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The Hypolipidemic Natural Product Guggulsterone Is a Promiscuous Steroid Receptor Ligand

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ABSTRACT

Guggulsterone (GS) is the active substance in guggulipid, an extract of the guggul tree, *Commiphora mukul*, used to treat a variety of disorders in humans, including dyslipidemia, obesity, and inflammation. The activity of GS has been suggested to be mediated by antagonism of the receptor for bile acids, the farnesoid X receptor (FXR). Here, we demonstrate that both stereoisomers of the plant sterol, (*E*)- and (*Z*)-GS, bind to the steroid receptors at a much higher affinity than to FXR. Both stereoisomers bind to the mineralocorticoid receptor (MR) with a K_i value of approximately 35 nM, which is greater than 100 times more potent than their affinity for FXR. Both (*E*)- and (*Z*)-GS also displayed high affinity for other steroid receptors, including the androgen (AR), glucocorticoid (GR), and progesterone receptors (PR) with K_i values ranging from 224 to 315 nM. In cell-based functional cotransfection assays, GSs behaved as antagonists of AR, GR, and MR, but as agonists of PR. Agonist activity was also demonstrated with estrogen receptor (ER) α; however, the potency was very low ($EC_{50} > 5000$ nM). In addition, GS displayed activity in functional assays in cell lines expressing endogenous AR, GR, ER, and PR. These data suggest that the variety of pharmacological effects exhibited by GS may be mediated by targeting several steroid receptors.

Resin of the gum of the guggul tree, *Commiphora mukul*, has been used in Ayurvedic medicine to treat a variety of diseases for several thousand years (Urizar and Moore, 2003). The active substances from the resin have been demonstrated to be the plant sterols (*E*)- and (*Z*)-GS (Beg et al., 1996), and the ethyl acetate extract of the resin, which is enriched for these plant sterols is currently available as an over-the-counter herbal remedy, guggulipid. Although guggulipid has been suggested to have beneficial effects for the treatment of dyslipidemia, obesity, arthritis, and inflammation, animal and clinical efficacy data have focused primarily on the effects on lipid metabolism. A number of studies, both in animal models and human clinical trials, have shown that guggulipid has beneficial effects on serum lipoprotein profiles models (Satyavati et al., 1969; Agarwal et al., 1986; Nityanand et al., 1989; Singh et al., 1994; Chander et al., 1996).

Two recent studies suggest that GSs exert their hypolipidemic activity via antagonism of the farnesoid X receptor (FXR; NR1H4), a nuclear hormone receptor that functions as a receptor for bile acids (Urizar et al., 2002; Wu et al., 2002). Despite the fact that GS displayed relatively low-potency antagonism for FXR, this receptor has been shown to play an essential role in cholesterol and fatty acid homeostasis in addition to its role in bile acid metabolism, suggesting a possible mechanism of action (Francis et al., 2003). However, at least two lines of evidence conflict with the hypothesis that

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ABBREVIATIONS: GS, guggulsterone; FXR, farnesoid X receptor; PXR, pregnane X receptor; PR, progesterone receptor; ER, estrogen receptor; AR, androgen receptor; MR, mineralocorticoid receptor; GR, glucocorticoid receptor; LXRα, liver X receptor α; TR, thyroid hormone receptor; PPAR, peroxisome proliferator-activated receptor; HEK, human embryonic kidney; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TAT, tyrosine amino transferase; bDNA, branched chain DNA; PSA, prostate-specific antigen; RU486, mifepristone; LY427697, 2-(4-{2-[3-(2,4-difluoro-phenyl)-1-heptyl-ureido]-ethyl}-phenoxyl-2-methyl-butyric acid; LY509547, 2-methyl-2-(4-{3-[propyl-(5-pyridin-2-yl-thiophene-2-sulfonyl)-amino]-propyl}-phenoxy)-proprionic acid; GW4064, 3-(2,6-dichlorophenyl)-4-(3′-carboxyl-2-chlorostilbene-4-yl)-oxymethyl-5-isopropyl-isoxazole; ICI 182,780, faslodex.

the hypolipidemic effects of GS are mediated by FXR. First, the FXR null mice display increased serum total cholesterol, triglyceride, and phospholipid levels (Sinal et al., 2000), which is inconsistent with antagonism of this receptor exhibiting beneficial effects on lipid metabolism. Second, consistent with the FXR null mice, a selective synthetic FXR agonist (GW4064) decreases plasma triglycerides and increases plasma high-density lipoprotein levels in rats (Maloney et al., 2000; Willson et al., 2001). Thus, it becomes apparent that the pharmacological effects of GS may be mediated via additional pathways.

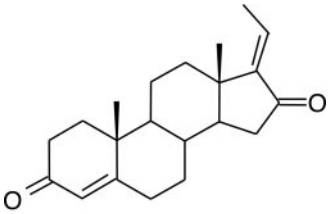
Previous studies demonstrated that GS also activates the pregnane X receptor (PXR; NR1I2), a nuclear receptor that functions as a xenobiotic receptor (Wu et al., 2002; Brobst et al., 2004). However, this is not unusual given the promiscuity of PXR and would not explain the pharmacological effects of GS given the number of compounds that activate this receptor that lack the activity of GS. Brobst et al. (2004), using a cotransfection assay, recently described the ability of GS to activate the progesterone receptor (PR; NR3C3) and estrogen receptor α (ERα; NR3A1), indicating that GS may be more promiscuous than originally expected. In the current study, we found that both (*E*)- and (*Z*)-GS are potent steroid receptor ligands binding to the androgen receptor (AR; NR3C4), mineralocorticoid receptor (MR; NR3C2), glucocorticoid receptor (GR; NR3C1), and PR at affinities as much as 100 times greater than the affinity for FXR. GS primarily functions as an antagonist of these receptors with the exception of PR, where it behaves as a partial agonist. Thus, the wide range of activities that GS has been purported to display may be caused by polypharmacological effects targeting the steroid receptor subclass of the nuclear receptor superfamily.

