the probe is held a single methylene from the backbone and can reasonably be assumed to adopt configurations similar to those of other large side chains within the structural contexts. This permits a spatial resolution important for understanding the physiological functions of proteins.

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Supporting Online Material

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Methods
Figs. S1 to S4

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A Natural Product That Lowers Cholesterol As an Antagonist Ligand for FXR

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Extracts of the resin of the guggul tree (*Commiphora mukul*) lower LDL (low-density lipoprotein) cholesterol levels in humans. The plant sterol guggulsterone [4,17(20)-pregnadiene-3,16-dione] is the active agent in this extract. We show that guggulsterone is a highly efficacious antagonist of the farnesoid X receptor (FXR), a nuclear hormone receptor that is activated by bile acids. Guggulsterone treatment decreases hepatic cholesterol in wild-type mice fed a high-cholesterol diet but is not effective in FXR-null mice. Thus, we propose that inhibition of FXR activation is the basis for the cholesterol-lowering activity of guggulsterone. Other natural products with specific biologic effects may modulate the activity of FXR or other relatively promiscuous nuclear hormone receptors.

Cholesterol metabolism is tightly regulated at multiple levels, including its release in the form of bile acids. A negative feedback loop that decreases the rate of bile acid production by the liver when bile acid levels are high is an important component of this regulation. The bile acid receptor FXR (*1–3*) (NR1H4) mediates this and a number of other bile acid–dependent regulatory processes (*4*), establishing its central role in cholesterol metabolism. FXR is a promiscuous nuclear hormone receptor that can be activated by a number of other compounds not structurally related to bile acids (*5–8*). On the basis of this apparent flexibility, we hypothesized that at least a subset of compounds reported to affect cholesterol metabolism via unknown mechanisms could act by modulating FXR activity.

The gum resin of *Commiphora mukul* (guggulu in Sanskrit) has been used in Ayurvedic medicine since at least 600 BC to treat a wide variety of ailments, including obesity and lipid disorders (*9*, *10*). An ethyl acetate extract of this resin has been found to lower LDL cholesterol and triglyceride levels in humans (*11*, *12*). Since receiving regulatory approval in India in 1987, this extract, termed gugulipid, has been widely and effectively used to treat hyperlipidemia (*9*, *10*). Among a number of compounds present in this extract, the stereoisomers E- and Z-guggulsterone (*cis*- and *trans*-4,17(20)-pregnadiene-3,16-dione, respectively) (Fig. 1A) have been shown directly to decrease hepatic cholesterol levels in rodent models (*13*).

The effect of this plant product on FXR activity was initially assessed using transient transfections with a synthetic FXR responsive reporter plasmid (*14*). Z-guggulsterone alone had no effect on FXR activity, but it strongly inhibited FXR activation by chenodeoxycholic acid (CDCA), the most potent of the bile acid agonist ligands (Fig. 1B). Essentially identical results were obtained with E-guggulsterone, but the Z isomer was used for the studies described here. The inhibition of CDCA activation was dose dependent and efficacious. In the presence of 100 μM CDCA, a concentration approximately threefold above that required for half-maximal activation of FXR, 10 μM guggulsterone decreased FXR transactivation by nearly 50%

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and 100 μM guggulsterone resulted in 90% inhibition. Very similar results were observed with the promoter of the orphan receptor SHP (Fig. 1C), which contains an FXR–retinoid X receptor (FXR-RXP) heterodimer binding site and has recently been found to be induced by bile acids (*15*, *16*). Although the in vivo levels of guggulsterone associated with therapeutic effects in humans are not known, the concentrations required to inhibit FXR transactivation are consistent with levels that might be achieved by commonly used doses (75 mg guggulsterone per day).

Guggulsterone did not affect specific DNA binding by FXR-RXR heterodimers in vitro (data not shown) but did inhibit transactivation by a chimera consisting of the DNA binding domain of GAL4 and the ligand-binding domain of FXR (Fig. 1D). Thus, the inhibition requires the FXR ligand-binding domain and is not a consequence of an indirect effect on DNA binding.

