British Journal of Pharmacology

British Journal of Pharmacology (2016) **173** 1219–1235 1219

RESEARCH PAPER

Madecassoside ameliorates bleomycin-induced pulmonary fibrosis in mice through promoting the generation of hepatocyte growth factor via PPAR-γ in colon

Correspondence Yue Dai and Zhi-Feng Wei, Department of Pharmacology of Chinese MateriaMedica, China Pharmaceutical University, 24 Tong Jia Xiang, Nanjing 210009, China. E-mail: yuedaicpu@hotmail.com; zhifeng-wei@hotmail.com

Received 3 April 2015; **Revised** 04 January 2016; **Accepted** 7 January 2016

Ying Xia*, Yu-Feng Xia*, Qi Lv, Meng-Fan Yue, Si-Miao Qiao, Yan Yang, Zhi-Feng Wei and Yue Dai

Jiangsu Key Laboratory of Drug Discovery for Metabolic Diseases, Department of Pharmacology of Chinese Materia Medica, China Pharmaceutical University, 24 Tong Jia Xiang, Nanjing 210009, China

*These authors equally contributed to this paper.

BACKGROUND AND PURPOSE

Madecassoside has potent anti-pulmonary fibrosis (PF) effects when administered p.o., despite having extremely low oral bioavailability. Herein, we explored the mechanism of this anti-PF effect with regard to gut hormones.

EXPERIMENTAL APPROACH

A PF model was established in mice by intratracheal instillation of bleomycin. Haematoxylin and eosin stain and Masson's trichrome stain were used to assess histological changes in the lung. Quantitative-PCR and Western blot detected mRNA and protein levels, respectively, and cytokines were measured by ELISA. Small interfering RNA was used for gene-silencing. EMSA was applied to detect DNA-binding activity.

KEY RESULTS

Administration of madecassoside, p.o., but not its main metabolite madecassic acid, exhibited a direct anti-PF effect in mice. However, i.p. madecassoside had no anti-PF effect. Madecassoside increased the expression of hepatocyte growth factor (HGF) in colon tissues, and HGF receptor antagonists attenuated its anti-PF effect. Madecassoside facilitated the secretion of HGF from colonic epithelial cells by activating the PPAR-γ pathway, as shown by an up-regulation of PPAR-γ mRNA expression, nuclear translocation and DNA-binding activity both *in vitro* and *in vivo*. Also GW9662, a selective PPAR-γ antagonist, almost completely prevented the madecassoside-induced increased expression of HGF and amelioration of PF.

CONCLUSIONS AND IMPLICATIONS

The potent anti-PF effects induced by p.o. madecassoside in mice are not mediated by its metabolites or itself after absorption into blood. Instead, madecassoside increases the activity of PPAR-γ, which subsequently increases HGF expression in colonic epithelial cells. HGF then enters into the circulation and lung tissue to exert an anti-PF effect.

Abbreviations

α-SMA, α-smooth muscle actin; aP2, adipocyte fatty acid-binding protein; BLM, bleomycin; BMP-7, bone morphogenetic protein-7; ECM, extracellular matrix; H&E, haematoxylin and eosin; HGF, hepatocyte growth factor; HT-29, human colon carcinoma cells line; PF, pulmonary fibrosis; PPREs, peroxisome proliferator response elements; Q-PCR, Quantitative-PCR; siRNA, small interfering RNA

DOI:10.1111/bph.13421

Tables of Links

TARGETS
Other protein targets[a]
Adipocyte fatty acid binding protein 4 (aP2)
Nuclear hormone receptors[b]
PPAR-γ
Catalytic receptors[c]
c-MET

LIGANDS	
Collagen type 1	IL-10
GW9662	PHA-665752
HGF	SU11274
IFN-γ	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c]Alexander *et al.*, 2015 a,b,c).

Introduction

Pulmonary fibrosis (PF) is a chronic, progressive and irreversible lung disease, and has a poor prognosis with a mean survival of 2 to 5 years after diagnosis (King *et al.*, 2011). Nevertheless, there are few effective treatments currently available for PF (Spagnolo *et al.*, 2015). Although pirfenidone and nintedanib have recently been approved by the Food and Drug Administration (FDA) for treating idiopathic PF, they have limited therapeutic efficacy and significant side effects. Therefore, it is imperative to develop satisfactory therapeutic drugs for PF.

Centella asiatica (Umbelliferae) is an annual herbaceous plant that has been widely used in Asia for its pleiotropic bioactivities. Triterpenoid compounds constitute its main active ingredients (Hashim *et al.*, 2011), of which madecassoside has been demonstrated to possess anti-inflammatory effects (Liu *et al.*, 2008a; Li *et al.*, 2009), to facilitate burn wounds (Liu *et al.*, 2008b) and inhibit keloid formation (Sampson *et al.*, 2001; Song *et al.*, 2012). Our recent studies showed that p.o. administration of madecassoside could markedly ameliorate mouse PF induced by intratracheal instillation of bleomycin (BLM), and this was accompanied by reduced deposition of extracellular matrix (ECM) and attenuated inflammation in the lung tissues (Lu *et al.*, 2014). However, the detailed mechanisms by which madecassoside produces these effects have not been elucidated.

As a triterpenoid saponin, madecassoside is extremely hard to absorb after p.o. administration (Wang *et al.*, 2014). It is mainly excreted in faeces in its original form and as aglycone madecassic acid, which is the main product of its metabolism in the intestine. The plasma or tissue concentration is much lower than the minimal effective concentration required for inhibition of the activation of lung fibroblasts, which are the key effector cells for PF (Leng *et al.*, 2013; Lu *et al.*, 2014). Such a pharmacokinetic-pharmacodynamic disconnection implies that the mode of action of madecassoside is distinct from currently used anti-PF drugs. With regard to this paradox, there might be two possibilities: (i) madecassoside functions via its metabolite madecassic acid; (ii) madecassoside exerts its anti-PF effect by enhancing the generation of endogenous anti-PF factors from the intestinal tract, which is the largest endocrine organ in the body that can secrete various growth factors and cytokines [*e.g.* hepatocyte growth factor (HGF), IFN-γ and IL-10] capable of interfering with fibrosis in many organs (Dignass and Sturm, 2001; Porowski *et al.*, 2009; Luzina *et al.*, 2014).

In the present study, we designed experiments to verify these two hypotheses with regard to the mechanism of the anti-PF effect of madecassoside and have provided evidence for a reasonable explanation for the anti-PF activity of other compounds with pharmacokinetic characteristics similar to those of madecassoside.

Methods

Animals

Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). Female ICR mice, 6–8 weeks-old and weighing 22–26 g, were purchased from the Comparative Medicine Centre of Yangzhou University (Yangzhou, China). The animal experiments were conducted with the approval of the Animal Ethics Committee of China Pharmaceutical University and conformed to the National Institute of Health guidelines on the ethical use of animals. All animals were housed under a 12 h light/12 h dark cycle (lights on from 7:00 to 19:00 h) with controlled room temperature (21–25°C) and humidity (50–65%) in the cages (290 × 178 × 160 mm), and allowed *ad libitum* access to a diet of standard laboratory chow and water.

BLM-induced PF model and treatment

On day 0, the mice were anaesthetized by administration of chloral hydrate hydrochloride (350 mg/kg, i.p.) and then PF was established in mice by the intratracheal instillation of BLM (5 $mg \cdot kg^{-1}$ in saline). Madecassoside (40 $mg \cdot kg^{-1}$ in saline) was administered p.o. or i.p. daily from day 1 to day 21; madecassic acid (10, 20 $mg \cdot kg^{-1}$ in 0.5% CMC-Na) was given p.o. daily from day 1 to day 21; SU11274 (0.18 $mg \cdot kg^{-1}$ in DMSO with a final concentration lower than 1 ‰); PHA-665752 (0.825 $mg \cdot kg^{-1}$ in DMSO with a final concentration lower than 1 ‰) and GW9662 (1 $mg \cdot kg^{-1}$ in DMSO with a final concentration lower than 1 ‰) were injected i.p. 0.5 h before madecassoside administration daily from day 1 to day 21 (Puri *et al.*, 2007; Gao *et al.*, 2013b, 2013a). Mice in the normal and model groups were given an equal volume of vehicle.