Materials and Methods

Radioligand Binding Assays. Radioligand binding assays were performed using either the charcoal separation or scintillation proximity technology as described previously (Palmer et al., 2000; Bramlett et al., 2003). Tritiated radioligands were used for all assays with the exception of thyroid hormone receptor (TR) and included dexamethasone (GR), methyltrienolone (R1881) (AR), aldosterone (MR), promegestone (R5020) (PR), GW4064 (FXR), T1317 (liver X receptor α, LXRα), 9-cis retinoic acid, 17β-estradiol (ER), peroxisome proliferator-activated receptor (PPAR) α and PPARδ (LY427697), and PPARγ (LY509547) (Brooks et al., 2001; Xu et al., 2004). Iodinated triiodothyronine was used for TRα and TRβ. Radioligands were purchased from Amersham Biosciences Inc. (Piscataway, NJ). (*E*)- and (*Z*)-GS were evaluated in each assay with 10 concentration-point displacement curves. Assays were performed with each receptor a minimum of three independent times, and the K_i value was determined using the Cheng and Prusoff (1973) equation after determination of the IC_{50} value by fitting the curve to a four-parameter logistical equation.

Cell-Based Transfection Assays. Human embryonic kidney (HEK)293 cells or C2C12 cells were cotransfected using FuGENE reagent (Roche Diagnostics, Indianapolis, IN). A reporter plasmid containing two copies of probasin ARE (GGTTCTTGGAGTACT) and TK promoter upstream of the luciferase reporter cDNA was transfected with a plasmid constitutively expressing human AR using viral CMV promoter. A reporter plasmid containing two copies of GRE (TGTACAGGATGTTCT) and TK promoter upstream of the luciferase reporter cDNA was transfected with a plasmid constitutively expressing either human GR, human MR, or human PR, using viral CMV promoter. A reporter plasmid containing five copies of the Gal4 UAS upstream of luciferase was used in transfections where the Gal4-AR chimeric receptor was used. The Gal4-AR chimeric receptor was created by replacing the endogenous AR DNA binding domain with the Gal4 DNA binding domain, thus leaving the AF-1 and AF-2 regions of AR intact. The reporter plasmid for FXR contained three copies of the IR1 element derived from the phospholipid transfer protein promoter (Urizar et al., 2000) upstream of a minimal promoter upstream of luciferase and was transfected into cells along with human FXR under the control of the CMV promoter. Cells were transfected in T150-cm^2 flasks in DMEM media with 5% charcoal-stripped fetal bovine serum (FBS). After an overnight incubation, transfected cells were trypsinized, plated in 96-well dishes in DMEM media containing 5% charcoal-stripped FBS, incubated for 4 h, and then exposed to test compounds. Transfection assays for ERα and ERβ were performed in PC-3 human prostatic adenocarcinoma cells grown in RPMI 1640 medium (without phenol red) with 10% heat-inactivated FBS, 1 mM HEPES (Invitrogen, Carlsbad, CA), 100 units of penicillin, and 100 g/ml streptomycin. The PC-3 cells were transiently transfected using FuGENE transfection reagent along with full-length human ERα or human ERβ in pCMV vectors and an ERE-tkLUC reporter vector. In the antagonist assays, low concentrations of agonist for each respective receptors were added to the media (0.25 nM dexamethasone for GR, 0.3 nM methyltrienolone for AR, 0.05 nM progesterone for PR, 0.05 nM aldosterone for MR, and 0.1 nM 17β-estradiol for ER). After 24-h incubation with compounds, cells were lysed and luciferase activity was determined. Data were fit to a four-parameter logistical equation to determine EC_{50}/IC_{50} values. The percentage of efficacy was determined versus maximum stimulation obtained with 100 nM methyltrienolone for AR assay, with 30 nM progesterone for PR assay, with 30 nM aldosterone for MR assay, with 100 nM dexamethasone for GR assay, with 100 nM estradiol for ERα and ERβ, and with 1 μM GW4064 for FXR. Assays were performed a minimum of three times for each receptor in both the agonist and antagonist formats.

Steroid Receptor Functional Assays. Tyrosine amino-transferase (TAT) mRNA was measured using branched chain DNA



Guggulsterone

Fig. 1. Chemical structure of guggulsterone [4,17(20)-pregnadiene-3,16-dione].

