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Identification of a novel agonist of peroxisome proliferator-activated receptors α and γ that may contribute to the anti-diabetic activity of guggulipid in *Lep*^{ob}/*Lep*^{ob} mice☆,☆☆

Claire L. Cornick[a], Barbara H. Strongitharm[b], Gary Sassano[b], Christopher Rawlins[b], Andrew E. Mayes[b], Alison N. Joseph[b], Jacqueline O'Dowd[a], Claire Stocker[a], Ed Wargent[a], Michael A. Cawthorne[a], A. Louise Brown[b], Jonathan R.S. Arch[a,*]

[a]*Clore Laboratory for Life Sciences, University of Buckingham, MK18 1EG Buckingham, UK*
[b]*Biosciences Division, Unilever Research, Colworth Laboratory, Sharnbrook, MK44 1LQ Bedfordshire, UK*

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Abstract

The ethyl acetate extract of the gum of the guggul tree, *Commiphora mukul* (guggulipid), is marketed for the treatment of dyslipidaemia and obesity. We have found that it protects *Lep*^{ob}/*Lep*^{ob} mice from diabetes and have investigated possible molecular mechanisms for its metabolic effects, in particular those due to a newly identified component, commipheric acid. Both guggulipid (EC_{50}=0.82 μg/ml) and commipheric acid (EC_{50}=0.26 μg/ml) activated human peroxisome proliferator-activated receptor α (PPARα) in COS-7 cells transiently transfected with the receptor and a reporter gene construct. Similarly, both guggulipid (EC_{50}=2.3 μg/ml) and commipheric acid (EC_{50}=0.3 μg/ml) activated PPARγ and both promoted the differentiation of 3T3 L1 preadipocytes to adipocytes. Guggulipid (EC_{50}=0.66 μg/ml), but not commipheric acid, activated liver X receptor α (LXRα). E- and Z-guggulsterones, which are largely responsible for guggulipid's hypocholesterolaemic effect, had no effects in these assays. Guggulipid (20 g/kg diet) improved glucose tolerance in female *Lep*^{ob}/*Lep*^{ob} mice. Pure commipheric acid, given orally (960 mg/kg body weight, once daily), increased liver weight but did not affect body weight or glucose tolerance. However, the ethyl ester of commipheric acid (150 mg/kg, twice daily) lowered fasting blood glucose and plasma insulin, and plasma triglycerides without affecting food intake or body weight. These results raise the possibility that guggulipid has anti-diabetic activity due partly to commipheric acid's PPARα/γ agonism, but the systemic bioavailability of orally dosed, pure commipheric acid appears poor. Another component may contribute to guggulipid's anti-diabetic and hypocholesterolaemic activity by stimulating LXRα.

Keywords: Guggulipid; Commipheric acid; Peroxisome proliferator-activated receptor; Liver X receptor α; *Lep*^{ob}/*Lep*^{ob} mouse; Diabetes

1. Introduction

The World Health Organisation has estimated that about three quarters of the world's population relies on traditional remedies as its main source of medicine [1], and many drugs are synthetic analogues of herbal products [2]. The gum resin guggul, also known as gum guggul or guggulu, is secreted from the Indian shrub *Commiphora mukul* and is used in Ayurvedic medicine as a standardised extract, guggulipid, to treat various disorders, including hyperlipidaemia, obesity, diabetes [3] and inflammation, such as in osteoarthritis. Its use in hyperlipidaemia is supported by a number of clinical studies, although one well-designed study has cast doubt on this utility [4]. Further studies are required to support its use in other indications [5–7].

The hypolipidaemic and hypocholesterolaemic effects of guggul have been attributed to E- and Z-guggulsterone [7]. It was suggested that these compounds act by antagonising the farnesoid X receptor (FXR), thereby preventing feedback inhibition of bile acid synthesis [8]. Others, however, have reported that guggulsterones can enhance the effects of FXR

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☆☆ Unilever has applied for patents on commipheric acid but does not market commipheric acid or guggulipid.
* Corresponding author. Tel.: +44 1280 820306; fax: +44 1280 820135.
E-mail address: jon.arch@buckingham.ac.uk (J.R.S. Arch).

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agonists, thereby enhancing transcription of the bile acid export pump [9]. Moreover, guggulsterones activate or inhibit the pregnane X receptor [8,10,11], are inverse agonists of the constitutive androstane receptor [12] and are promiscuous steroid receptor ligands [13]. This promiscuity does not, however, extend to peroxisome proliferator-activated receptors (PPARs) or to the liver X receptor (LXR) [8,13].

Preliminary studies conducted at our laboratory showed that guggulipid reduced body weight gain and improved glucose homeostasis in genetically obese and insulin-resistant Lep^{ob}/Lep^{ob} mice [14]. Here we describe the identification of a novel component in guggulipid, commipheric acid, and compare it with guggulipid as an activator of PPARα, PPARγ and LXRα. Studies on guggulipid and the ethyl ester of commipheric acid in Lep^{ob}/Lep^{ob} mice are described.

2. Methods and materials

Guggulipid (Gukkaselect) was obtained from Indena (Milan, Italy); rosiglitazone and ciglitazone were from Cayman Chemicals (Michigan, USA); WY14643 was from BIOMOL International (Exeter, UK); and E- and Z-guggulsterones from Steraloids (Newport, RI, USA). T0901317 was a gift from Dr Mike Sierra (Carex, Strasbourg, France).

Commipheric acid was prepared by fractionation of guggulipid [15]. The ethyl ester of commipheric acid was prepared by dissolving 50 g of commipheric acid in 500 ml of absolute ethanol. Twenty-five milliliters of concentrated (36%) hydrochloric acid was added and the mixture was stirred at 65°C. The course of the reaction was followed by titration of the unreacted acid and was stopped after 40 h. The reaction product was extracted into ether and washed with water until the water was neutral to pH paper. The solvent was evaporated and the ester was dissolved in an equal volume of ether. It was separated from commipheric acid by adding it to an alkali alumina column and eluting it with ether. The structure was confirmed by ^{13}C NMR.