On day 21, the blood from each mouse was collected from the abdominal aorta. Then, mice were killed with an excess

dose of chloral hydrate hydrochloride. Lungs, small intestine and colon tissues were removed for further assay.

Group sizes

Group sizes were equal for each *in vivo* experiment and did not vary. In all experiments, the intestine and lung samples were taken from eight mice for each different group. In addition, all *in vitro* experiments were repeated three times.

Randomization

All the mice were divided into different groups according to the random comparison group methods in pharmacology. Vehicles and tested samples were randomly assigned to each group prior to the start of the experiment.

Blinding

All investigators conducting and analysing experiments were blinded to the respective groups. Data files were labelled with a date and sample identifier (*e.g.* number or time of the experiment). In addition, data were analysed in this format and then subsequently reassigned to their experimental conditions using lab records.

Normalization

Data were subjected to statistical analysis prior to normalization. Data sets were presented as mean of normal to allow for comparison of the results obtained from control and tested groups, in which there might be significant variation in the magnitude of the expression of different genes and proteins observed in the control responses of samples.

Statistical comparison

Data are presented as means ± SEM Statistical analysis was performed using SPSS statistical software (SPSS, Chicago, IL, USA). Statistical differences were assessed by one-way ANOVA followed by a *post hoc* Tukey's test. In cases where the latter condition was violated, non-parametric Games–Howell *post hoc* tests were used, and P values less than 0.05 ($P < 0.05$) were accepted as a significant difference. The correlation between two variables was evaluated by Spearman's nonparametric correlation analysis. P values less than 0.05 ($P < 0.05$) were considered statistically significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Lung index analysis

Body wt (g) of each mouse was recorded, and lung wet wt (mg) was determined immediately after its excision. The lung index ($mg \cdot g^{-1}$) was calculated by dividing the wet lung wt by the body wt (Niu *et al.*, 2013; Gao *et al.*, 2013b, 2013a).

Hydroxyproline assay

On day 21, the upper lobes of left lung tissues of mice were collected. The content of hydroxyproline in each lung lobe was measured by a commercial kit according to the manufacturer's instruction.

Histopathological examination

On day 21, the lower lobes of left lung tissues of mice were taken, and fixed in 10% neutral buffered formalin, embedded in paraffin and serially sectioned (5 μm) for haematoxylin and eosin (H&E) and Masson's trichrome staining respectively. The extent of the inflammation and fibrosis were scored by a pathologist blinded to the experimental groups. Pathological parameters mainly included 1, intra-alveolar congestion; 2, emphysema; 3, intra-alveolar epithelial hyperplasia; 4, interstitial and intra-alveolar infiltration of inflammatory cells; 5, collagen deposition. The results of H&E staining were graded according to parameters 1–4. The results of Masson's trichrome staining were graded according to parameter 5 (the bright blue part represented the mature collagen in the photograph). The score of each parameter was on a scale of 0–3 (criteria for grading were as follows: grade 0, normal; grade 0.5, slight; grade 1, mild; grade 2, moderate; and grade 3, severe). The score of each parameter was summed up for each mouse (Ji *et al.*, 2013; Lu *et al.*, 2014).

ELISA detection

On day 21, mouse blood was taken from the abdominal aorta. It was centrifuged for 15 min at about 700 g. The serum was obtained for immediate use or store at −80°C. The colon and right lung tissues of 100 mg samples were rinsed and homogenized with PBS, and stored overnight at −20°C. After two freeze–thaw cycles to break the cell membranes, the homogenates were centrifuged at about 3000 g for 5 min at 4°C. The relative factors in serum, colon and lung tissue homogenates were detected by ELISA.

Cell culture

Human colon carcinoma cells (HT-29 cells) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in DMEM supplemented with 10% FBS and maintained at 37°C in 5% CO_2 humidified air. The cells (4×10^5 $cells \cdot mL^{-1}$) were seeded into 96-well plates and treated with madecassoside for 24 h. Supernatants were collected and the levels of HGF were assayed by ELISA.

Cell viability assay

The viability of HT-29 cells was detected by use of the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. HT-29 cells (4×10^5 $cells \cdot mL^{-1}$) seeded in 96-well plates were incubated with madecassoside for 24 h. Four hours before the end of the incubation, 20 μL of MTT solution (5 $mg \cdot mL^{-1}$) was added into each well. Then, the supernatants were removed, 150 μL DMSO was added to dissolve the formazan crystal. The optical absorbance at 570 nm was read with a Model 1500 Multiskan spectrum microplate Reader (Thermo, Waltham, MA, USA).

EMSA

The DNA-binding activity of PPAR-γ was detected by use of an EMSA assay using a commercial kit (Pierce, Appleton, WI, USA). HT-29 cells (4×10^5 $cells \cdot mL^{-1}$) were treated with madecassoside for 12 h, and the nuclear proteins were extracted. Biotin-labelled PPAR-γ-specific oligonucleotides were prepared as the labelled probe according to the manufacturer's instructions. Nuclear extracts were mixed with poly (dI-dC), labelled probe, binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM PMSF, 1 $\mu g \cdot \mu L^{-1}$ aprotinin and

1 μg·μL^{-1} leupeptin) and incubated at room temperature for 10 min. Then, 10 μL of protein-DNA complex was subsequently fractionated on a gel electrophoresis at 10 V·cm^{-1} for 1 h at 4°C with 6.5% polyacrylamide gel and transferred to a nylon membrane. The biotin end-labelled DNA was detected using a Streptavidin-HRP conjugate and a chemiluminescent substrate. The membrane was then exposed to X-ray film and finally analysed using QUANTITY ONE software (Bio-Rad, CA, USA).

Western blot assay

The right lung tissues of mice were prepared. The α-SMA level in lung tissues and PPAR-γ level in HT-29 cells were detected by Western blot. The proteins were extracted using a nuclear and cytoplasmic protein extraction kit (KeyGEN Biotech, Nanjing, China) and quantified by the Bradford assay. Samples were separated on a 10% SDS-PAGE gel and transferred to PVDF membranes. Membranes were blocked for 2 h at room temperature with 9% skimmed milk in Tris-HCl buffer saline (TBS)-Tween-20 and incubated with the corresponding antibodies. After being rinsed, the membranes were incubated with the secondary antibodies for 2 h and with enhanced chemiluminescent reagent for 2–10 min respectively. Then, they were exposed to an X-ray film.

Q-PCR assay

Total RNA from small intestines, colons, lung tissues and HT-29 cells were extracted using TRIzol reagent (Takara Bio, Otsu, Japan). The purity and quality of the RNA samples were examined with an UV spectrophotometer at 260 and 280 nm. Then, reverse transcriptase reaction and real-time PCR were performed by using commercial kits (Vazyme Biotech, Nanjing, China). The mRNA concentrations of the genes detected were normalized to that of GAPDH in each sample. The details of the primers (Sangon Biotech, Shanghai, China) are listed in Table 1.

Gene-silencing by siRNA

Three pairs of siRNA targeting human PPAR-γ together or one pair of scrambled RNA were designed and synthesized by RiboBio Co. (RiboBio, Guangzhou, China). The transfection of siRNA was performed using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 50 nM. After transfection for 24 h, cells were incubated with madecassoside for another 24 h. The supernatants and mRNA extracts were harvested for further analysis.