TABLE 1
Binding affinities (K_i) of (*E*)- and (*Z*)-guggulsterone to the steroid receptors

	(*E*)-GS	(*Z*)-GS
	nM	
GR	252 ± 6	224 ± 26
MR	37 ± 2	39 ± 4
AR	315 ± 13	240 ± 21
PR	224 ± 6	201 ± 18
ERα	>5000	>5000
ERβ	>5000	>5000
FXR	>5000	>5000
LXRα	>5000	>5000
TRα	>5000	>5000
TRβ	>5000	>5000
PPARα	>5000	>5000
PPARδ	>5000	>5000
PPARγ	>5000	>5000
RXRα	>5000	>5000

(bDNA; QuantiGene) as described previously (Burris et al., 1999). The branched chain-DNA (bDNA; QuantiGene) assay for TAT mRNA was performed according to the manufacturer's protocol (Genospectra, Fremont, CA). For QuantiGene TAT mRNA measurement in H4IIE cells, cells were seeded in 96-well plates at 25,000 cells/well in DMEM with 10% FBS and allowed to attached overnight. The following day, cell media were replaced with serum-free DMEM and serum starved for 24 h before treatment with dexamethasone, (*E*)-guggulsterone, (*Z*)-guggulsterone, or RU486 for 24 h. At the conclusion of treatment, cells were lysed with 50 μl of lysis buffer (Genospectra). After a 15-min incubation at 37°C, 50 μl of the lysate from each well was added to capture plates (Genospectra) containing either rat glyceraldehyde-3-phosphate dehydrogenase or rat TAT-specific oligonucleotides in 50 μl of lysis buffer totaling 100 μl. The capture plate was sealed and incubated overnight at 53°C in a Fisher Labline plate incubator. After overnight incubation, the bDNA and label probes were annealed as directed by the manufacturer. Finally, upon addition of luminescent alkaline phophatase substrate, dioxitane, luminescence was quantitated using a TopCount microplate scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences, Boston, MA).

T-47D cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium with 10% FBS, 1% minimal essential medium nonessential amino acids (Invitrogen), 1% sodium

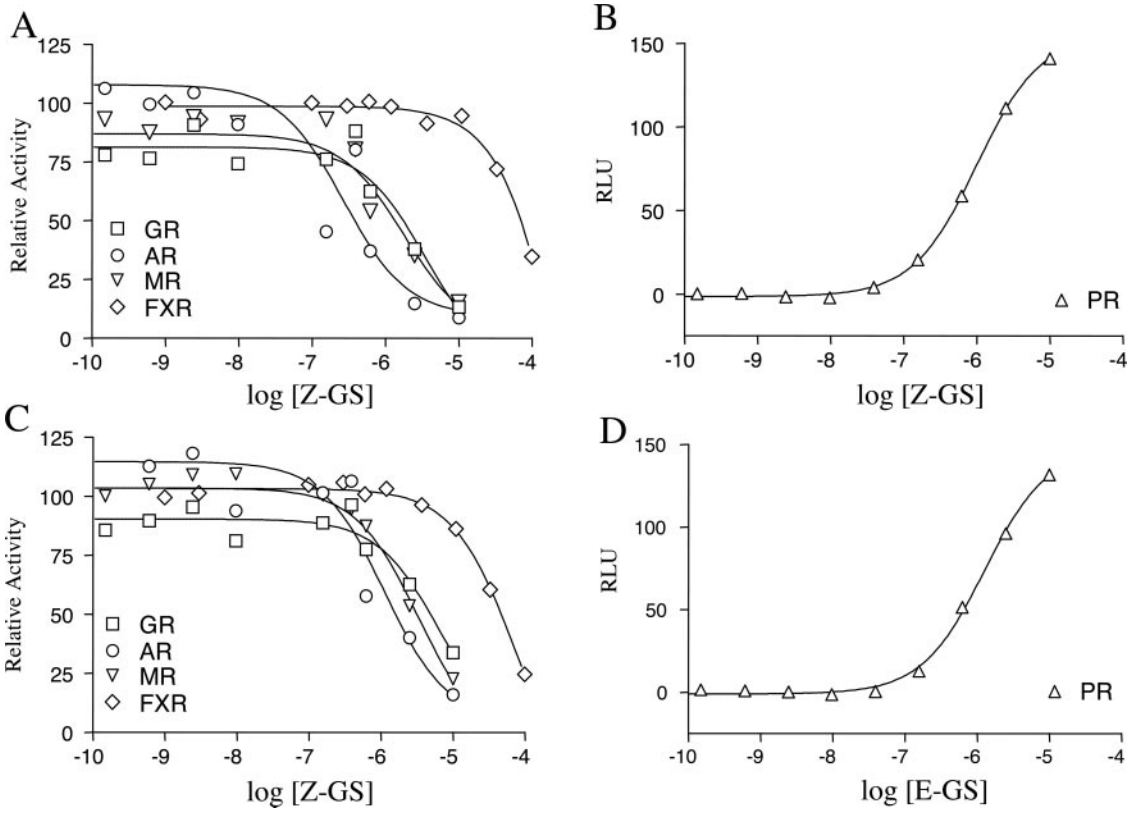


Fig. 2. Functional analysis of the activity of (*E*)- and (*Z*)-GS in HEK293 cells transfected with MR, GR, AR, or MR. A, (*Z*)-GS is an MR, GR, AR, and FXR antagonist. HEK293 cells transiently transfected with vectors directing the expression of MR, GR, AR, or FXR and the appropriate reporter vector were treated with a cognate agonist and the ability of (*Z*)-GS to antagonize their activity was examined. B, (*Z*)-GS is a PR agonist. HEK293 cells transiently transfected with a vector directing the expression of PR and a reporter vector were treated with varying doses of (*Z*)-GS. C, (*E*)-GS is a MR, GR, AR, and FXR antagonist. HEK293 cells transiently transfected with vectors directing the expression of MR, GR, AR, or FXR and the appropriate reporter vector were treated with a cognate agonist and the ability of (*E*)-GS to antagonize their activity was examined. D, (*E*)-GS is a PR agonist. HEK293 cells transiently transfected with a vector directing the expression of PR and a reporter vector were treated with varying doses of (*E*)-GS. For each receptor, a representative of at least three independent experiments is shown. Table 2 provides the potency and efficacy data that includes statistics on all experiments performed. Relative activity reflects the activity relative to induction of activity with a fixed amount of appropriate agonists as described under *Materials and Methods*.