The compounds were dissolved in ethanol at concentrations of 10 mM, or in the case of guggulipid at 10 μg/ml, except for rosiglitazone and the guggulsterones, which were dissolved in dimethyl sulphoxide. Appropriate vehicles were used as controls in all experiments. Other reagents were from Sigma-Aldrich (Poole, Dorset, UK), unless otherwise stated.

2.1. Fractionation of guggulipid

The fractionation of guggulipid is described in United States Patent 6737442 [15]. Briefly, 10 g guggulipid was fractionated through a column that contained 100 g silica gel using 17 solvents ranging from hexane (Fraction 1), through hexane/ethyl acetate mixtures (Fractions 2 to 11), ethyl acetate (Fraction 12) and ethyl acetate/methanol mixtures (Fractions 13 to 16) to methanol (Fraction 17). Subfractionation of Fraction 5 by high-performance liquid chromatography and solid-phase extraction led to the isolation of the bicyclic triterpenoid 13-(decahydro-2-hydroxy-2,5,5,8a-tetramethyl-6-oxo-1-naphthalenyl)-2,6,10-trimethyl-[1R-[1α(2E,6E,10E)2β,4aβ,8aα]]-2,6,10-tridecatrienoic acid (Fig. 1), which was characterised and structurally determined by gas chromatography-mass spectrometry and ^{13}C NMR, respectively. This compound was named commipheric acid. It constituted 9.5% of guggulipid (Table 1). Two bicyclic triterpenoids previously identified by others [16,17] were also found in Fraction 5. These were octahydro-6-hydroxy-5-(13-hydroxy-4,8,12-trimethyl-3,7,11-tridecatrienyl)-1,1,4a,6-tetramethyl-[4aS-[4aα,5α(3E,7E,11E),6-β,8aβ]]-2(1H)-naphthalenone (also termed myrrhanone A or commipherol) and decahydro-5-[(3E,7E,11E)-13-hydroxy-4,8,12-trimethyl-3,7,11-tridecatrienyl]-1,1,4a,6-tetramethyl-(2S,4aS,5R,6R,8aS)-2,6-naphthalenediol (termed myrrhanol A) (Fig. 1).

A subsequent, larger scale (500 g starting material) fractionation outsourced to Molecular Nature (Berkshire, UK) resulted in Fractions 3, 4 and 5 containing similar components to Fractions 4, 5 and 6 in the original fractionation. Commipheric acid was most highly represented (27.5%) in the new Fraction 3. Commipherol/myrrhanone A constituted a similar proportion of guggulipid to commipheric acid but was most highly represented in the new Fraction 5. Myrrhanol A constituted only 0.5% of guggulipid (Table 1).

2.2. Vectors

Expression vectors for human RXRα (pRSV/hRXRα) and PPARγ1 (pcDNA4/hPPARγ1) were obtained from Professor VKK Chatterjee (Addenbrooke's Hospital, Cambridge, UK). The vector for human LXRα (pRSV/hLXRα) was purchased from Invitrogen (Paisley, UK) and that for *Renilla luciferase* (pRLTK) from Promega (Southampton, UK). The vector for human PPARα (pcDNA3/PPARα) was prepared by removing the human PPARα cDNA insert from pUC18/hPPARα [18] as a *Nru*I/*Bam*HI fragment and ligating it into *Eco*RV/*Bam*HI-cleaved pcDNA3.1(−) (Invitrogen). The ligated DNA was transformed into competent JM109 *E. coli* cells (Promega).

The firefly luciferase reporter gene vector for PPARα and PPARγ (pPRE3TK-luc) was prepared as described previously [15]. Briefly, the vector was prepared by replacing the NF-κB enhancer element of pNF-κB-luc (Clontech, California, USA) with a cassette of three PPAR response elements (PPREs). The double-stranded PPRE cassette [15] was prepared using the Klenow fill-in technique, digested with *Mlu*I and *Bgl*II, and ligated into pNF-κB-luc that had been cleared with the same restriction endonucleases. Digested vector and insert DNA were ligated and the ligated DNA was used to transform competent *E. coli* JM109 cells (Promega). The firefly luciferase reporter gene vector for LXRα (LXRETK-luc) was similarly created by cleaving the NF-κB enhancer element of NF-κB-luc and replacing it with three direct repeats of the DNA response element for the