Chemicals and reagents

Madecassoside and madecassic acid (purities > 95%) were purchased from Nanjing Jing Zhu Biological Technology Co., Ltd. (Nanjing, China); BLM hydrochloride was purchased from Nippon Kayaku Co., Ltd. (Tokyo, Japan); a commercial kit for detection of hydroxyproline was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); FBS was purchased from Zhejiang Tianhang Biotechnology Co., Ltd. (Hangzhou, China); GW9662, a selective PPAR-γ antagonist, was purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA); PPAR-γ and α-smooth muscle actin (α-SMA) monoclonal antibodies were purchased from Epitopmics Inc. (Burlingame, CA, USA); β-actin monoclonal antibody was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA); IFN-γ ELISA kit was

Table 1

Primers used in Q-PCR

Primers		Sequence (5′ → 3′)
Collagen type I	Forward	TGTTGGCCCATCTGGTAAAGA
	Reverse	CAGGGAATCCGATGTTGCC
PPAR-γ	Forward	TCTCAGTGGAGACCGCCCAGG
	Reverse	GCTGCACGTGCTCTGTGACGAT
HGF	Forward	AGCACCATCAAGGCAAGGT
	Reverse	GACCAGGAACAATGACACCA
IFN-γ	Forward	CTGCTGATGGGAGGAGATGT
	Reverse	TGTCATTCGGGTGTAGTCACA
IL-10	Forward	GCCTTATCGGAAATGATCCA
	Reverse	AGGGTCTTCAGCTTCTCACC
Klotho	Forward	GTTGGGTCACTGGGTCAATC
	Reverse	CGCCCTAAACTTGTCATCGT
BMP-7	Forward	GTACGTCAGCTTCCGAGACC
	Reverse	GGTGGCGTTCATGTAGGAGT
CD36	Forward	CTCATGCCAGTCGGAGACATGC
	Reverse	GCTGTTCTTTGCCACGTCATCTGG
aP2	Forward	AAATCACCGCAGACGACAG
	Reverse	TCATAACACATTCCACCACCA
GAPDH	Forward	GACATTTGAGAAGGGCCACAT
	Reverse	CAAAGAGGTCCAAAACAATCG

purchased from Dakewe Biotech Co., Ltd. (Shanghai, China); mouse HGF ELISA kit was purchased from CUSABIO Biotech Co., Ltd. (Wuhan, China); human HGF ELISA kit was purchased from ExCell Biology, Inc. (Shanghai, China); specific HGF receptor antagonists (SU11274/PHA-665752) were purchased from ApexBio Technology Co., Ltd. (Houston, TX, USA); enhanced chemiluminescent plus reagent kit was purchased from DiZhao Biotech Co., Ltd. (Nanjing, China); TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA); HiScript™ reverse transcriptase system and SYBR® green master mix were purchased from Vazyme Biotech Co., Ltd. (Nanjing, China); small interfering RNA (siRNA) PPAR-γ was purchased from RiboBio Co. (RiboBio, Guangzhou, China); lipofectamine 2000 was purchased from (Invitrogen, Carlsbad, CA, USA).

Results

Madecassoside but not madecassic acid attenuated BLM-induced PF in mice

When madecassoside is administered p.o. it is eventually hydrolysed to madecassic acid by intestinal flora (Leng *et al.*, 2013). To identify its active form, madecassoside (40 mg·kg^{-1}) and madecassic acid (10, 20 mg·kg^{-1}) were given p.o. from day 1 to day 21 after instillation of BLM, and their anti-PF effects were observed and compared. As shown in Figure 1A, madecassoside decreased the lung index of mice, but madecassic acid seemed to be ineffective.

The deposition of collagen, the major component of ECM, is the most important factor for tissue fibrosis. Hydroxyproline is an indicator of collagen as it is maintained as a constant amount in collagen. Figure 1B, C show that the hydroxyproline content and mRNA level of collagen type I markedly increased in the lung tissues of PF mice. Madecassoside decreased the levels of hydroxyproline and collagen type I: in contrast, madecassic acid had no marked effect on levels of hydroxyproline and collagen type I.

As shown in Figure 1D, E, the lungs of mice in the model group displayed collapsed alveolar spaces, emphysema, severe infiltration of inflammatory cells and fibroblast proliferation, as well as excessive collagen deposition. Madecassoside, but not madecassic acid, was able to protect lung tissues from structural damage, inflammation and collagen deposition.

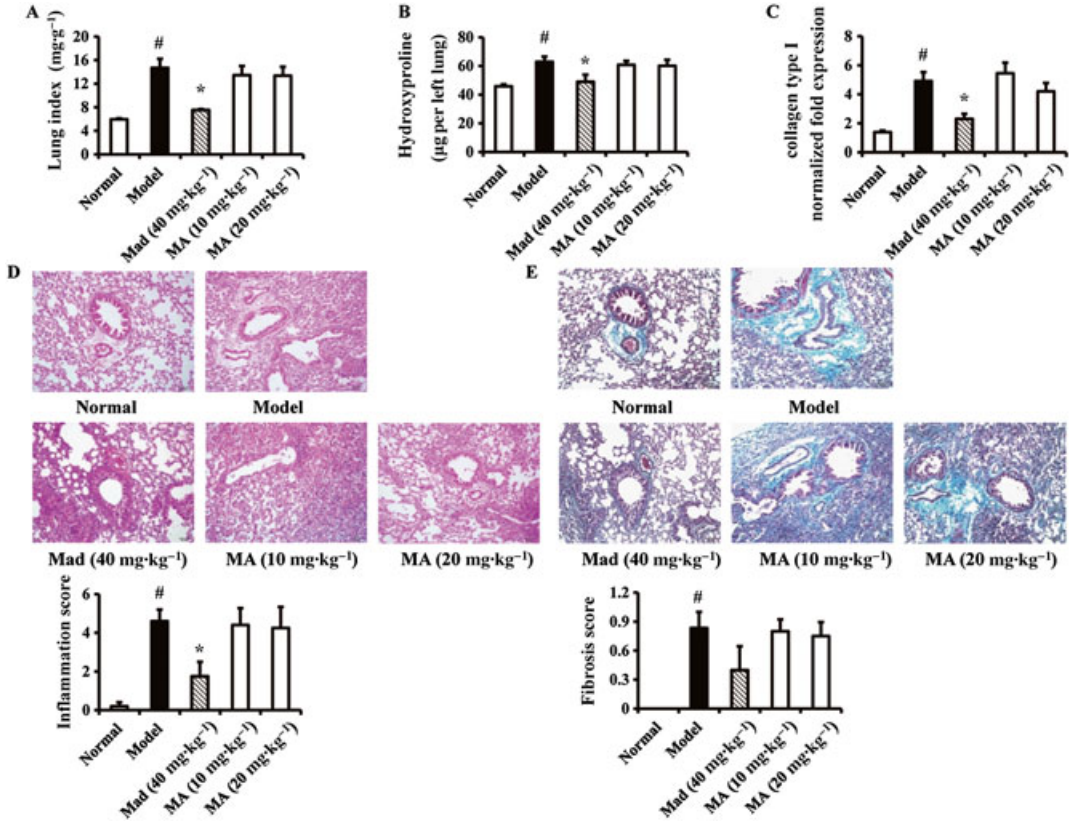

Figure 1

Effects of madecassoside (Mad) and madasiatic acid (MA) on BLM-induced PF in mice. (A) Body wt (g) of each mouse was recorded, and lung wet wt (mg) was determined immediately after it had been removed. The lung index (mg·g^{-1}) was calculated by dividing the wet lung wt by the body wt. (B) The hydroxyproline concentrations in the upper lobes of left lung tissues were measured by using kits according to manufacturer's instructions. (C) The mRNA expression of collagen type I in the right lung tissues was measured by using Q-PCR assay. The relative expression of transcription factors was normalized to that of GAPDH in each sample. (D, E) Histopathological changes in the lower lobes of left lung tissues were examined by H&E stain and Masson's trichrome stain (original magnification 200×) respectively. The histological scores of all groups were calculated. Data are expressed as means ± SEM, n = 6–8. $^{\#}P < 0.05$ versus normal; $^{*}P < 0.05$ versus model.

Furthermore, to exclude the possibility that the anti-PF effect of madecassoside was due to inhibition of early inflammation, we administered madecassoside to mice p.o. daily from day 7 to day 21 after instillation of BLM. The results showed that madecassoside was also effective as anti-PF agent even when treatment started 7 days after BLM (data not shown). These findings indicate that madecassoside, but not its metabolite madecassic acid, exerts a direct anti-PF effect in mice.

The anti-PF effect of madecassoside was dependent on an intact intestinal tract

The oral bioavailability of saponins is generally low because of their poor permeability and microflora hydrolysis in intestines, and they are likely to be detained in the intestinal tract (Gao *et al.*, 2012; Feng *et al.*, 2014). To identify the importance of the intestine in the action of madecassoside, a parenteral administration was performed. The results showed that madecassoside had little effect in mouse PF when given by an i.p. injection for 21 days (Supporting Information Fig. S1e), which suggests that p.o. madecassoside does not exert its anti-PF effect by direct absorption into the circulation, but the intestinal tract might be its primary action site.