TABLE 2
Functional activity of (*E*)- and (*Z*)-guggulsterone in cotransfection assays

Receptor	IC_{50}		EC_{50} (% Eff)	
	(*E*)-GS	(*Z*)-GS	(*E*)-GS	(*Z*)-GS
	nM		*nM*	
GR	6060 ± 310	1740 ± 150	N.A.	N.A.
MR	1880 ± 390	1000 ± 310	N.A.	N.A.
AR	660 ± 240	220 ± 70	N.A.	N.A.
PR	N.A.	N.A.	740 ± 220 (63)	1200 (64)
ERα	N.A.	N.A.	>5000 (41)	>5000 (59)
ERβ	N.A.	N.A.	>5000 (6)	>5000 (15)
FXR	>50,000	>50,000	N.A.	N.A.

Eff, efficacy; N.A., not applicable.

pyruvate (Invitrogen), and 1% antibiotic/antimycotic (Invitrogen). Cells were trypsinized and seeded into 96-well plates at a density of 20,000 cells/well in culture media containing 10% charcoal/dextran-treated, heat-inactivated FBS (Hyclone Laboratories, Logan, UT) and allowed to attach overnight. To evaluate agonist or antagonist activity, compounds were tested in the absence or presence of 0.25 nM promegestone (R5020; PerkinElmer Life and Analytical Sciences, Boston, MA). After 24 h of treatment, the cells were washed with Dulbecco's phosphate-buffered saline (Invitrogen), all liquid was removed, and plates were frozen at −80°C overnight. Alkaline phosphatase activity was evaluated using the 1-Step *p*-nitrophenol phosphate assay (Pierce Chemical, Rockford, IL).

Human prostate cancer LNCaP cells were seeded into 96-well culture plates at 20,000 cells/well in DMEM-F12 (3:1) (Invitrogen) supplemented with 5% charcoal-stripped serum (Hyclone Laboratories), 1% penicillin/streptomycin, 1% HEPES buffer, and 1% L-glutamine. The next day, cells were treated with compounds for 48 h. R1881 was used as a standard AR agonist, and relative efficacy of GS was calculated based on the maximal efficacy of R1881 (Bonne and Raynaud, 1975). After treatment, 5 μl/well media was removed and assayed for the presence of human prostate specific antigen by enzyme-linked immunosorbent assay according to the manufacturer's protocol (Diagnostic Systems Laboratories, Inc., Webster, TX).

The MCF-7 breast adenocarcinoma cell line proliferation assay for estrogen activity was performed as described previously (Dodge et al., 1996).

Results

GS has previously been reported to be a specific FXR antagonist (Urizar et al., 2002; Wu et al., 2002); however, we noted upon analysis of its effect on FXR target genes that additional pharmacological activity might be associated with GS. The steroidal structure of GS (Fig. 1) suggested that its ability to bind to steroid receptors should be examined. Radioligand binding assays for all the steroid receptors within the nuclear receptor superfamily (AR, GR, PR, MR, ERα, and ERβ) were performed and indicated that both stereoisomers of GS [(*E*)-GS and (*Z*)-GS] display high affinity for AR, GR, MR, and PR relative to FXR (Table 1). The affinity of (*E*)-GS and (*Z*)-GS for the steroid receptors was impressive considering that both stereoisomers bound to MR with greater than 125-fold selectivity versus FXR, whereas the selectivity for AR, GR, and PR versus FXR was greater than 20-fold. No significant stereoisomer selectivity was detected for any of the receptors. It is clear that the highest affinity for GS was against MR with a K_i value of 37 to 39 nM, whereas affinity constants for AR, GR, and PR were in the 200 to 320 nM range. GS was able to displace a radiolabeled FXR ligand; however, it required significantly greater than 5000 nM GS for this to occur and never reached 50% displacement even at concentrations reaching 40,000 nM. Radioligand binding assays were also performed for a variety of other nuclear hormone receptors, including the LXRα, TRα, TRβ, PPARα, PPARδ, PPARγ, and the RXRα. No significant binding was detected for any of these receptors (Table 1).