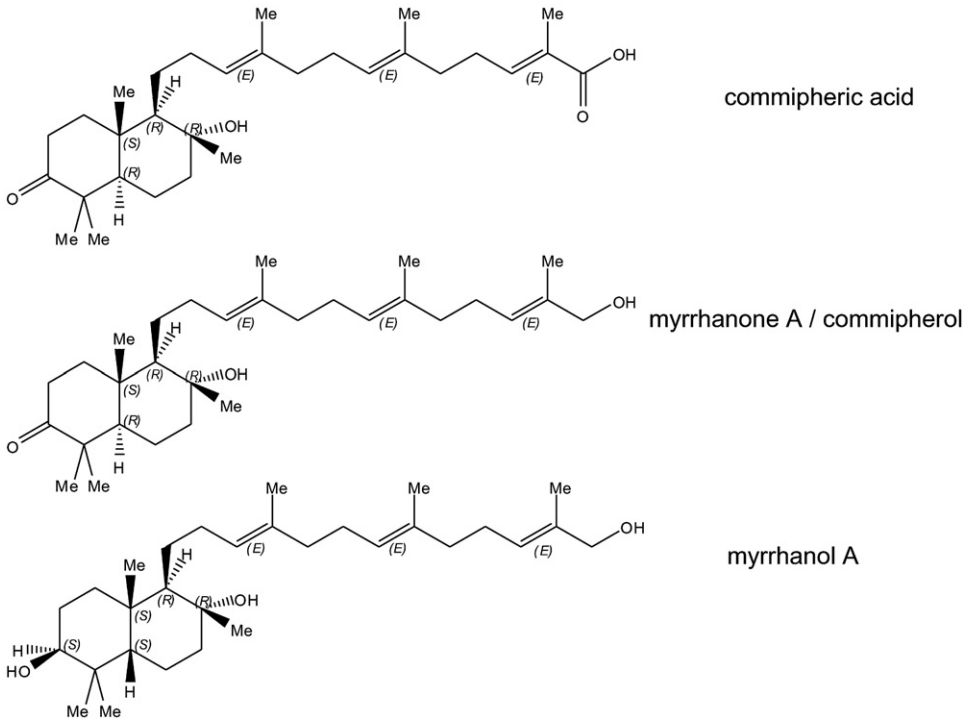

Fig. 1. Chemical structures of commipheric acid, myrrhanone A/commipherol and myrrhanol A.

LXR (LXRE). The double-stranded LXRE cassette prepared using the Klenow fill-in technique had the sequence: 5′-GCATTCACGCGTCCAGGGTTTAAATAAGTTCAG-TTCACAGGGTTTAAATAAGTTCAGTTCACAGGGTT-TAAATAAGTTCAGGCAACAGATCTTACGTG-3′.

2.3. Reporter gene assays

COS-7 cells (ECACC No 87021302) were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin in a 5% CO_2 atmosphere. They were plated in 24-well plates at a density of 7.5×10^4 cells/well, left overnight, washed with serum-free medium (DMEM plus 2 mM L-glutamine) and transfected using Lipofectamine as directed by the manufacturer (Invitrogen). They were transfected in 250 μl serum-free medium with the following expression vectors: *Renilla* luciferase (0.03 μg/well), which was used to control for transfection efficiency; human RXRα (0.02 μg/well); human PPARα, PPARγ or LXR, depending on the assay (each 0.02 μg/well); firefly luciferase containing response elements for the PPARs or LXRα (both 0.4 μg/well), as appropriate. Mevalonic acid (1 μM) and simvastatin (5 μM) were included in the medium for the LXRα assay to control the rate of cholesterol synthesis and prevent high background levels of activation of LXRα by endogenous cholesterol, as described by others [19–21]. The choice of these particular concentrations was made following preliminary studies on activation of LXRα by T0901317 using a range of concentrations of first simvastatin (0.5 to 50 μM) and then mevalonic acid (1 to 1000 μM).

Five hours after transfection, 250 μl serum-free medium containing guggulipid, commipheric acid, standard nuclear receptor agonists or the guggulsterones was added to the cells. WY14643 (25 μM), ciglitazone (5 μM) or T0901317 (1 μM) was included in each PPARα, PPARγ or LXRα assay, respectively, to control for inter-experiment variability and so that the maximal effects of guggulipid and commipheric acid could be expressed relative to these compounds. Cell lysates were prepared after 48 h using 100 μl 1× passive lysis buffer (Promega) and frozen overnight. Firefly and *Renilla* luciferase assays were measured using a Dual Luciferase Assay kit (Promega). Measurements were performed on an MLX microtitre plate luminometer (Dynex Technologies, Worthing, UK). Each concentration of guggulipid or compound was tested in triplicate using cells from three flasks and then the experiment was repeated with cells from another three flasks, so that n=6 flasks. The highest concentrations of guggulipid and commipheric acid were toxic to the cells and these were excluded from the analysis.

Table 1
Percentages of commipheric acid, commipherol/myrrhanone A and myrrhanol A in guggulipid and 'new' Fraction 3

	Percentages in	
	Guggulipid	Fraction 3
Commipheric acid	9.5	27.5
Commipherol/myrrhanone A	9.9	3.0
Myrrhanol	0.5	1.2

2.4. Adipocyte differentiation

Murine 3T3 L1 preadipocytes (ECACC No. 86052701) were seeded into 12-well plates and maintained at 37°C in DMEM supplemented with 10% newborn fetal bovine serum, 2 μM L-glutamine, 1000 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 atmosphere. The experiment was initiated by further supplementing the medium with insulin (5 μg/ml), and with guggulipid (5 μg/ml), commipheric acid (5 μM) or the 1% ethanol vehicle. The medium was replenished every 2 days. On Day 10, accumulation of lipid was assessed by staining with Oil Red O dye. The cells were washed with phosphate-buffered saline and then fixed for 10 min with neutral-buffered formalin before washing again and staining for 15 min with 125 μl 1×Oil Red O. They were kept in 500 μl phosphate-buffered saline before photography. In a separate experiment, cells were treated with guggulipid, commipheric acid, ciglitazone, rosiglitazone, E-guggulsterone or Z-guggulsterone (0.15, 0.3, 0.6, 1.25, 2.5, 5, 10 μM; μg/ml for guggulipid), and staining was quantified by extracting Oil Red O for 10 min with 300 μl isopropanol per well. The isopropanol was transferred to a 96-well plate and absorbance was measured at 540 nm [22].

2.5. Animal studies

Studies were conducted in female C57Bl/6 Lep^{ob}/Lep^{ob} mice (Harlan Olac, Bicester, UK) maintained at 23±1°C with lights on from 0700 to 1900. Procedures were conducted in accordance with the University of Buckingham project licence under the Animals (Scientific Procedures) Act 1986 and as agreed by the University of Buckingham Ethical Review Board.

In an initial study, guggulipid was incorporated into pellets (prepared by Unilever, Netherlands) at a concentration of 20 g/kg chow. Mice were fed on standard chow or on pellets that contained guggulipid for 7 weeks. During Week 6, the mice were fasted for 4.5 h from 0700 and blood (100 μl) was taken from the tip of the tail for the measurement of plasma insulin (mouse standard, Crystal Chemistry, Downers Grove, IL, USA). The mice were given glucose (2 g/kg body weight) by oral gavage 30 min later. Blood (20 μl) was taken for measurement of glucose [Sigma Enzymatic (Trinder) colorimetric method] 30 min and immediately before, and 30, 60, 90 and 120 min after dosing glucose. The plasma triglyceride concentration was measured at the end of the study (Infinity Triglycerides reagent, Serosep, Limerick, Ireland).