Madecassoside promoted the expressions of endogenous anti-PF factors in BLM-induced PF mice

The intestinal tract can generate various anti-fibrosis factors, including HGF, IFN-γ, IL-10, Klotho and bone morphogenetic

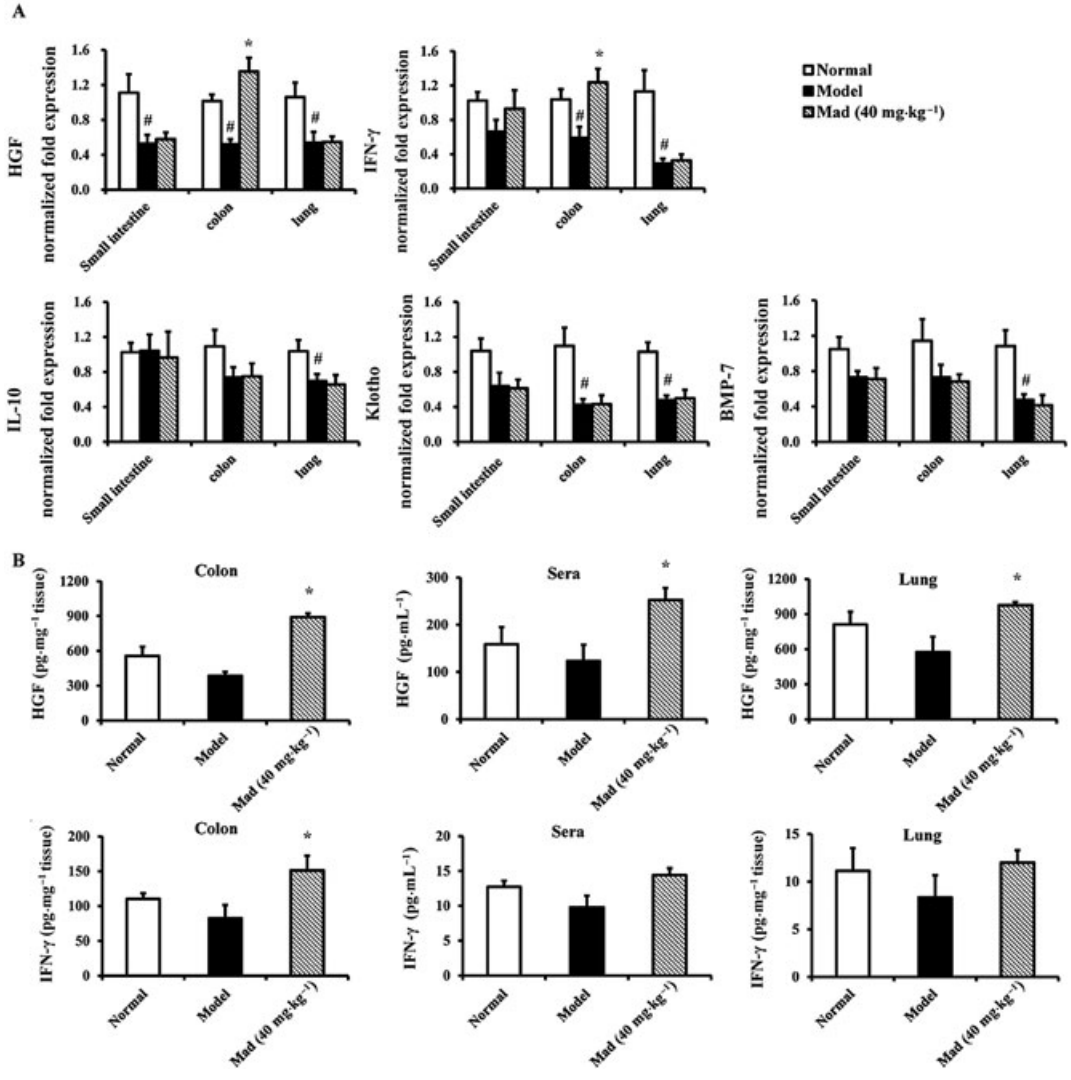

Figure 2

Effect of madecassoside (Mad) on the expressions of HGF, IFN-γ, IL-10, Klotho and BMP-7 in sera, small intestines, colons and lung tissues of BLM-treated mice. (A) The mRNA expressions of HGF, IFN-γ, IL-10, Klotho and BMP-7 in small intestines, colons and lung tissues were measured by using Q-PCR assay. The relative expression of transcription factors was normalized to that of GAPDH in each sample. (B) The contents of HGF and IFN-γ in colons, serum and lung tissues were determined by using ELISA assay. Data are expressed as means ± SEM, $n = 6$. $^{\#}P < 0.05$ versus normal; $^{*}P < 0.05$ versus model.