The radioligand binding data suggest that both (*E*)- and (*Z*)-GS display broad-spectrum steroid receptor binding activity. To investigate the functional significance of the binding activity, we assessed the activity of both GS stereoisomers in cell-based functional assays in which we coexpressed AR, GR, MR, or PR in HEK293 cells along with a luciferase reporter under the direction of a promoter with multiple copies of a steroid receptor response element inserted. Both GS stereoisomers were tested in agonist and antagonist format. As illustrated in Fig. 2, both (*E*)- and (*Z*)-GS antagonized AR, GR, and MR. GS was most potent targeting AR, which (*Z*)-GS antagonized with an IC_{50} value of 220 nM (Table 2). The activity of GS against GR and MR was significantly less with IC_{50} values generally in the 1 to 2 μM range. (*Z*)-GS displayed greater potency than (*E*)-GS for AR, GR, and MR in the cell-based assay. Although (*E*)- and (*Z*)-GS were antagonists of AR, GR, and MR, both stereoisomers behaved as partial agonists of PR. As shown in Fig. 2, B and D, both stereoisomers increased reporter expression with EC_{50} values ranging from 740 to 1200 nM. Maximal efficacy (versus progesterone) for both stereoisomers was 63 to 64%. Agonist activity was also detected for ERα and ERβ in transfected PC-3 cells; however, the EC_{50} was greater than 5000 nM. At the highest concentration tested (10 μM), GS displayed selectivity for ERα (maximal efficacy 41–59% versus 17β-estradiol) versus ERβ (6–15% versus 17β-estradiol) (Table 2).

To confirm the functional activity of GS as a steroid receptor ligand, we assessed the activity of both GS stereoisomers in several cell lines endogenously expressing GR, AR, PR, or ER. The ability of GS stereoisomers to affect expression of a GR-regulated gene, TAT, was examined in the H4IIE hepatocarcinoma cell line. TAT is a well characterized glucocorticoid responsive gene directly responsive to GR via a glucocorticoid response element located in its promoter (Jantzen et al., 1987). As illustrated in Fig. 3, A and B, the GR agonist dexamethasone increased expression of TAT mRNA 5- to

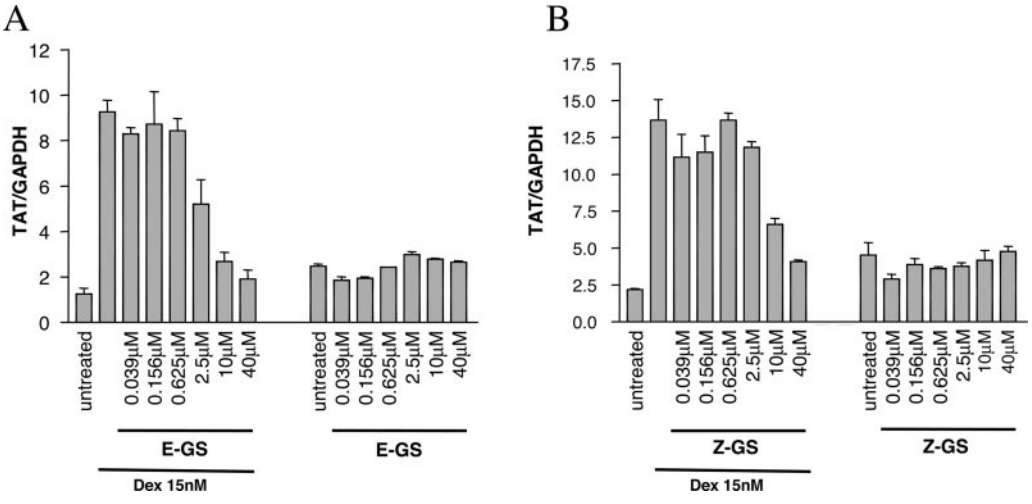


Fig. 3. (*E*)- and (*Z*)-GS antagonize dexamethasone-induced TAT expression from H4IIE cells. A, (*E*)-GS antagonizes dexamethasone-induced TAT expression. H4IIE rat hepatoma cells were treated with either (*E*)-GS alone or in the presence of the GR agonist dexamethasone followed by assessment of TAT mRNA expression. B, (*Z*)-GS antagonizes dexamethasone-induced TAT expression. H4IIE rat hepatoma cells were treated with either (*Z*)-GS alone or in the presence of the GR agonist dexamethasone followed by assessment of TAT mRNA expression.

6-fold, whereas either (*E*)- or (*Z*)-GS alone did not affect TAT expression. However, either (*E*)- or (*Z*)-GS effectively antagonized the activity of dexamethasone. As was the case for the other cell-based assays, the potency of (*E*)- and (*Z*)-GS was lower than indicated by the radioligand binding assay; however, both the transfection assay and TAT expression assay indicated single-digit micromolar potency [EC_{50} (*E*)-GS = 3.0 μM; EC_{50} (*Z*)-GS = 9.9 μM].