The two best-characterized FXR target genes encode SHP and the ileal bile acid binding protein (I-BABP), which also has a single FXR-RXR heterodimer binding site in its promoter (*2*, *17*). Bile acid induction of expression of the orphan receptor SHP, a potent transcriptional repressor, has been proposed as the basis for the negative feedback regulation of expression of bile acid production in the liver via repression of CYP7A expression (*15*, *16*). In primary mouse hepatocytes, SHP messenger RNA (mRNA) expression was induced approximately sevenfold by CDCA treatment, as measured by quantitative real-time polymerase chain reaction (PCR). As expected from the transient transfection results, this response was inhibited by guggulsterone (Fig. 1E). Bile acid activation of the endogenous FXR in the intestinal derived CACO-2 cell line results in a stronger induction of I-BABP mRNA levels, and this induction was also blocked by guggulsterone (Fig. 1F). Thus, the effects of guggulsterone do not require FXR overexpression and can be observed with native genes and endogenous receptors in both primary cultured cells and cell lines.

The simplest explanation of these results is that guggulsterone is an antagonist ligand of FXR. A fluorescence resonance energy transfer (FRET)–based coactivator binding assay was used to test this directly. In the presence of the agonist ligand CDCA, a peptide containing the receptor binding domain of the coactivator SRC-1 is specifically recruited to the FXR li-