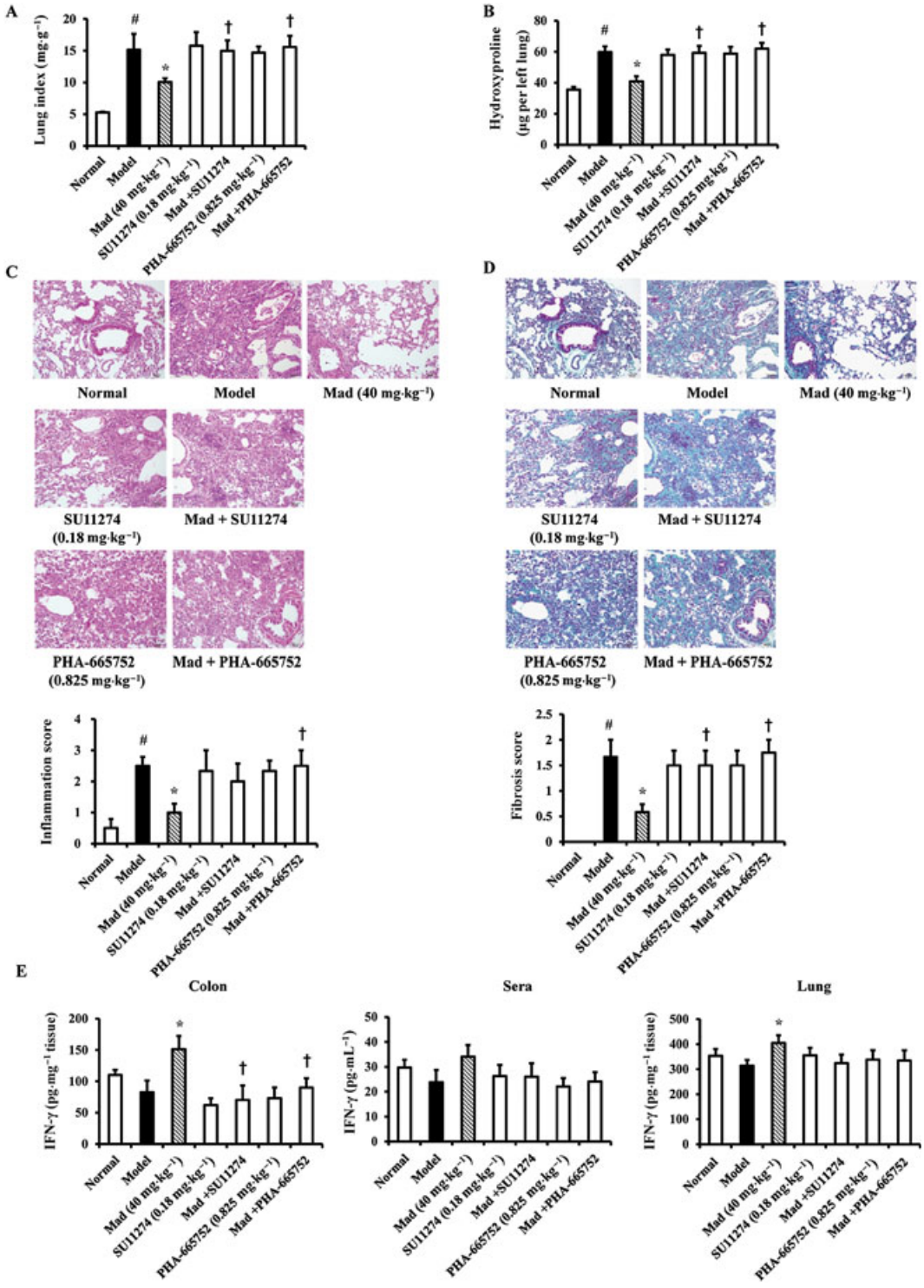

Figure 3

Influence of specific HGF receptor inhibitors SU11274 and PHA-665752 on the inhibition of madecassoside (Mad) against PF induced by BLM in mice. (A) Body wt (g) of each mouse was recorded, and lung wet wt (mg) was determined immediately after being removed. The lung index ($mg \cdot g^{-1}$) was calculated by dividing the wet lung wt by the body wt. (B) The contents of hydroxyproline in the upper lobes of left lung tissues were measured by using kits according to manufacturer's instructions. (C, D) Histopathological changes of the lower lobes of left lung tissues were examined by H&E stain and Masson's trichrome stain (original magnification 200×) respectively. The histological scores of all groups were calculated. (E) Effects of SU11274 and PHA-665752 on Mad-induced promotion of the content of IFN-γ in colons, sera and lung tissues. The content of IFN-γ was measured by using ELISA assay. Data are expressed as means ± SEM, $n = 6–8$. $^{\#}P < 0.05$ versus normal; $^{*}P < 0.05$ versus model; $^{\dagger}P < 0.05$ versus Mad.

protein-7 (BMP-7) (Luzina *et al.*, 2014). To further confirm the primary site of action for madecassoside and its underlying mechanisms, we compared its effects on the expressions of inhibitory factors in the small intestine, colon and lungs of PF mice. Results from the Q-PCR assay demonstrated that the mRNA expressions of the above factors, except for IL-10, decreased to different extents in small intestines, colons and lungs of mice with PF. Madecassoside showed little effect on the mRNA expressions of IL-10, Klotho and BMP-7 in the three organs examined, or on the expressions of HGF and IFN-γ in intestine and lungs. But it strikingly elevated mRNA levels of HGF and IFN-γ in the colons of mice (Figure 2A).

Next, the protein levels of two potential endogenous anti-PF factors HGF and IFN-γ in various organs were detected by ELISA. In contrast the levels of mRNA expressed, the protein levels of HGF and IFN-γ in the colon, sera and lung of PF mice were all increased by madecassoside treatment. Of note, the increases in the colon were markedly higher than that in sera and lungs, and the % increase of HGF was significantly higher than that of IFN-γ in the colon (Figure 2B). These findings suggest that the systemically elevated HGF and IFN-γ induced by madecassoside might originate from the colon, and IFN-γ might be secondary to HGF.

In addition, we employed Spearman bivariate correlation analysis to explore the relationship between the levels of HGF and IFN-γ in lungs and the anti-PF effect of madecassoside. The data show that collagen deposition (scores of Masson stain) shows a strong negative correlation with HGF levels ($rs = -0.949$, $P = 0.014$) and moderately correlated with IFN-γ levels ($rs = -0.738$, $P = 0.155$) in the lungs of PF mice treated with madecassoside (Supporting Information Fig. S2b). Interestingly, IFN-γ levels positively correlated with HGF levels in the colons and lungs of PF mice treated with madecassoside ($rs = 0.891$, $P = 0.037$ and $rs = 0.930$, $P = 0.037$ respectively) (Supporting Information Fig. S2). As HGF is able to promote the generation of IFN-γ (Benkhoucha *et al.*, 2010), we hypothesized that the increase in IFN-γ levels induced by madecassoside might be secondary to the up-regulation of HGF. Overall, these data indicate that the amelioration by madecassoside of collagen deposition and fibrosis in mouse lungs is closely correlated with elevated HGF levels.

Furthermore, when normal mice were given madecassoside (40 mg·kg^{-1}, p.o.) for 21 days, the mRNA and protein expressions of HGF in colon tissues were only slightly increased (data not shown). Therefore, it is likely that madecassoside only up-regulates the expression of HGF in the colons of mice treated with BLM.

Specific HGF receptor inhibitors attenuated the amelioration by madecassoside of BLM-induced PF in mice

To further define whether or not HGF plays a crucial role in the therapeutic effect of madecassoside, two different specific HGF receptor inhibitors SU11274 and PHA-665752 were used. As shown in Figure 3A–D, p.o. madecassoside (40 mg·kg^{-1}) was indeed able to attenuate BLM-induced PF in mice. Both

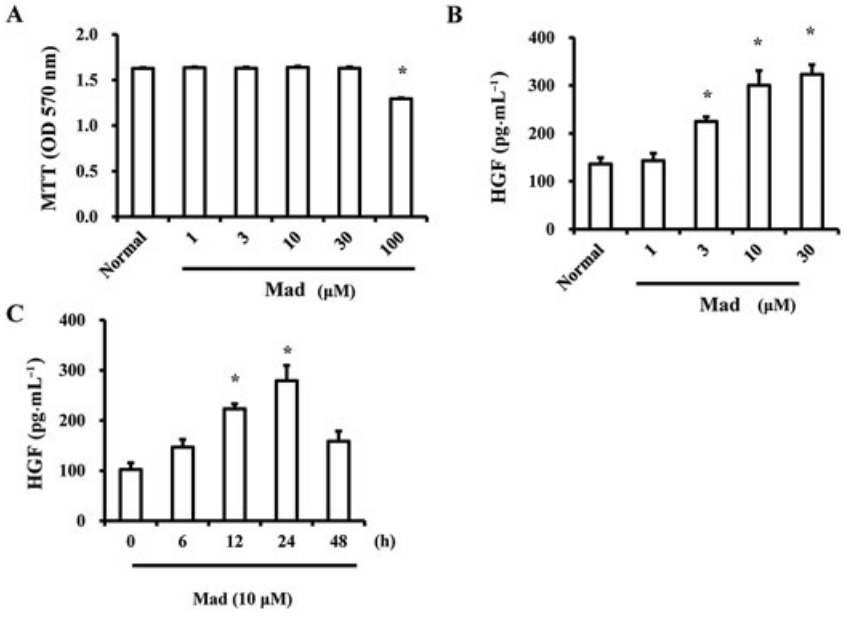

Figure 4

Effect of madecassoside (Mad) on HGF secretion from HT-29 cells. (A) Effect of Mad on the cell viability of HT-29 cells. Cells were incubated with or without Mad for 48 h. Cell viability was determined by MTT assay. (B) Concentration dependence of the HGF secretion from HT-29 cells. HT-29 cells were exposed to different concentrations of Mad for 24 h, and the level of HGF was assessed by using ELISA assay. (C) Time course of the HGF secretion from HT-29 cells. Cells were incubated with or without Mad (10 μM), and supernatants were collected at the indicated time points. The level of HGF was determined by using ELISA assay. Data are expressed as means ± SEM of three independent experiments. $^{*}P < 0.05$ versus normal.

SU11274 (0.18 mg·kg^{-1}) and PHA-665752 (0.825 mg·kg^{-1}) themselves did not affect the progress of PF, but they almost completely prevented the anti-PF effect of madecassoside. Moreover, the two inhibitors markedly decreased the contents of IFN-γ in colons, sera and lungs of PF mice (Figure 3E). These findings confirm that HGF is involved in the anti-PF effect of madecassoside, and the madecassoside-mediated increase in IFN-γ levels is secondary to HGF generation.

Madecassoside promoted HGF generation in colons of PF mice by activating PPAR-γ

There are reports suggesting that the gene expression of HGF is tightly regulated by the upstream regulator PPAR-γ because a peroxisome proliferator response element (PPRE) is present in the promoter region of the HGF gene (Jiang *et al.*, 2001; Li *et al.*, 2006). Recent studies showed that asiaticoside (an analogue of madecassoside) protected septic mice from lung injury probably through up-regulating the expression of PPAR-γ (Zhang *et al.*, 2011). Asiatic acid (the aglycone of asiaticoside) inhibited TGF-β-induced collagen synthesis in human keloid fibroblasts by activation of PPAR-γ (Bian *et al.*, 2013). These findings suggest that madecassoside might promote HGF generation in colons of PF mice through activation of PPAR-γ. *In vitro* and *in vivo* studies were then performed to identify the effect of madecassoside on the expression and activation of PPAR-γ.