Also consistent with the transfection results, assessment of activity of (*E*)- and (*Z*)-GS in the MCF-7 breast adenocarcinoma cell proliferation assay for estrogen activity indicated that both stereoisomers were weak agonists with (*Z*)-GS providing significantly more activity than (*E*)-GS (Fig. 4). The activity of both (*E*)- and (*Z*)-GS was blocked by the selective ER antagonist ICI 182,780 in these cells (Fig. 4, inset). AR activity was confirmed first in the LNCaP human prostate cancer cell line. Expression of prostate-specific antigen (PSA) protein was used to monitor AR activity because PSA is directly responsive to AR action via androgen response elements localized in the promoter of the gene (Murtha et al., 1993; Luke and Coffey, 1994; Cleutjens et al., 1996). As shown in Fig. 5A, both (*E*)- and (*Z*)-GS stimulated PSA expression in LNCaP cells. This is in contrast to the cell-based transfection assay where both GS stereoisomers antagonized AR activity. However, LNCaP cells have been shown to respond to both AR agonists and antagonists by increasing PSA expression (Wolf et al., 1992). The potency of induction of PSA expression in the LNCaP cells [EC_{50} (*Z*)-GS = 260 nM; EC_{50} (*E*)-GS = 550 nM] was consistent with potency in cell based transfection assays (Table 2). An AR antagonist that retains the ability to block AR activity in the LNCap cells, bicalutamide, was able to suppress the agonist activity of both GS stereoisomers, indicating that the effect is mediated by AR (Fig. 5A, inset). To further examine the functional AR activity of the GSs, we tested their activity in two additional assay systems. We created a chimeric AR protein by replacing the wild-type AR DNA binding domain with that of the yeast transcription factor, GAL4. This chimeric AR retained the AF-1 domain amino-terminal to the GAL4 DNA binding domain and the AF-2 domain carboxy-terminal to the GAL4 replacement. This chimeric receptor was then used in a co-transfection system in HEK293 cells along with a reporter containing five copies of a GAL4 UAS upstream of luciferase. As shown in Fig. 5B, both GSs effectively antagonized the activity of the AR agonist R1881 in a dose-responsive manner. The IC_{50} values of the stereoisomers were similar: (*E*)-GS IC_{50} = 1.0 μM and (*Z*)-GS IC_{50} = 1.5 μM. We also examined the activity of the GSs using identical constructs as

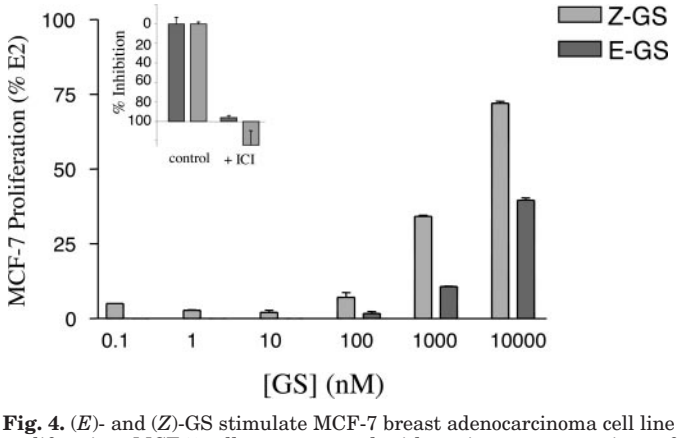


Fig. 4. (*E*)- and (*Z*)-GS stimulate MCF-7 breast adenocarcinoma cell line proliferation. MCF-7 cells were treated with various concentrations of (*E*)- or (*Z*)-GS for 48 h and proliferation was assessed by [^{3}H]thymidine incorporation. Efficacy is indicated as a percentage of stimulation of proliferation relative to maximal stimulation by 17β-estradiol (E2). The inset illustrates the ability of the specific ER antagonist ICI 182,780 (1 nM) to inhibit (*E*)- and (*Z*)-GS-induced cellular proliferation.

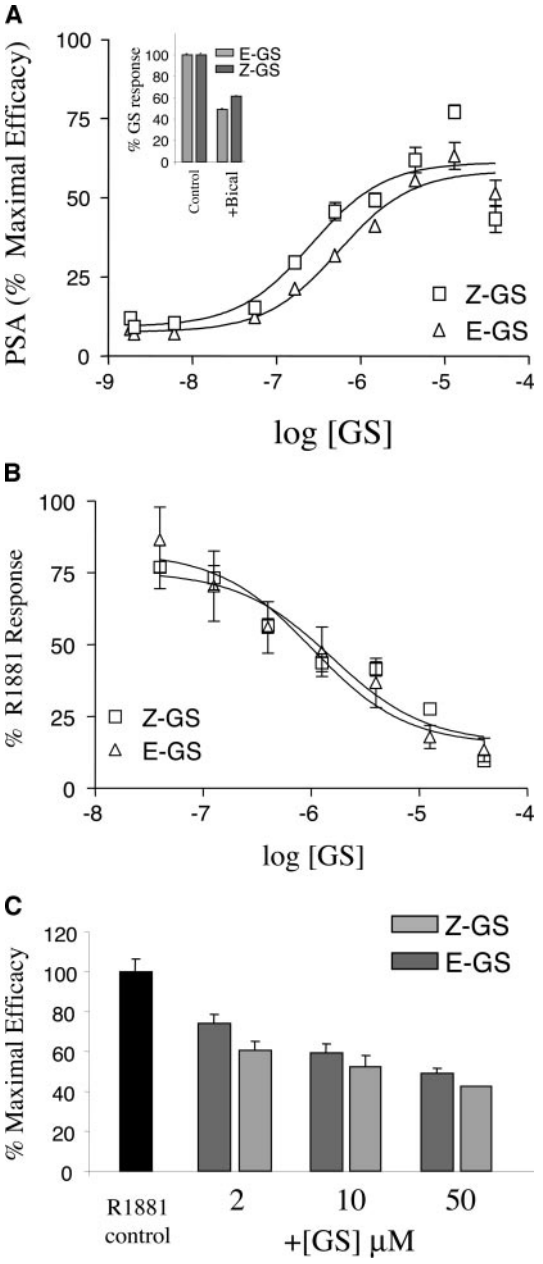


Fig. 5. Functional AR activity of guggulsterones. A, (*E*)- and (*Z*)-GS stimulate PSA expression in the human prostate cancer cell line LNCaP. LNCaP cells were treated with (*E*)- or (*Z*)-GS for 48 h followed by assessment of PSA secretion by enzyme-linked immunosorbent assay. Efficacy is indicated as a percentage of stimulation of PSA relative to maximal stimulation by R1881. The inset illustrates the ability of the specific AR antagonist bicalutamide (2 μM) to inhibit (*E*)- and (*Z*)-GS-induced PSA expression. B, (*E*)- and (*Z*)-GS antagonize R1881 stimulated GAL4 reporter activity in HEK293 cells transfected with a GAL4 AR chimeric receptor in which the DNA binding domain of AR as been replaced with the GAL4 DNA binding domain. Both the AF-1 and AF-2 domains of AR are intact in the chimeric receptor. C, (*E*)- and (*Z*)-GS antagonize R1881-stimulated ARE reporter activity in C2C12 cells transfected with wild-type AR.