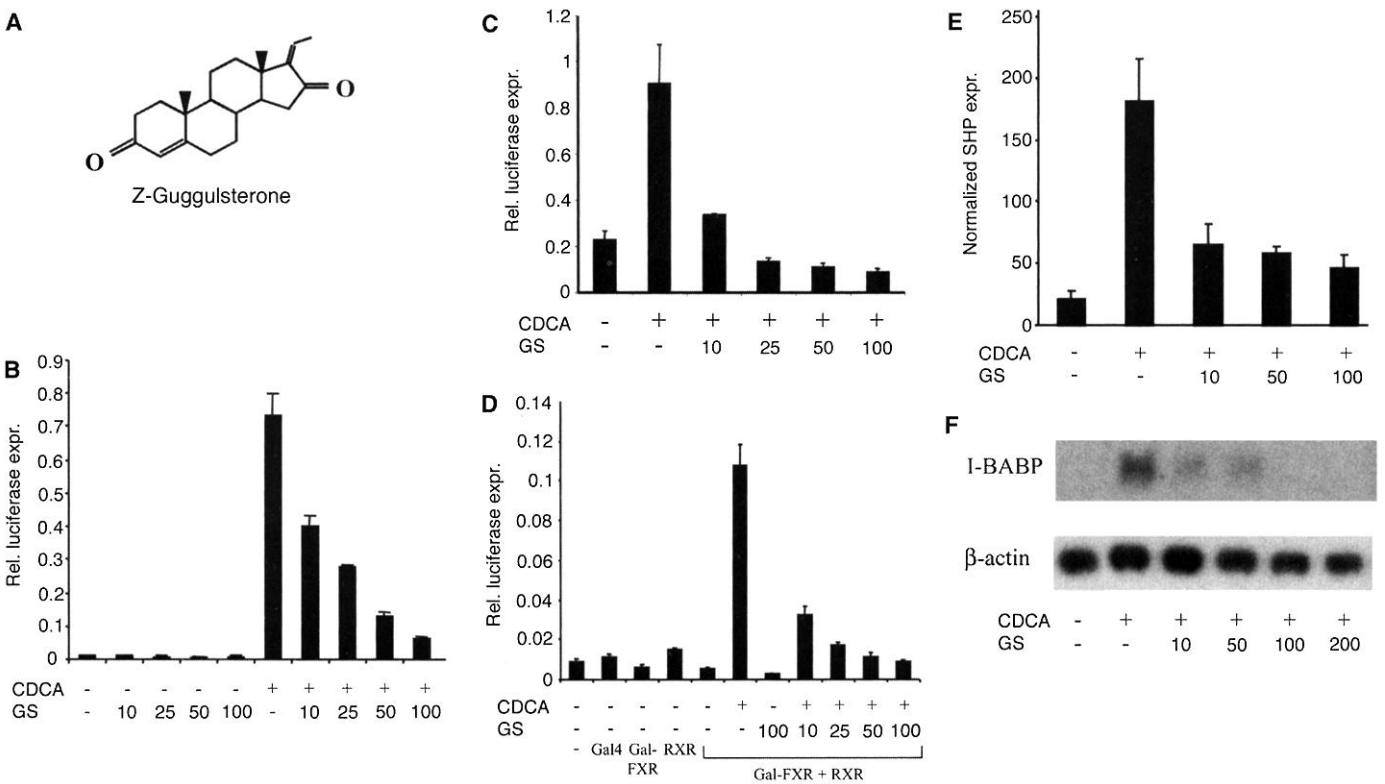

Fig. 1. Guggulsterone inhibits FXR transactivation. (**A**) Structure of Z-guggulsterone [*trans*-4,17(20)-pregnadiene-3,16-dione]. (**B**) HepG2 cells were maintained and transfected as described (*14*). Cells were cotransfected with a luciferase reporter plasmid [$(PLTP)_2$TKluc] containing two copies of the FXR response element from the phospholipid transfer protein (PLTP) promoter upstream of the thymidine kinase (TK) promoter and expression vectors for FXR and RXR, along with a CMX-β-gal internal control (*14*). Cells were treated with vehicle alone or 100 μM CDCA with or without Z-guggulsterone (GS) (*33*), as indicated. Results are luciferase expression normalized using the β-gal internal control. Murine FXR (NR1H4, accession number NP 033134) was used for these and other transfection studies. (**C**) HepG2 cells were cotransfected as in (B) with a human SHP promoter (*34*) (bases −482 to +29, GenBank Accession Number AF044316) luciferase reporter plasmid. GS alone modestly decreased promoter activity. Cells were treated as in (B). (**D**) HepG2 cells were cotransfected as in (B) with a luciferase reporter plasmid containing five copies of the Gal4 binding site and an expression vector for a Gal4 DNA binding domain FXR ligand-binding domain fusion protein, along with a CMX-β-gal internal control. (**E**) Guggulsterone inhibits FXR activation of target genes. Primary mouse hepatocytes were prepared (*35*, *36*) and cultured (*37*) as described. Twenty-four hours after isolation, hepatocytes were treated for 48 hours with vehicle alone or 100 μM CDCA with or without GS, as indicated. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). The TaqMan one-step reverse transcriptase–PCR Master Mix Reagent Kit was used to determine relative SHP mRNA expression levels using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) (*38*). Results are the means ± SD of two independent experiments measured in triplicate. (**F**) Caco-2 cells were cultured for 4 days after reaching confluence and then treated with 100 μM CDCA or 100 μM CDCA and GS, as indicated. 20 μg total RNA isolated using Trizol (Invitrogen) was used for Northern blot analysis as previously described (*14*). The probe for human I-BABP was prepared by PCR using primers (5′-TCCAGCGATGTAATCGAAAAGG-3′) and (5′-GATAGTTGGGGAAATTCACCACC-3′). The blot was sequentially hybridized with I-BABP and β-actin probes.

gand-binding domain. Guggulsterone alone does not result in such recruitment (data not shown). However, addition of increasing amounts of guggulsterone specifically blocked the agonist-dependent coactivator binding to FXR (Fig. 2). Quantitation of additional results varying both CDCA and guggulsterone concentrations indicates that the two compounds compete for binding the FXR ligand-binding domain (data not shown). We conclude that guggulsterone acts as an FXR antagonist both in vitro and in vivo.