Effect of madecassoside on cell viability of HT-29 cells. HT-29 cells were chosen for *in vitro* studies. To exclude the possibility that the effect of madecassoside was due to cytotoxicity, the viability of HT-29 cells was tested. It was shown that madecassoside (1–30 μM) did not affect the viability of HT-29 cells, while madecassoside (100 μM) obviously inhibited the viability (Figure 4A).

Effect of madecassoside on the expression of HGF in HT-29 cells. We examined the effect of madecassoside on HGF

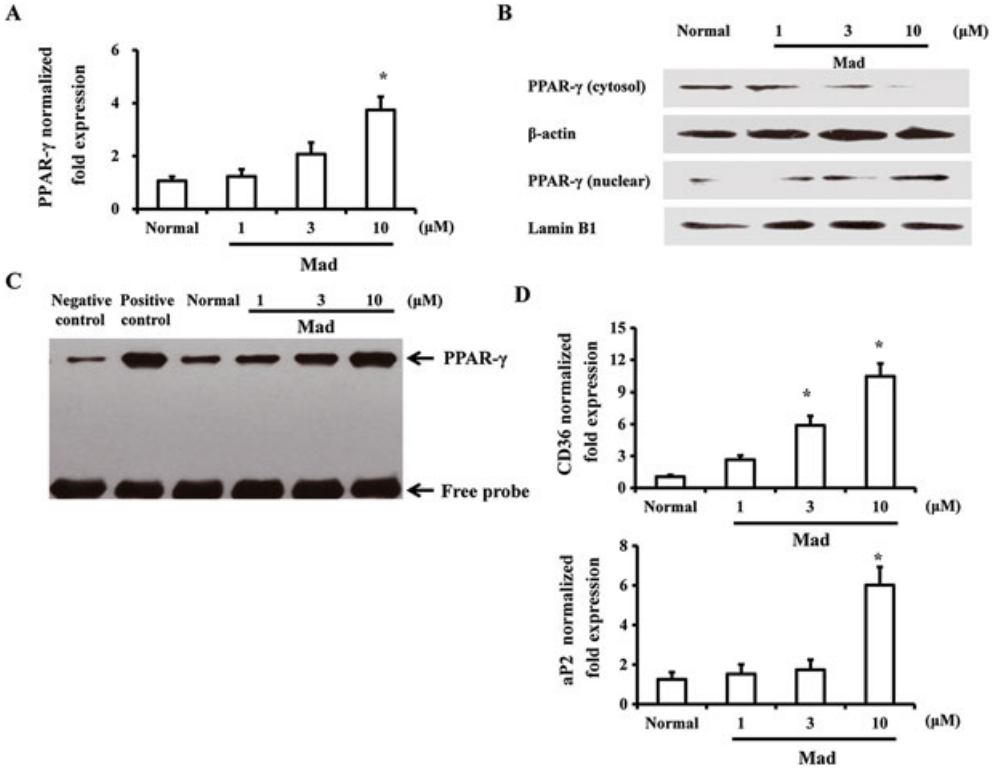

Figure 5

Effect of madecassoside (Mad) on the expression and activation of PPAR-γ in HT-29 cells. (A) HT-29 cells were treated with or without Mad for 12 h, and the mRNA expression of PPAR-γ was determined by using Q-PCR assay. (B) Effect of Mad on the nuclear translocation of PPAR-γ in HT-29 cells. HT-29 cells were treated with or without Mad for 24 h and then lysed. Protein of PPAR-γ in cytosol and nucleus was extracted for Western blot assay. (C) Effect of Mad on the DNA-binding activity of PPAR-γ was assessed by using EMSA assay. Lane 1 (negative control) contains only labelled DNA probe with no PPAR-γ protein; Lane 2 (positive control) contains a mix of PPAR-γ proteins and labelled DNA probe, plus 200-fold unlabelled DNA probe; Lanes 3, 4, 5 and 6 contain labelled DNA probe and PPAR-γ proteins extracted from HT-29 cells treated with or without Mad (1, 3, 10 μM) for 24 h respectively. (D) Effect of Mad on mRNA expressions of CD36 and aP2 in HT-29 cells. Cells were treated with or without Mad (1, 3, 10 μM) for 12 h, and the cell lysates were collected at indicated time points. The mRNA expressions of CD36 and aP2 were detected by using Q-PCR assay. The relative expressions of transcription factors were normalized to that of GAPDH in each sample. Data are expressed as means ± SEM of three independent experiments. $^{*}P < 0.05$ versus normal.

secretion from HT-29 cells. As depicted in Figure 4B, madecassoside (1, 3, 10, 30 μM) treatment up-regulated HGF secretion by 2.4, 64.7, 119.3 and 133.7% respectively.

We then examined the time course for madecassoside's promotion of HGF secretion in HT-29 cells. HT-29 cells were treated with or without madecassoside (10 μM) for 6, 12, 24, 48 h respectively. Madecassoside substantially increased HGF levels in cell supernatants with a peak at 24 h (Figure 4C). In the subsequent experiments, the incubation time of 24 h was used.

Effect of madecassoside on the expression and activation of PPAR-γ in HT-29 cells and BLM-induced PF mice. HT-29 cells were treated with madecassoside for 12 h, and the expression of PPAR-γ mRNA was studied. As shown in Figure 5A, madecassoside (10 μM) markedly increased PPAR-γ mRNA expression.

In unstimulated cells, PPAR-γ is evenly distributed between the cytosolic and nuclear compartments, and it will enter into the nucleus of cells after being activated. To assess the effect of madecassoside on the activation of the PPAR-γ signalling pathway, we firstly examined the nuclear translocation of PPAR-γ by Western blot analysis. As shown in Figure 5B, madecassoside treatment for 24 h concentration-dependently decreased the cytosol protein level of PPAR-γ but increased the nuclear level of PPAR-γ, suggesting that madecassoside enhanced the nuclear translocation of PPAR-γ in HT-29 cells.

Moreover, the effect of madecassoside on the DNA-binding ability of PPAR-γ was assessed by EMSA. The nuclear proteins were isolated from HT-29 cells treated with or without madecassoside (1, 3, 10 μM) for 24 h. The data show that madecassoside concentration-dependently increased the DNA-binding of PPAR-γ to PPRE in HT-29 cells (Figure 5C). In combination with findings obtained from the nuclear translocation investigation, madecassoside was considered to increase the transcriptional activity of PPAR-γ.

CD36 and adipocyte fatty acid-binding protein 4 (aP2) are the two key target genes of PPAR-γ (Han *et al.*, 2002). In HT-29 cells, madecassoside (1, 3, 10 μM) dramatically enhanced the expressions of CD36 mRNA by 169.8, 421.9 and 958.8%. At the concentration of 10 μM, madecassoside also enhanced the expression of aP2 mRNA (Figure 5D).

To further verify the ability of madecassoside to enhance PPAR-γ, we studied its effects on the expression and activation of PPAR-γ in BLM-induced PF mice. In normal mice, PPAR-γ mRNA was highly expressed in colons and lungs as compared with small intestines (duodenum, jejunum and ileum). In PF mice, the expression of PPAR-γ mRNA in various tissues strikingly decreased, and madecassoside (40 mg·kg^{-1}) treatment dramatically elevated PPAR-γ expression in colons but not in other tissues (Figure 6A, B), which also supports our aforementioned theory that the colon is the primary site of action of madecassoside. Moreover, our data showed that madecassoside markedly facilitated the mRNA expressions of CD36 and aP2 in colons of PF mice (Figure 6C).

