings. Two singlets below 1 ppm are from the geminal methyl. The taurine protons are at 3 to 4 ppm, and the ring protons are between 1 to 2 ppm. (B) Saturation transfer difference NMR spectra: (a) 100 μM tauro-**2** with 5 μM hPLA2; (b) 100 μM tauro-**2** with 5 μM hPLA2 + 5 μM cembren-1-ol (**8**).

feeding increases production of **3**, whereas the synthesis of **1** is not influenced.[23] Such changes in the **1** to **3** ratio could determine the extent of cholesterol catabolism in the liver and absorption in intestine.

As specific targets for small molecules, systemic FXR and extrasystemic PLA2 offer two well-separated control points to regulate cholesterol homeostasis. A role for bile salts in cholesterol homeostasis is suggested by structural specificity of their effects on such targets. Also as compared in Table 1, certain cembranoids (Table 1) specifically lowered the cholate-activated PLA2-catalyzed rate, whereas guggulsterone (**7**) is an FXR antagonist. On the other hand, ezetimibe, which is prescribed to lower cholesterol absorption, and cembranoids **8** and **13** showed no noticeable FXR agonistic or antagonistic effects. However, the FXR antagonist guggulsterone (**7**) enhanced bile salt efflux pump promoter transactivation through an FXR-independent mechanism, and it may also influence transcription of enzymes involved in bile salt synthesis.

When taken in combination, the results at hand showed that bile salts and guggul components may coordinately regulate cholesterol homeostasis both in the intestine for absorption and in the liver for catabolism. These kinetic consequences of the chenodeoxycholate (**3**)/cholate (**1**) ratio on PLA2-catalyzed hydrolysis influenced by cembranoids, and also the effect of guggulsterone (**7**) and bile salts on FXR in the liver, could comodulate cholesterol and fat homeostasis in a complementary fashion. Therapeutic drugs targeting cholesterol biosynthesis and absorption have been developed. Among these, statins decrease cholesterol biosynthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase in the liver. A meta-analysis has shown that, compared to the mortality of 18.7% in the control group, statins reduce all-cause mortality in the over 65 group to 15.6% over 5 years.[24] Since all the test subjects are exposed to statins and due to the reported side effects of the long-term use of statins,[25] therapies with better safety profiles are needed for the preventive control of cholesterol levels. However, if guggul exerts a hypolipidemic effect through the combined effects of cembranoids (**8**−**12**) on PLA2 and of guggulsterone (**7**) on FXR, the pharmacological potential of such comodulation of cholesterol homeostasis would be promising.

Experimental Section

Cembranoids and Other Compounds. Bile salts (**1**−**6**) were obtained from Sigma. *Z*-Guggulsterone (**7**) and cembranoids (**8**−**12**) were isolated from a hexane extract of guggul fractionated by monitoring the PLA2 rate-lowering activity,[11] 2-*epi*-16-deoxysarcophine (**13**) and sarcophine (**14**) from the Red Sea soft coral *Sarcophyton glaucum*,[26] and (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol) (**15**) and (1*S*,2*E*,4*R*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (**16**) from tobacco (*Nicotiana tabacum*) leaf.[27] Identity of these compounds was based on extensive analysis of their spectral and chromatographic data and comparison with literature.[11,26,27] Purity of the baseline-separated cembranoids obtained by high-pressure liquid chromatography was estimated to be >95% from their NMR spectra.

Assay for the Rate-Lowering Effect of Test Compounds on hPLA2-Catalyzed Hydrolysis of 1,2-Dimyristoyl-*sn*-3-glycerophosphocholine (DMPC) Vesicles. Protocols to characterize kinetic and spectroscopic effects of bile salts on pPLA2[2] were adopted for the effects of test compounds on hPLA2 recombinantly expressed in *E. coli*.[28] Briefly, reaction progress initiated with 2 to 10 pM hPLA2 was measured using a pH-stat titration (Radiometer PHM titrator) with 1 mM 2-aminopropanediol base added to 4 mL of reaction mixture in the nitrogen-purged thermostated glass reaction vessel, containing sonicated vesicles of 1 mM DMPC, 5 mM $CaCl_2$, 1 mM NaCl, and 0.1 mM EDTA at pH 8.0 and 24 ± 0.1 °C. Progress curves with or without added bile salts are complex, and the preburst region of the minimum slope during the delay was used for monitoring the rate-lowering effect.[2,29] Sequential and replicate points in Figures 1A and 1B show ranges typically within 95% of the calculated parameters. The overall statistical uncertainty in the reported parameters was estimated to be less than 10%.

Saturation Transfer Difference (STD) NMR Measurements. Measurements were carried out on a Bruker spectrometer operating at 600.13 MHz and equipped with a triple resonance CryoProbe under conditions given in the legend of Figure 2. As established in our laboratory,[14] the ^{1}H NMR resonance range for hPLA2 is −0.2 to 9.5 ppm, and 0.5 to 5 ppm for bile salts. The pulse sequence and other details for the STD spectra were optimized for the amphiphile binding to PLA2. Typically, a train of Gaussian-shaped rf saturation pulses (50 ms with irradiation power of 87 Hz) was used to saturate nuclear magnetization of the protein resonances. The saturation time of 0.5 s

was used unless noted otherwise. The rf saturation pulse train was followed by a hard 90° pulse, a $T_{1\rho}$ filter with strength of 4960 Hz (40 ms) for removing residual protein signals, and a Watergate sequence to suppress the solvent signal. The frequency of the Gaussian pulse train was set to the off-resonance frequency at 30 ppm and the on-resonance frequency at 6.7 ppm unless noted otherwise. The STD-NMR signal is the difference between the signals with on- and off-resonance irradiation. The subtraction was carried out by a phase-cycling scheme. The STD spectra were coadded and averaged from 6144 scans.

Assay for FXR Agonists and Antagonists. Bile acids transcriptionally regulate the expression of the human bile salt export pump (BSEP) through activation of FXR.[30,31] In our assay, human BSEP promoter reporter pBSEP(−2.6 kb) was used to evaluate FXR activation by a test compound.[32] Human hepatoma Huh 7 cells obtained from ATCC were maintained in DMEM medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1× nonessential amino acids. Cells were plated in 24-well plates at a density of 8×10^4 cells per well and cultured overnight. Transient transfection was conducted by lipofection with LipofectAMINE and Plus Reagent (Invitrogen).[32] Cells were transfected with 100 ng of BSEP promoter reporter pBSEP(−2.6 kb), 100 ng of human FXR expression plasmid, and 10 ng of the null-*Renilla* luciferase plasmid as the internal control. Sixteen hours after transfection, cells were treated with 10 μM test compound for 30 h.

The luciferase activity was assayed with a dual-luciferase reporter assay system.[32] Briefly, treated Huh 7 cells were washed once with PBS and lysed with 100 μL of passive lysis buffer (Promega, Madison, WI) with gentle rocking for 30 min. Cell lysates (10 μL) were transferred to a 96-well reader plate, and luciferase luminescence was measured by using an E.G. & G. Berthold microplate luminometer (Perkin-Elmer, Boston, MA). The luminescence was normalized relative to the *Renilla* signal, and the ratio of the treated sample over the control served as fold activation. Data are presented as means (±SD < 10%) of at least two separate experiments, each in triplicate. Cells treated with vehicles (0.1% DMSO and methanol) served as controls. Antagonistic activity was measured against the **3**-mediated activation of FXR, where transfected cells were treated for 30 h with 10 μM test compound in the presence of 10 μM chenodeoxycholate (**3**).

References and Notes

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