those used in the HEK293 cell cotransfections but transfecting C2C12 cells instead. Both GSs displayed the ability to antagonize the activity of the R1881; however, the C2C12 cells seemed to be less sensitive with the IC_{50} values of GSs near 10 μM. Thus, these additional models confirm the AR antagonist activity of the GSs.

Induction of alkaline phosphatase activity in the T-47D breast cancer cell line is a commonly used assay to assess the activity of compounds with PR activity (Palmer et al., 2000). Thus, we used this assay to assess the activity of both (*E*)- and (*Z*)-GS in a cell line expressing endogenous levels of PR. As shown in Fig. 6, both (*E*)- and (*Z*)-GS functioned as PR agonists inducing alkaline phosphatase activity. Maximal induction for both stereoisomers was in the range of 80% of the maximal level induced by the PR agonist R5020. The potencies of the stereoisomers were consistent with that found in the cell-based transfection experiments. (*Z*)-GS displayed an EC_{50} value of 1.6 μM, whereas (*E*)-GS displayed an EC_{50} of 1.2 μM. The activity of both stereoisomers was blocked by the PR antagonist RU486 (Fig. 6, inset). No antagonist activity for either stereoisomer was detected in this assay (data not shown).

Discussion

The plant sterols (*E*)- and (*Z*)-GS have been identified as the active agents in guggulipid (Satyavati, 1988; Beg et al., 1996), which is an ethyl acetate extract from the gum resin of the tree *C. mukul*. An agent derived from gugglu (gum resin of *C. mukul*) used in ancient Ayurvedic medicine, guggulipid has been shown to have activity as a hypolipidemic agent both in animal models (Chander et al., 1996; Satyavati et al., 1969; Urizar and Moore, 2003) and in humans (Agarwal et al., 1986; Nityanand et al., 1989; Singh et al., 1994; Urizar and Moore, 2003). Although the efficacy of guggulipid has met with some recent controversy (Firenzuoli and Gori, 2003; Karuparthy and Vepachedu, 2003; Szapary et al., 2003), it is commonly used in India for treatment of hyperlipidemia and obesity and is widely available worldwide as an herbal dietary supplement.

The pharmacological activity of GS has been suggested to be mediated by the nuclear hormone receptor FXR based on its ability to antagonize this receptor (Urizar et al., 2002; Wu et al., 2002). (*Z*)-GS has been shown to antagonize the activity of either a natural bile acid ligand (chenodeoxycholic acid) or a synthetic one (GW4064) in cell-based cotransfection assays as well as in FXR target gene induction assays in primary hepatocytes and in the Caco-2 and HepG2 cell lines (Urizar et al., 2002; Wu et al., 2002). In addition, both studies demonstrated that GS antagonizes coactivator recruitment in biochemical assays, indicating that GS is a direct FXR ligand, albeit a weak one with an IC_{50} value in the range of 5 to 50 μM (Urizar et al., 2002; Wu et al., 2002; Cui et al., 2003). A very recent study indicated that GS activates Gal4-DNA binding domain PR and ERα ligand binding domain chimeric receptors in a cell-based cotransfection assay (Brobst et al., 2004). Our analysis of the activity of GS not only confirms this but also provides significant insight into the broad steroid receptor binding activity that is probably associated with the pharmacological activity of this