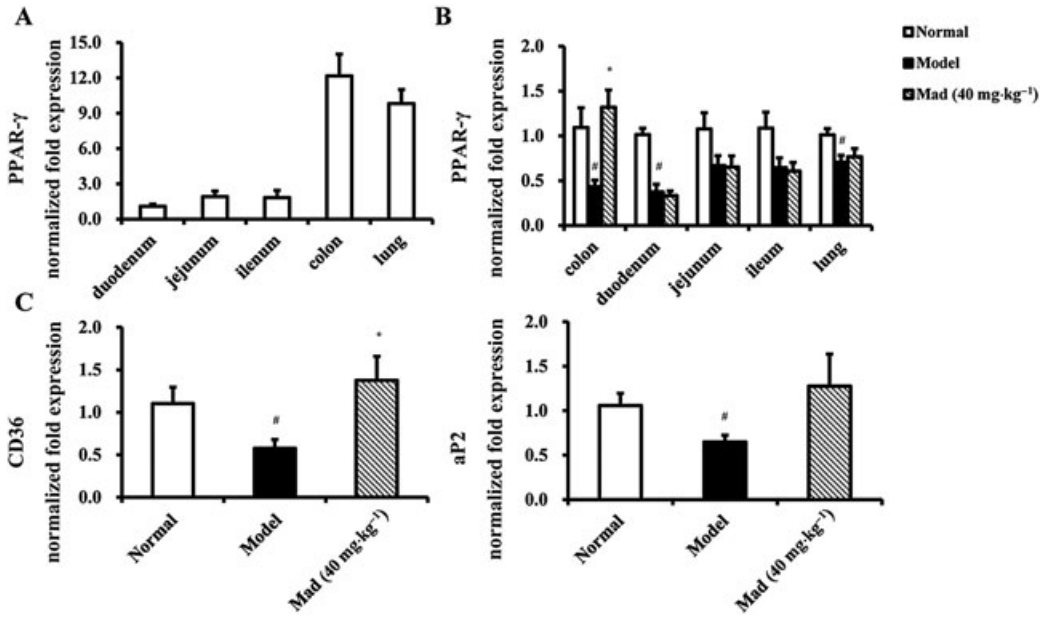

Figure 6

Effect of madecassoside (Mad) on the expression and activation of PPAR-γ in intestinal tracts and lung tissues of mice with PF induced by BLM. (A) Relative mRNA expression of PPAR-γ in duodenum, jejunum, ileum, colon and lung of normal mice was measured by using Q-PCR assay. (B) Effect of Mad on relative mRNA expression of PPAR-γ in duodenums, jejunums, ileums, colons and lung tissues of mice. Relative mRNA expression of PPAR-γ was measured by using a Q-PCR assay. (C) Effect of Mad on relative mRNA expressions of CD36 and aP2 in colons of mice. The relative expressions of transcription factors were measured by using a Q-PCR assay. Data are expressed as means ± SEM, $n = 6$. $^{\#}P < 0.05$ versus normal; $^{*}P < 0.05$ versus model.

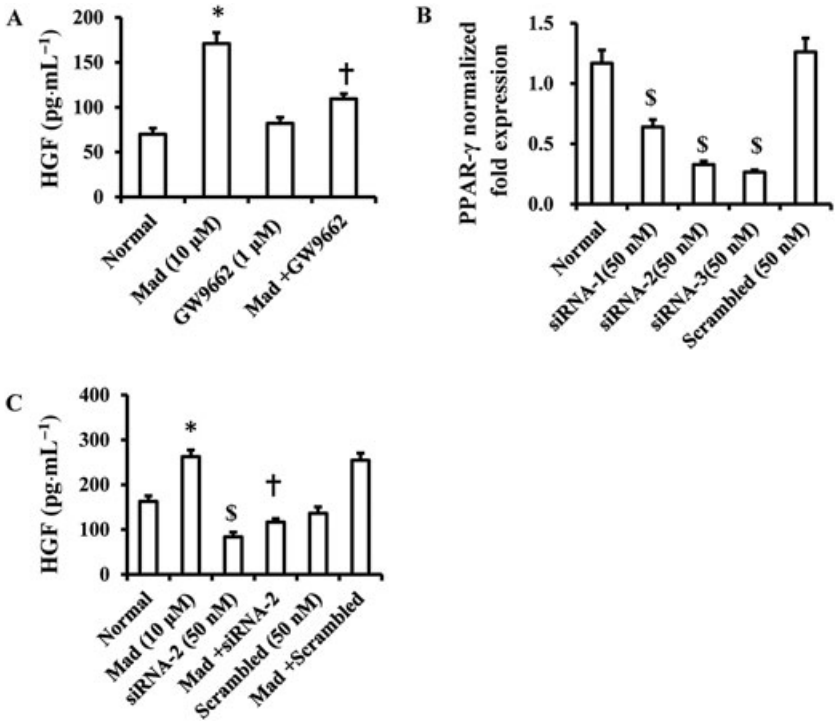

Figure 7

Effects of PPAR-γ antagonist GW9662 and siRNA gene-silencing on the up-regulation of HGF in HT-29 cells by madecassoside (Mad) treatment. (A) The PPAR-γ antagonist GW9662 attenuated the up-regulation by Mad of HGF secretion from HT-29 cells. Cells were treated with GW9662 (1 μM), Mad (10 μM) or Mad (10 μM) + GW9662 (1 μM) for 24 h, then HGF concentration in the supernatants was determined by using an ELISA assay. (B) PPAR-γ mRNA expression in HT-29 cells was determined by using a Q-PCR assay after being incubated with or without three pairs of siRNA or scrambled RNA for 12 h. (C) PPAR-γ silencing attenuated the Mad-induced increase in HGF secretion from HT-29 cells. Cells were treated with scrambled RNA (50 nM), siRNA-2 (50 nM), Mad (10 μM), Mad (10 μM) + scrambled RNA (50 nM) or Mad (10 μM) + siRNA-2 (50 nM) for 24 h, then HGF concentration in the supernatant was determined by using an ELISA assay. Data ware expressed as means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{\$}P < 0.05$ versus normal; $^{\dagger}P < 0.05$ versus Mad.

Taken together, these results indicate that madecassoside enhances the expression and activation of PPAR-γ in the colonic epithelial cells of mice with PF.

Effect of a PPAR-γ antagonist and siRNA gene-silencing on madecassoside-mediated expression of HGF in HT-29 cells. The above findings demonstrated that madecassoside can facilitate HGF expression and PPAR-γ activation in colonic epithelial cells. The internal relationship between these two activities was verified by using the specific antagonist GW9662 and siRNA gene-silencing of PPAR-γ. GW9662 (1 μM) itself did not affect HGF expression in HT-29 cells but markedly attenuated madecassoside-mediated HGF expression (Figure 7A). Furthermore, we silenced the PPAR-γ gene in HT-29 cells with siRNA. As shown in Figure 7B, C, all three pairs of siRNA successfully interfered with the expression of PPAR-γ to different degrees. Pair 2 and Pair 3 were similarly effective, and Pair 2 was chosen randomly. Notably, PPAR-γ silencing prevented the increased HGF expression in HT-29 cells induced by madecassoside, in contrast scrambled RNA had little effect.

Effect of a PPAR-γ antagonist on madecassoside-mediated generation of HGF and amelioration of PF in BLM-treated mice

Finally, the correlation between activation of PPAR-γ, consequent generation of HGF and eventual anti-PF effect of madecassoside was verified. Mice were intratracheally instilled with BLM, and administered madecassoside (40 $mg \cdot kg^{-1}$, p.o.) and injected with the PPAR-γ antagonist GW9662 (1 $mg \cdot kg^{-1}$) i.p. for 21 days. The data show that GW9662 itself lacked significant bioactivity. However, it almost completely prevented the increased HGF levels caused by madecassoside in the colons and sera of mice (Figure 8A, B), which accords well with our *in vitro* findings (Figure 7A). Moreover, Figure 8C–H shows that GW9662 was able to reverse the anti-PF effect of madecassoside, as evidenced by lung index, hydroxyproline content, mRNA expression of collagen type I, histopathological changes and the level of α-SMA (a molecular hallmark of myofibroblasts). These findings strongly suggested that madecassoside attenuates BLM-induced PF by activation of PPAR-γ and subsequent generation of HGF in the colon.