The specificity of the guggulsterone effect was examined using GAL4 fusions to the ligand-binding domains of a number of other nuclear hormone receptors. Guggulsterone did not activate or inhibit transactivation by several other receptors associated with lipid metabolism, including liver X receptor α (LXRα), peroxisome proliferator activated receptor γ (PPARγ), and RXRα (Fig. 3A). A modest (<35%) inhibition of activated PPARα and a somewhat more substantial inhibition of activated pregnane X receptor (PXR) were observed. This latter inhibition is a consequence of an apparent partial agonist activity of guggulsterone, which activates PXR approximately 50% as effectively as the specific PXR agonist pregnenolone 16α-carbonitrile (PCN) (Fig. 3B). A potential role of this xenobiotic receptor in cholesterol metabolism is suggested by the observations that PXR can be activated by a subset of toxic bile acids and that PXR activation in mice decreases CYP7A expression and also bile production via unknown mechanisms (*18–20*). The regulation of CYP7A expression differs in rodents and humans (*21–23*), however, and bile acid production was modestly increased in humans treated with the strong hPXR–steroid and xenobiotic receptor (hPXR-SXR) agonist rifampicin (*24*). Several studies have demonstrated that even high doses of rifampicin do not alter serum cholesterol levels in humans (*25–27*). Thus, PXR activation cannot explain the cholesterol-lowering effect of guggulsterone.

To determine whether the activity of guggulsterone as an FXR antagonist is required for its cholesterol-lowering effects, we asked whether these effects are observed in FXR-null animals. Wild-type and FXR-null mutant mice were fed a normal diet or a high-cholesterol diet for 1 week. Each group included control and guggulsterone-treated animals. The cholesterol diet substantially increased hepatic cholesterol levels in both wild-type and FXR-null animals (Fig. 4). Guggulsterone alone had no effect on the control animals but, in agreement with previous results (*13*), decreased hepatic cholesterol levels in the cholesterol-fed wild-type animals. This cholesterol-lowering effect of guggulsterone was absent in the FXR-null animals. In contrast to the results with guggulsterone, similar treatments with the PXR agonist PCN had no effect on hepatic cholesterol levels (data not shown).

We conclude that guggulsterone is an FXR antagonist and that FXR is required for the cholesterol-lowering activity of this natural product. It is interesting that such an effect might not have been predicted for an FXR antagonist, because cholesterol levels are not decreased in mice completely lacking FXR [(*4*) and Fig. 4]. However, the lifelong absence of a major component of a tightly controlled and complex regulatory network is quite different from an acute and partial inhibition of such a component. As in a number of other cases (*28*, *29*), chronic compensatory mechanisms may result in abnormal metabolic effects in these FXR-deficient animals.

The results described here suggest that FXR, the PPARs, and other relatively promiscuous nuclear hormone receptors may be found to

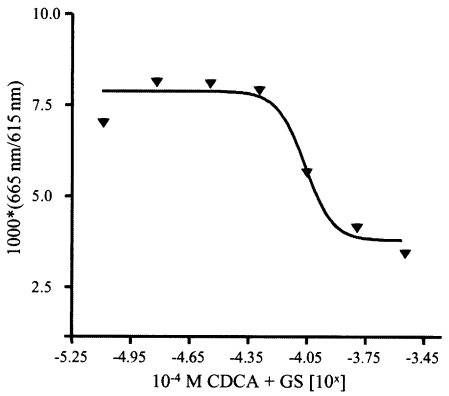

Fig. 2. Guggulsterone is an antagonist ligand for FXR in vitro. A FRET ligand-binding assay was carried out in antagonist mode (*39*) with 100 μM CDCA and increasing amounts of GS, as indicated. Binding reactions contained 8 nM Europium labeled GST-FXR ligand-binding domain fusion protein and 16 nM allophycocyanin-labeled SRC-1 receptor binding peptide. Results are expressed as 1000*(665 nm/615 nm).