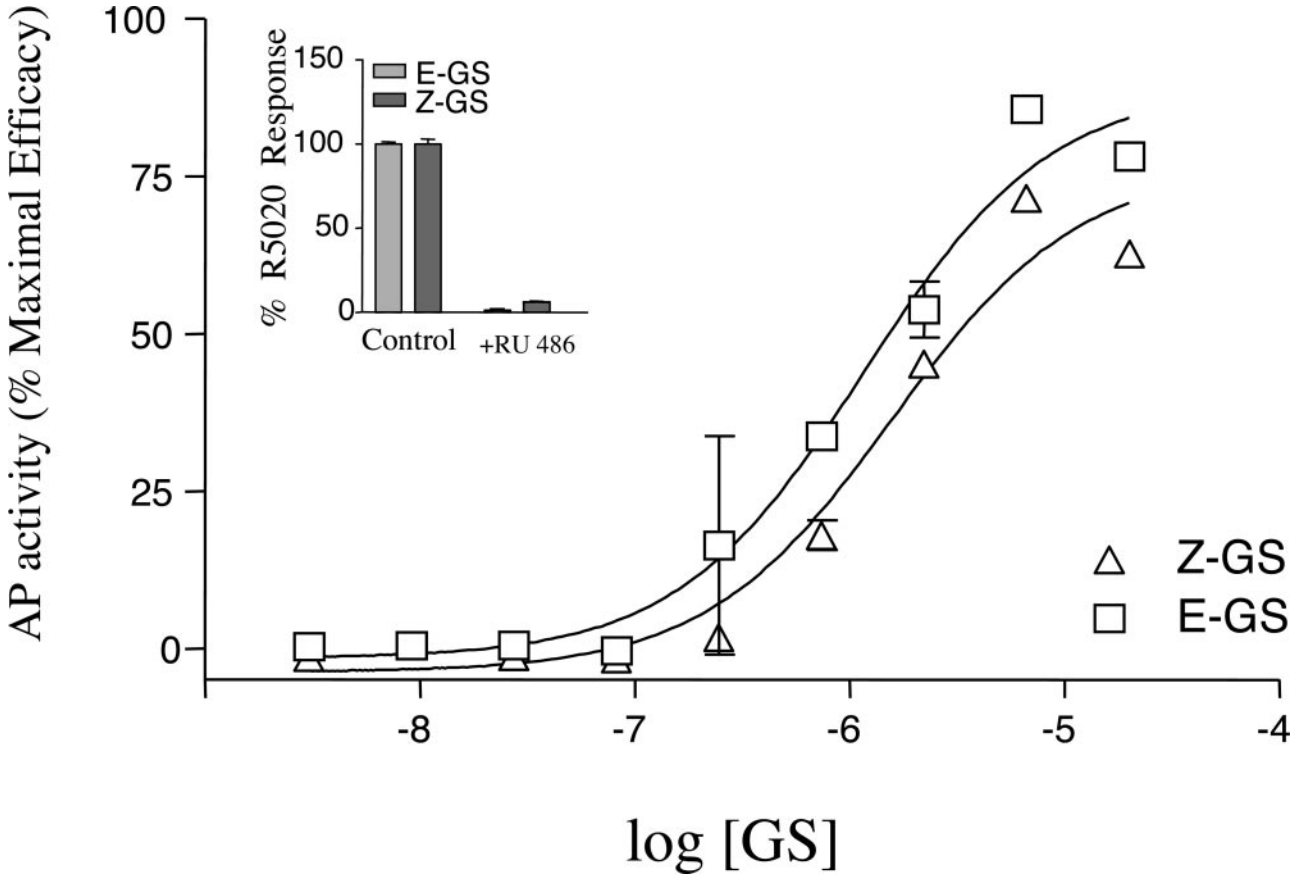


Fig. 6. (*E*)- and (*Z*)-GS stimulate alkaline phosphatase activity expression in the human breast cancer cell line T-47D. T-47D cells were treated with (*E*)- or (*Z*)-GS for 48 h followed by assessment of alkaline phosphatase activity. Efficacy is indicated as a percentage of maximal stimulation by R5020. The inset illustrates the ability of the PR antagonist RU486 (30 nM) to inhibit (*E*)- and (*Z*)-GS-induced alkaline phosphatase activity.

unique natural product. Profiling of both stereoisomers of GS in radioligand binding assays for a wide range of nuclear receptors indicated that the primary targets for the natural product were the steroid receptors: AR, GR, MR, and PR. The GSs bound to MR with the highest affinity with K_i values in the 35 nM range, whereas the K_i values for AR, GR, and PR were all in the 200 to 320 nM range. A radioligand binding assay for FXR indicated that the GSs behaved as FXR ligands but did not displace radiolabeled GW4064 until concentrations well beyond 5000 nM. Thus, GSs display significant selectivity for the steroid receptors over FXR in the range of >20- to 125-fold. Functional analysis of (*E*)- and (*Z*)-GS in cell-based assays demonstrated that the natural product antagonizes MR, GR, and AR and activates PR. Consistent with Brobst et al. (2004), ER agonist activity was also detected in two functional assays, one of which suggests ERα selectivity, although the potency was very low.

Thus, both (*E*)- and (*Z*)-GS function as antagonists of MR, GR, and AR, and agonists of PR at concentrations well below those required to block FXR, indicating that the actions of GS may be caused by a polypharmacological profile of activity targeting the steroid receptor members of the nuclear hormone receptor superfamily in addition to FXR. Nuclear receptors such as FXR require significantly higher levels of their natural ligands (micromolar levels) for activation than do the steroid receptors (nanomolar levels). However, based on our data indicating that GSs display considerably higher affinity for several steroid receptors than for FXR, it is reasonable to assume that doses of GS required to act on FXR in vivo would be well above those required to affect steroid receptor activity. Much of the activity that we have characterized for the GSs is consistent with the clinical pharmacology associated with its use. It is clear that compounds with similar steroid receptor agonist/antagonist profiles, such as cyproterone acetate, which functions as an AR and GR antagonist and PR agonist, have been demonstrated to have hypolipidemic activity (Damgaard-Pedersen and Fogh, 1980; Wallentin and Varenhorst, 1980). Likewise, spironolactone that functions as a MR and GR antagonist and PR agonist has been demonstrated to have beneficial effects on hypertension and congestive heart failure (Pitt et al., 1999). In addition, there are a variety of other pharmacological activities that have been associated with guggulipid use such as reduction of acne (Urizar and Moore, 2003), which based on our data may be associated with the antiandrogen activity of the GSs. The range of receptors targeted by the GSs indicates that the pharmacology of these plant sterols will require greater examination to understand their activity and clinical significance.

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