Studies were subsequently conducted with commipheric acid (60, 240 and 960 mg/kg body weight, once daily at 0900, as an emulsion in sodium caseinate) and the ethyl ester of commipheric acid (150 mg/kg body weight, twice daily at 0900 and 1700, in sunflower oil), given by oral gavage. Control animals were given the vehicles (10 ml/kg body weight). There were six mice in each treatment group. Oral glucose tolerance tests were performed after giving commipheric acid for 2 weeks and 1 week, respectively. The plasma triglyceride concentration was measured after 2 weeks' treatment with the ethyl ester of commipheric acid, and the non-esterified fatty acid concentration after 4 weeks (colorimetric enzymic assay kit; Wako Chemicals, Neuss, Germany). Liver weight was measured after 3 weeks' treatment with commipheric acid.

2.6. Data analysis

Areas under the glucose tolerance curve and other manipulations and analyses of the data were performed using Prism software, version 4.03 (GraphPad Software, San Diego, CA, USA). Results from the animal experiments and intrinsic activities relative to standards in the reporter gene assays are presented as arithmetic means±S.E.M. EC_{50} values in the reporter gene assays were calculated relative to each compound's own maximum effect achieved. The maximum effects achieved were at 3 μg/ml for guggulipid and 3 μM for commipheric acid. It is unclear whether higher effects would have been achieved in the PPARγ and LXRα assays if cell toxicity had not precluded the use of higher concentrations. None of the compounds, with the possible exception of rosiglitazone, achieved a maximum effect in the adipocyte differentiation assay, and EC_{50} values are given relative to each compound's effect at 10 μM or, for guggulipid, 10 μg/ml. EC_{50} values are presented as geometric means with 95% confidence intervals because EC_{50} values typically follow a normal distribution only when logged [23]. Analysis (using logged values for the EC_{50} values) was by one-way analysis of variance followed by Bonferroni's post-test. The post-tests in the reporter gene assays were restricted to comparisons with the standard, and between guggulipid and commipheric acid.

3. Results

3.1. Reporter gene assays

Preliminary concentration curves (Fig. 2A) showed that 25 μM WY14643 and 5 μM ciglitazone elicited maximal responses for these compounds in the PPARα and PPARγ assays, respectively (Fig. 2A). These concentrations of the standard agonists were included in the PPARα and PPARγ assays conducted on guggulipid and commipheric acid. Rosiglitazone was much more potent and more efficacious than ciglitazone, guggulipid or commipheric acid. Although it did not elicit a maximal effect, 1 μM T0901317 was used as a standard in the LXRα assays because higher concentrations elicited variable effects. With the exception of commipheric acid in the PPARα assay, guggulipid and commipheric acid were toxic to the cells at concentrations greater than 3 μg/ml or 3 μM, respectively.

We focussed on commipheric acid because in a preliminary experiment, Fraction 3 (5 μg/ml) from the second fractionation, which contained 27.5% commipheric acid compared to 9.5% in guggulipid (Table 1), stimulated luciferase expression in a PPARγ reporter gene assay by

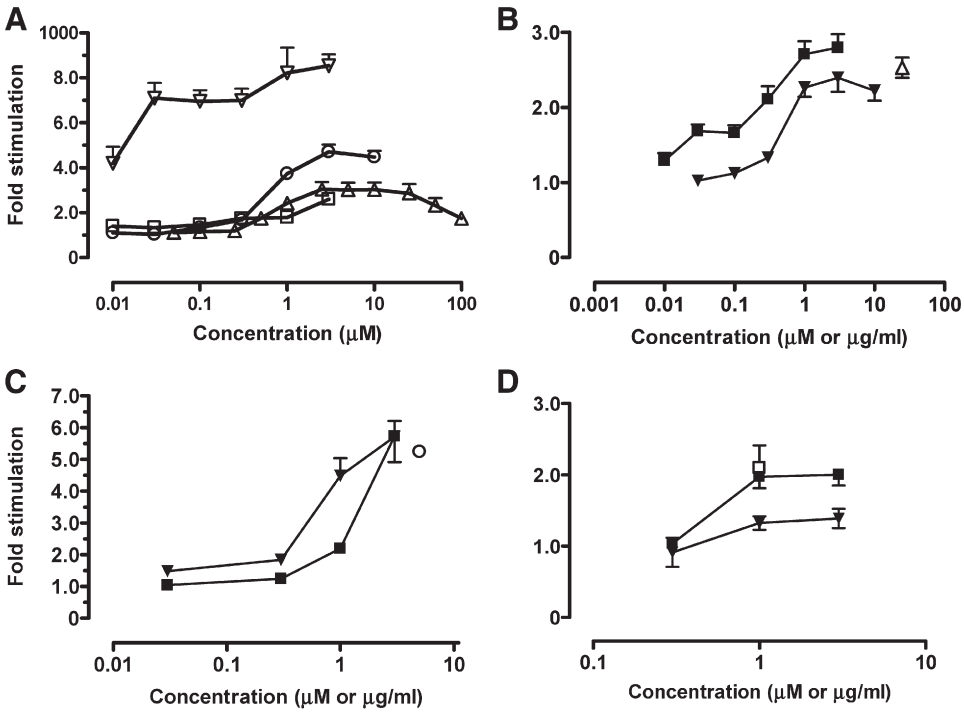

Fig. 2. Stimulation of luciferase expression by genes with response elements for PPARα, PPARγ and LXRα. (A) Concentration–response curves for stimulation of PPARα reporter gene expression by WY14643 (Δ), PPARγ reporter gene expression by ciglitazone (○) and rosiglitazone (▽), and LXRα reporter gene expression by T0901317 (□). (B–D) Concentration–response curves for stimulation of (B) PPARα, (C) PPARγ and (D) LXRα reporter gene expression by guggulipid (■) and commipheric acid (▼). Responses to single concentrations of WY 14643 (25 μM; Δ), ciglitazone (5 μM; ○) and T0901317 (1 μM; □) are shown in Panels (B), (C) and (D), respectively (n=6).