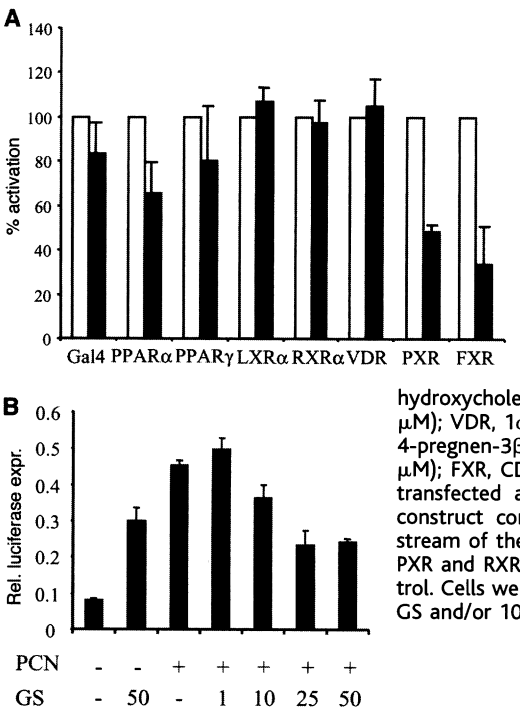

Fig. 3. Specificity of the guggulsterone effect. (**A**) HepG2 cells were cotransfected as in Fig. 1B with the Gal4 luciferase reporter and a series of chimeras in which the Gal4 DNA binding domain is fused to the indicated nuclear hormone receptor ligand-binding domain. Cells were treated with the appropriate agonist (open bars) or agonist plus GS (10 μM) (filled bars). Results in the presence of GS are presented as percent activation relative to the normalized luciferase expression in the presence of agonist (100%). Activation was fourfold with LXRα and higher with other receptors. The ligands used were PPARα, clofibrate (300 nM); PPARγ, BRL 49653 (1 μM); LXR, 22(R)-hydroxycholesterol (10 μM); RXR, 9-cis-retinoic acid (1 μM); VDR, 1α,25-dihydroxyvitamin D3 (100 nM); PXR, 4-pregnen-3β-ol-20-one-16α-carbonitrile (PCN, 10 μM); FXR, CDCA (100 μM). (**B**) HepG2 cells were cotransfected as in Fig. 1B with a luciferase reporter construct containing an LXRE response element upstream of the TK promoter and expression vectors for PXR and RXR, along with the CMX-β-gal internal control. Cells were treated with vehicle alone, 1 to 50 μM GS and/or 10 μM PCN, as indicated.

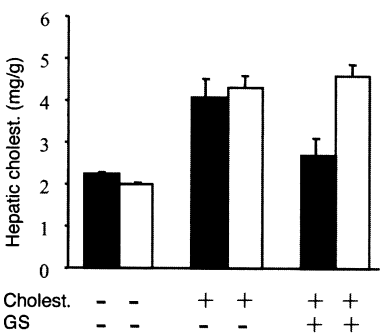

Fig. 4. Absence of guggulsterone effects in FXR-null mice. Wild-type (+/+; black bars) and FXR (–/–; open bars) null mice (*4*) were fed a control diet (n = 3 per group) or a diet supplemented with 2% cholesterol (n = 3 per group) and/or guggulsterone (100 mg/kg body weight) (n = 5 per group) for 7 days (*40*). Total lipids were extracted from the liver as previously described (*41–44*). Total hepatic cholesterol was measured from 100-μl aliquots using the Roche Cholesterol Enzymatic Reagent (Roche Diagnostics, Hoffmann-La Roche, Ltd., Basel, Switzerland). The hepatic cholesterol level in the cholesterol- and GS-fed wild-type animals was significantly different from that of both the wild-type animals fed cholesterol alone (P < 0.02) and the FXR (–/–) animals fed cholesterol plus GS (P < 0.01).

mediate the biological activities of other natural products with metabolic effects. For example, the activation of hPXR-SXR by the hyperforin present in the herbal antidepressant St. John's Wort results in undesirable effects on drug metabolism (*30*, *31*). It is an intriguing possibility that further characterization of the effects of natural products on such receptors will identify additional agents that, like guggulsterone, have more desirable activities.