5.4-fold. Moreover, this fraction improved glucose tolerance in Lep^{ob}/Lep^{ob} mice (results not shown).

Guggulipid and commipheric acid elicited similar maximal increases in PPARα activity to 25 μM WY14643 (Fig. 2B; Table 2). The EC_{50} value of commipheric acid (0.55 μM) was similar to that of WY14643 (0.73 μM). Guggulipid appeared (though on the basis of the 0.03 μg/ml data point only) to have a biphasic concentration–response curve. This raises the possibility that, in addition to commipheric acid, guggulipid contained a higher potency but lower efficacy PPARα agonist. The EC_{50} value of guggulipid in micrograms per milliliter was about three times greater than that of commipheric acid (Table 2). E- and Z-guggulsterones (0.3 to 10 μM) had no effect (data not shown).

Guggulipid (3 μg/ml) and commipheric acid (3 μM) elicited similar increases in PPARγ activity to the maximal effect of 5 μM ciglitazone (Fig. 2C; Table 2). The EC_{50} value of commipheric acid (0.64 μM) was similar to that of ciglitazone (0.74 μM). The EC_{50} value of guggulipid in micrograms per liter was 7.6-fold greater than that of commipheric acid (Table 2). E- and Z-guggulsterones (0.3 to 10 μM) again had no effect (data not shown).

Guggulipid (3 μg/ml) caused a similar increase in LXRα reporter gene activity to 1 μM T0901317. Commipheric acid had only a small effect (Fig. 2D; Table 2). E- and Z-guggulsterones (0.3 to 10 μM) had no effect (data not shown).

3.2. Adipocyte differentiation

Guggulipid, commipheric acid, ciglitazone and rosiglitazone stimulated the differentiation of 3T3-L1 preadipocytes

Table 2
Intrinsic activities (maximum fold increase relative to standard) and potencies of compounds in reporter gene assays

Assay	Compound	Intrinsic activity	EC_{50} (95% CI)
PPARα	WY14643	[1.0]	0.73 (0.63–0.83) μM
	Guggulipid	1.18±0.10	0.82 (0.78–0.86) μg/ml
	Commipheric acid	1.10±0.14	0.55 (0.47–0.63) μM
			0.26 (0.22–0.30)*** μg/ml
PPARγ	Ciglitazone	[1.0]	0.74 (0.49–1.12) μM
	Rosiglitazone	2.32±0.58*	0.005 (0.003–0.007)** μM
	Guggulipid	1.17±0.17	2.3 (1.8–2.9) μg/ml
	Commipheric acid	1.09±0.10	0.64 (0.53–0.77) μM
			0.30 (0.25–0.36)*** μg/ml
LXRα	T0901317	[1.0]	0.71 (0.53–0.83) μM
	Guggulipid	1.08±0.08	0.66 (0.59–0.73) μg/ml
	Commipheric acid	0.21±0.01***	–

The fold increases for the standards were as follows: WY14643 (25 μM), 2.88±0.41; ciglitazone (5 μM), 4.48±0.28; T0901317 (1 μM), 2.59±0.20, in the PPARα, PPARγ and LXRα assays, respectively. Effects of other compounds are given relative to standard in the same assay. CI=confidence interval. n=6 for all means.

* P<.05 for difference from ciglitazone.

** P<.01 compared to ciglitazone.

*** P<.001 compared to guggulipid.

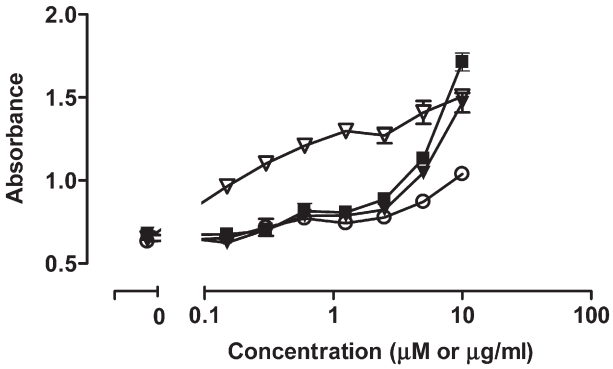

Fig. 3. Stimulation of 3T3-L1 preadipocyte differentiation: quantification by Oil Red O extraction into isopropanol after differentiation in the presence of rosiglitazone (▽), ciglitazone (○), guggulipid (■) or commipheric acid (▼) (n=6).

into lipid-containing adipocytes. Guggulipid and commipheric acid were similarly effective on a weight basis (Fig. 3, Table 3). They were more effective than ciglitazone over the range of concentrations used, but less effective than rosiglitazone. Fig. 4A–C shows the appearance of adipocytes after treatment with 5 μg/ml guggulipid, 5 μM commipheric acid or vehicle, respectively. E- and Z-guggulsterone (0.15 to 10 μM) had no effect (results not shown).

3.3. Animal studies

Guggulipid incorporated into pelleted diet improved oral glucose tolerance (P<.05). There was no significant effect of guggulipid on the fasting insulin concentration (Fig. 5C). Weight gain was decreased by guggulipid at the time of the glucose tolerance test (controls, 11.6±2.6 g; guggulipid, 2.6±1.1 g; P<.001), despite there being no effect on cumulative food intake (control groups, 56.1 and 61.3 g/mouse; guggulipid groups, 56.1 and 56.5 g/mouse). The terminal plasma triglyceride concentration was slightly but significantly reduced in the guggilipid-treated mice (controls, 2.70±0.06 mM; guggulipid, 2.50±0.08 mM; P<.05).

When commipheric acid was dosed orally, once daily for 3 weeks, it had no effect on food intake or body weight, and no effect after 2 weeks on oral glucose tolerance (results not shown). However, the highest dose (960 mg/kg body weight) increased liver weight (controls, 3.7±0.3 g; guggulipid, 4.5±0.2 g), consistent with activation of PPARα.

Treatment with the ethyl ester of commipheric acid (150 mg/kg body weight, twice daily for 7 days) did not affect food intake (control, 25.9 g; commipheric acid ester,

Table 3
Quantification of Oil Red O staining in preadipocyte differentiation assay

Compound	Maximum fold increase	EC_{50} (95% CI)
Ciglitazone	1.65±0.08	4.08 (1.55–6.60) μM
Rosiglitazone	2.34±0.12***	0.47 (0.28–0.66) μM***
Guggulipid	2.56±0.11*,***	5.92 (4.99–6.85) μg/ml
Commipheric acid	2.17±0.11**	6.47 (5.97–6.96) μM**
		3.05 (2.82–3.29) μg/ml

* P=.05 for difference from commipheric acid.
** P<.01 compared to ciglitazone.
*** P<.001 for difference from ciglitazone. n=6.

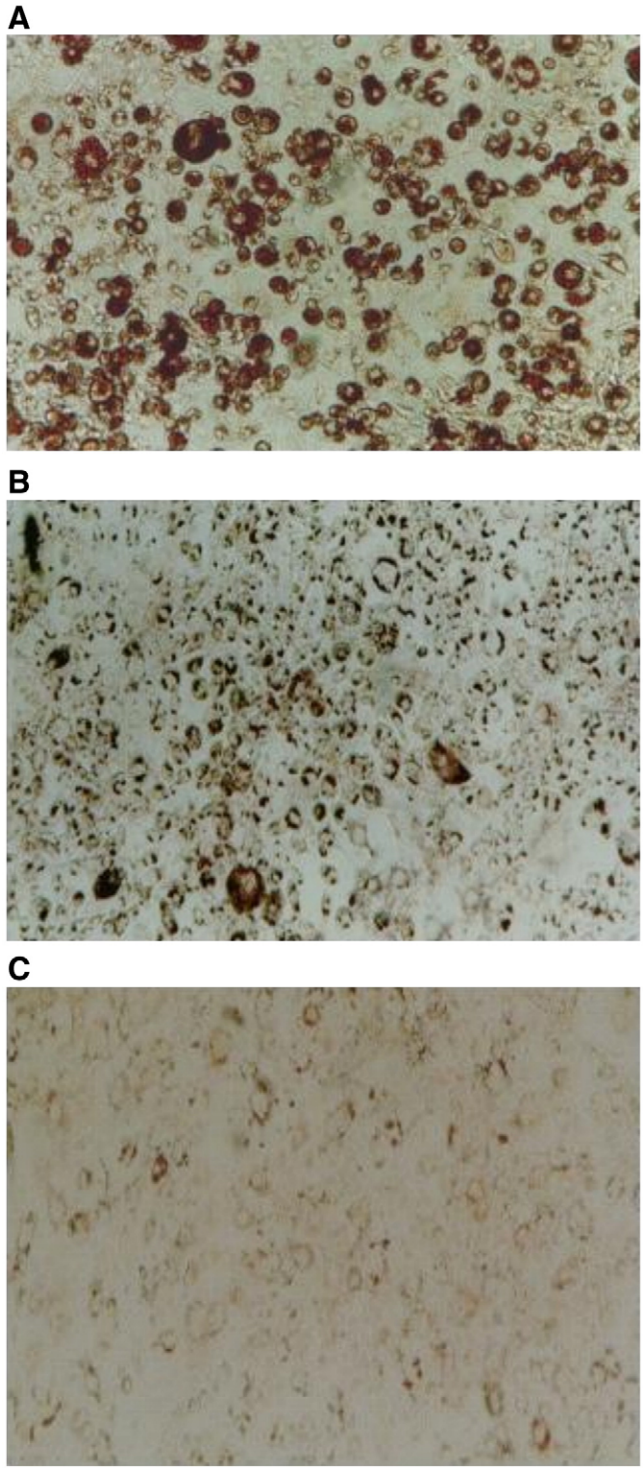

Fig. 4. Stimulation of 3T3-L1 preadipocyte differentiation: examples of appearance of cells after differentiation in the presence of (A) guggulipid (5 μg/ml), (B) commipheric acid (5 μM) or (C) vehicle.