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32. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
33. Guggulsterones [GS, *cis*- and *trans*-4,17(20)-pregnadiene-3,16-dione] were obtained from Steraloids (Newport, RI) and dissolved in dimethyl sulfoxide (DMSO).
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38. For real-time quantitative PCR, reaction mixes included a 200 nM final concentration of a SHP-specific TaqMan probe (5′ ATGTGCCAGGCCTCCGTGCCT) labeled with 6-carboxy fluorescein (FAM) reporter fluorescent dye, and a 50 nM and 300 nM final concentration of forward (5′ GTACCTGAAGGGCACGATCC) and reverse (5′ AGCCTCCTGTTGCAGGTGT) primers, respectively. For analysis, 1 ng of total RNA isolated from primary hepatocytes was used per reaction. The cycle parameters included a reverse transcription step at 48°C for 30 min, followed by 40 cycles of 95°C denaturation and 60°C annealing and extension. The 18S rRNA was used for the endogenous control.
39. For FRET analysis, the human FXR ligand-binding domain (LBD) (amino acids 244 to 472) was expressed as a GST-FXR-LBD fusion protein (glutathione S-transferase fused to FXR-LBD) in DH5α and purified using glutathione beads. The FRET assay was performed by incubating 8 nM of GST-FXR-LBD, 8 nM of Europium-labeled antibody to GST (Wallac, PerkinElmer Life Sciences, Boston, MA), 16 nM biotin-SRC-1 peptide [5′-biotin-CPSSHSSLTERHKILHRLLQEGSPS-CONH2] (*32*), 20 nM allophycocyanin conjugated streptavidin (APC-SA) (Wallac) in FRET assay buffer (20 mM KH_2PO_4/K_2HPO_4 (pH 7.3), 150 mM NaCl, 2 mM CHAPS detergent, 2 mM EDTA, 1 mM dithiothreitol (DTT) in the presence of the test compound(s) for 2 to 4 hours at room temperature. Data were collected using an LJL Analyst (Molecular Devices, Sunnyvale, CA). The results are expressed as 1000*(665 nm/615 nm).
40. Experimental diets consisted of control diet (TEKLAD 7001, Harlan Teklad, Madison, WI) supplemented with 2% cholesterol. Male 8- to 12-week-old mice were used for all experiments and were allowed water ad libitum. Z-Guggulsterone was resuspended in 0.2-ml saline and administered to mice by oral gavage. Control animals received the same amount of saline. At the end of the experiment, mice fasted for 4 hours, after which time livers were harvested and snap-frozen in liquid nitrogen and then stored at −80°C until use.
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Operant Reward Learning in *Aplysia*: Neuronal Correlates and Mechanisms

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Operant conditioning is a form of associative learning through which an animal learns about the consequences of its behavior. Here, we report an appetitive operant conditioning procedure in *Aplysia* that induces long-term memory. Biophysical changes that accompanied the memory were found in an identified neuron (cell B51) that is considered critical for the expression of behavior that was rewarded. Similar cellular changes in B51 were produced by contingent reinforcement of B51 with dopamine in a single-cell analog of the operant procedure. These findings allow for the detailed analysis of the cellular and molecular processes underlying operant conditioning.

Learning about relations between stimuli [i.e., classical conditioning (*1*)] and learning about the consequences of one's own behavior [i.e., operant conditioning (*2*)] constitute the major part of our predictive understanding of the world. Although the neuronal mechanisms underlying appetitive and aversive classical conditioning are well studied (e.g., *3*–*8*), a comparable understanding of operant conditioning is still lacking. Published reports include invertebrate aversive conditioning (e.g., *9*–*12*) and vertebrate operant reward learning (e.g., *13*). In several forms of learning, dopamine appears to be a key neurotransmitter involved in reward (e.g., *14*). Previous research on dopamine-mediated operant reward learning in *Aplysia* was limited to in vitro analogs (*15*–*18*). In this report, we overcome this limitation by developing both in vivo and single-cell operant procedures and describe biophysical correlates of the operant memory.

The in vivo operant reward learning paradigm was developed using the consummatory phase (i.e., biting) of feeding behavior in *Aplysia*. This model system has several features that we hoped to exploit. The behavior occurs in an all-or-nothing manner and is thus easily quantified (see supplemental video). The circuitry of the underlying central pattern generator (CPG) in the buccal ganglia is well characterized (*19*). The anterior branch of the esophageal nerve

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