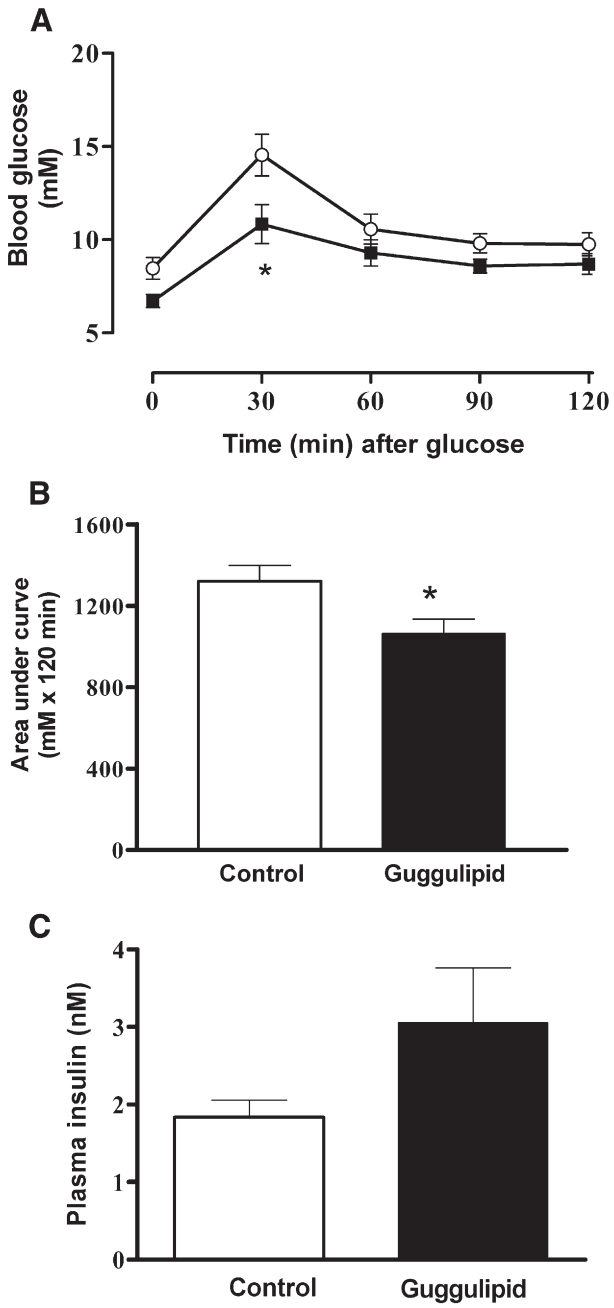

Fig. 5. (A) Oral glucose tolerance, (B) area under the glucose tolerance curve and (C) plasma insulin concentration prior to giving glucose in Lep^{ob}/Lep^{ob} mice fed on standard chow pellets (○) or chow pellets containing 2% guggulipid (■). The weight of the mice was 26.9±2.7 g at the start of treatment (means±S.D.). *P<.05 compared to control group. P=.06 for blood glucose at 0 min (n=10).

25.3 g), body weight gain (control, 4.5±0.7 g; commipheric acid ester, 4.3±0.3 g) or epidydimal, retroperitoneal or inguinal fat pad weights either (data not shown), but fasting blood glucose (Fig. 6A) and plasma insulin concentrations (Fig. 6C) were significantly reduced and there was a trend (P=.11) to an improvement in oral glucose tolerance (Fig. 6B). The control mice had higher glucose and insulin levels than those used for the guggulipid study, which was conducted 2 years previously. The mice used for the commipheric acid study, which was conducted between these studies, had intermediate glucose and insulin levels.

Treatment with the ethyl ester of commipheric acid for 13 days reduced the plasma triglyceride concentration (controls, 2.24±0.44 mM; commipheric acid, 1.15±0.22; P<.05). However, the plasma non-esterified fatty acid concentration was not significantly reduced after 28 days (controls, 0.91±0.02 mM; commipheric acid, 0.79±0.06; P=.20).

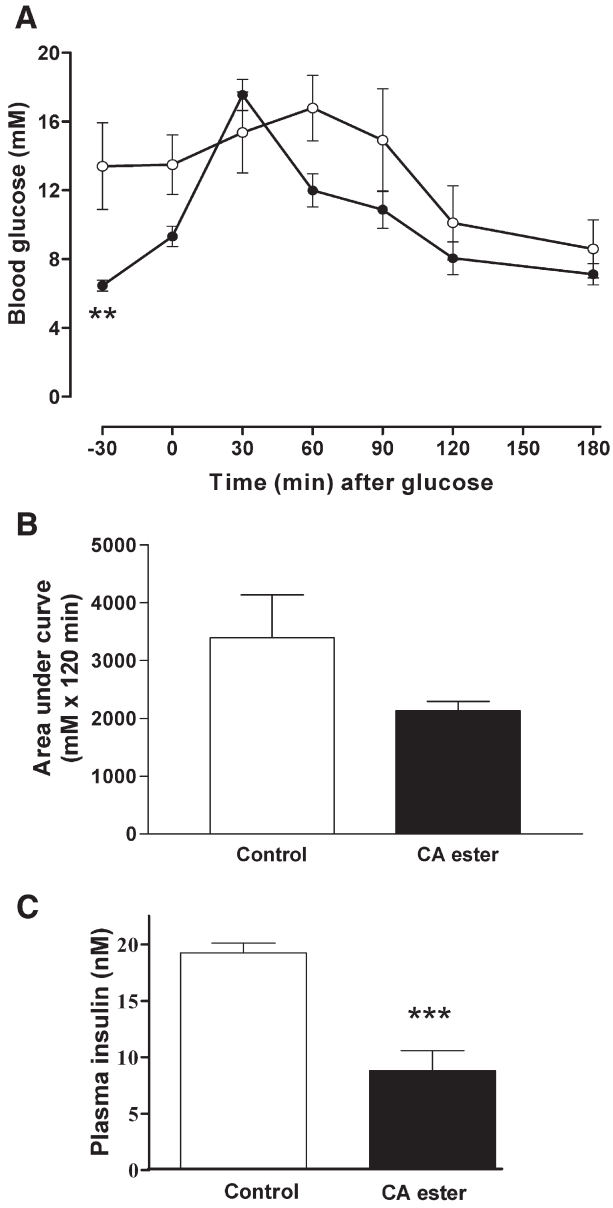

Fig. 6. (A) Oral glucose tolerance, (B) area under the glucose tolerance curve and (C) plasma insulin concentration 30 min prior to giving glucose in Lep^{ob}/Lep^{ob} mice fed on powdered chow diet and dosed twice daily with sunflower oil (10 ml/kg body weight; ○) or sunflower oil containing commipheric acid ethyl ester (150 mg/kg bodyweight; ●) for 7 days. The weight of the mice was 37.9±2.3 g on Day 0 and 41.9±3.0 g on Day 7 (means±S.D.). **P<.01; ***P<.001 compared to control mice (n=6).

4. Discussion

We have demonstrated that guggulipid has PPARα, PPARγ and LXRα agonist activity. PPARα and PPARγ agonism may contribute to the anti-diabetic activity that we observed when guggulipid was given in the diet of *Lep*^{ob}/*Lep*^{ob} mice, although this conclusion is complicated by the reduction in weight gain in response to guggulipid. PPARα and LXRα stimulation may contribute to the documented effects of guggulipid on dyslipidaemia. We have identified a novel activator of PPARα and PPARγ, commipheric acid, that may contribute to the anti-diabetic activity of guggulipid in *Lep*^{ob}/*Lep*^{ob} mice. Commipheric acid did not display anti-diabetic activity when given by oral gavage as the pure free acid, but its ethyl ester showed significant activity, reducing both the fasting blood glucose and plasma insulin concentrations after only 1 week of treatment with no effect on weight gain or food intake. Commipheric acid may be poorly absorbed, especially when given without food, and be better absorbed when given as the ethyl ester. We tried, unsuccessfully, to detect commipheric acid and its ester in plasma following administration of the ester. However, the detection limits of the assay for the acid and ester were 5.3 and 5.6 μg/ml, respectively, whereas the EC_{50} values for stimulation of PPARα and PPARγ by the acid were only 0.26 and 0.30 μg/ml, respectively. One caution when comparing between the animal studies is that, despite being of similar age and weight, the control mice in the different studies had rather different fasting glucose and insulin levels, apparently reflecting a drift to increased insulin resistance in the mice supplied over the course of the studies. PPARα agonism *in vivo* was supported by reductions in plasma triglyceride concentration following administration of guggulipid or the ethyl ester of commipheric acid.

A component of guggulipid other than commipheric acid activates LXRα. Previous investigations into the hypolipidaemic and hypocholesterolaemic effects of guggulipid have focussed on the interactions of E- and Z-guggulsterones with nuclear hormone receptors [13]. However, E- and Z-guggulsterone had no PPARα, PPARγ or LXRα agonist activity, in agreement with previous reports [8,13], nor did they promote fibroblast differentiation to adipocytes, consistent with their lack of PPARγ agonist activity.

4.1. PPARα and γ

Commipheric acid is a PPARα agonist of similar potency and efficacy to WY14643. It appears to make a significant contribution to the *in vitro* PPARα agonist activity of guggulipid, since it constitutes about one tenth of guggulipid and was about three times as potent as guggulipid on a weight basis in the PPARα reporter gene assay.

Commipheric acid is a PPARγ agonist of similar potency to the prototype PPARγ agonist ciglitazone. Commipheric acid appears to be responsible for much of the *in vitro* PPARγ agonist activity in guggulipid because it constitutes almost one tenth of guggulipid and was 7.6-fold more potent than guggulipid on a weight basis in the reporter gene assay. However, PPARγ agonists stimulate the differentiation of preadipocytes to adipocytes [24,25] and commipheric acid was only twice as potent as guggulipid in this assay — a non-significant difference in potency. It is possible that myrrhanone A/commipherol [16,17], which is present in guggulipid in a similar amount to commipheric acid (Table 1) and is also a PPARγ agonist [15], made a significant contribution to the PPARγ agonist activity of guggulipid.

PPARα agonists lower plasma triglyceride concentrations [26]. Guggulipid and the ethyl ester of commipheric acid reduced the plasma triglyceride concentration in our experiments. PPARα agonists also have insulin-sensitising effects in rodents, though not in humans [26,27]. PPARγ agonists have insulin-sensitising activity in both rodents and humans [28]. Therefore PPARα and possibly PPARγ agonism may be mechanisms by which guggulipid improved glucose tolerance in *Lep*^{ob}/*Lep*^{ob} mice.

PPARα agonists reduce, whereas PPARγ agonists usually promote, weight gain in *Lep*^{ob}/*Lep*^{ob} mice [29]. Some PPARγ agonists cause little or no weight gain in rodents, including *Lep*^{ob}/*Lep*^{ob} mice [30–32]. The combination of PPARα and PPARγ agonism, either in one compound or by administering separate PPARα and PPARγ agonists, may have little effect on, or reduce body weight in *Lep*^{ob}/*Lep*^{ob} mice [29,33–35]. In the experiment described here, commipheric acid ester did not affect food intake or weight gain. Guggulipid did not affect food intake, but it reduced body weight, possibly due to the presence of an additional component with PPARα activity.

4.2. Liver X receptor α

Guggulipid contains a compound or compounds that activate LXRα, but this is not commipheric acid, or E- or Z-guggulsterone. The presence of this component is another possible explanation for why commipheric acid was only twice as potent as guggulipid in the adipocyte differentiation (Oil Red O) assay (Table 3). Thus, although it is unclear whether LXR agonism stimulates preadipocyte differentiation [36,37], LXR agonists are able to augment fat accumulation in mature adipocytes differentiated in response to PPARγ receptor activation [38].

Despite increasing triglyceride synthesis in liver, muscle and adipose tissue [38,39], LXR agonists improve whole-body insulin sensitivity and glucose tolerance [40–43]. Thus the LXRα agonist activity of guggulipid may contribute to its anti-diabetic effects in *Lep*^{ob}/*Lep*^{ob} mice. This activity may also, together with the various interactions of E- and Z-guggulsterone with nuclear hormone receptors, contribute to the putative hypocholesterolaemic activity of guggulipid.

In conclusion, guggulipid contains compounds that activate PPARα, PPARγ and LXRα. These compounds are not E-guggulsterone or Z-guggulsterone, which interacts with other nuclear hormone receptors. Commipheric acid is at least partly responsible for the PPARα and PPARγ

activities, but not the LXRα activity. Commipheric acid appears to be poorly absorbed, but nevertheless these activities may contribute to the effects of guggulipid on glucose homeostasis in Lep^{ob}/Lep^{ob} mice.

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