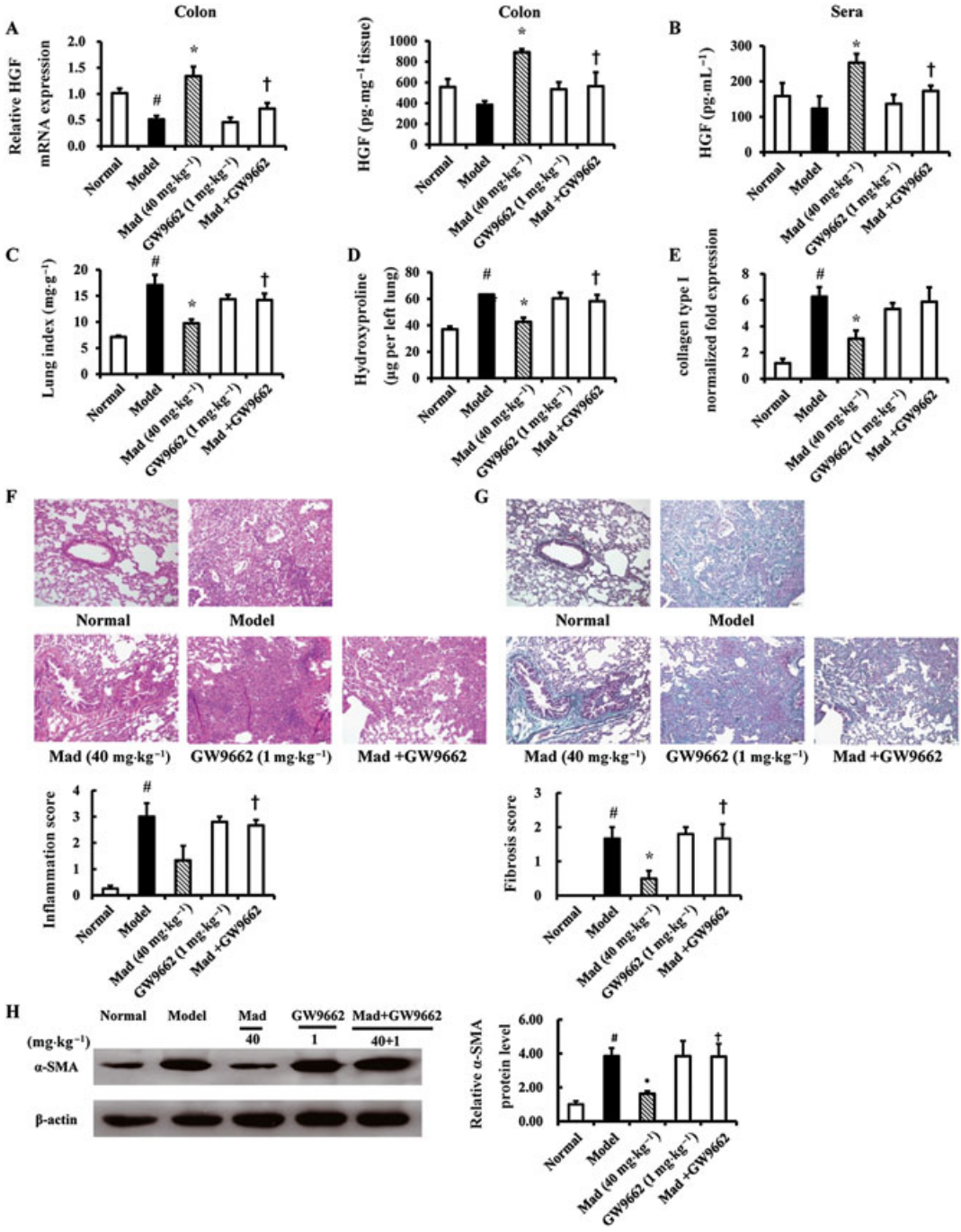

Figure 8

Effect of the PPAR-γ antagonist GW9662 on madecassoside (Mad)-mediated expression of HGF and amelioration of PF in BLM-treated in mice. (A) The relative mRNA and protein expression of HGF in colons were measured by using a Q-PCR and ELISA assay respectively. (B) Relative protein expression of HGF in serum was measured by using an ELISA assay. (C) The body wt (g) of each mouse was recorded and lung wet wt (mg) was determined immediately after its removal from the mouse. The lung index ($mg \cdot g^{-1}$) was calculated by dividing the wet lung wt by the body wt. (D) The content of hydroxyproline in the upper lobes of left lung tissues was measured by using kits according to the manufacturer's instructions. (E) Relative mRNA expression of collagen type I in the right lung tissues was measured by using a Q-PCR assay. (F, G) Histopathological changes in the lower lobes of left lung tissues were examined by H&E stain and Masson's trichrome stain (original magnification 200×) respectively. The histological scores of all groups were calculated. (H) The relative protein level of α-SMA in the right lung tissues was measured by using a Western blot assay. The relative expressions of proteins were normalized to that of β-actin in each sample. Data are expressed as means ± SEM, $n = 6–8$. $^{\#}P < 0.05$ versus normal; $^{*}P < 0.05$ versus model; $^{\dagger}P < 0.05$ versus Mad.

Discussion and conclusions

Our previous studies showed that p.o. madecassoside could strikingly ameliorate the pathological changes and reduce the collagen deposition in the lungs of mice with PF induced by BLM instillation. The effect of madecassoside was preliminarily attributed to inhibition of the inflammatory response, oxidative stress and consequent TGF-β overexpression. However, before madecassoside can be developed as an anti-PF drug, it is important to elucidate the mechanism and mode by which it mediates its anti-PF effects.

The triterpenoid saponin ingredients in plants are barely absorbed by the intestinal tract after p.o. administration. Meanwhile, they are easily degraded and metabolized by intestinal flora, and are transformed into secondary glycosides and aglycones (Hattori *et al.*, 1983; Han *et al.*, 2006; Paek *et al.*, 2006; Liang *et al.*, 2007). All these actions result in extremely low plasma level of saponins. Our previous studies demonstrated that madecassoside is eventually hydrolysed into its aglycone madecassic acid by rat intestinal flora (Leng *et al.*, 2013). In this study, we compared the anti-PF activities of madecassoside and madecassic acid. The data showed that madecassic acid had little effect on PF induced by BLM in mice, indicating that madecassoside might function by itself rather than by converting to madecassic acid. In addition, we demonstrated that the anti-PF effect of madecassoside was not due to inhibition of early inflammation. These findings indicate that madecassoside, but not its metabolite madecassic acid, exerts a direct anti-PF effect. Of course, our study could not exclude the possibility that the secondary glycosides might be the active forms of madecassoside.

There are few reports suggesting that the intestinal tract might be the target organ of natural products with low p.o. bioavailabilities (Lu *et al.*, 2009; Kang *et al.*, 2011). Our recent studies demonstrated that the anti-arthritic effect of p.o. madecassoside was probably achieved by its ability to increase IL-10 secretion from intestines (Wang *et al.*, 2015). Whether the anti-PF effect of madecassoside was also dependent on the intestinal tract was explored by observing its anti-PF activity when it was parenterally administered. Interestingly, when madecassoside was given by i.p. injection at the same dose as p.o., its anti-PF efficacy almost disappeared, suggesting that the anti-PF effect of madecassoside is dependent on an intact intestine.

The intestinal tract is the main local site that secretes growth factors and pleiotropic cytokines (Porowski *et al.*, 2009). TGF-β, connective tissue growth factor, platelet derived growth factor, HGF, EGFs, insulin-like growth factors, FGFs, ILs, IFNs, TNF, etc. are involved in the pathogenesis of PF, and can be roughly divided into profibrotic and anti-fibrotic agents (Atamas and White, 2003; Luzina *et al.*, 2014). TGF-β is the most powerful profibrotic growth factor that can stimulate the proliferation and differentiation of fibroblasts as well as epithelial-mesenchymal transition, and finally result in excessive production of ECM and an aberrant repair process in the lung tissues of patients (Border and Noble, 1994; Warburton *et al.*, 2013). In contrast, endogenous anti-fibrotic factors, such as IFN-γ, HGF, IL-10, Klotho and BMP-7, play negative regulatory roles in PF. IFN-γ-1b can improve the dyspnea symptoms of patients with idiopathic PF (Raghu *et al.*, 2004; Bouros *et al.*, 2006); HGF suppresses the fibrotic changes in the lung tissues of PF mice, and a small molecule mimetic of HGF has been used to treat patients with refractory PF (Yaekashiwa *et al.*, 1997; Dohi *et al.*, 2000; Chakraborty *et al.*, 2013); IL-10 gene delivered by rapid i.v. injection has been shown to attenuate BLM-induced PF in mice (Nakagome *et al.*, 2006); Klotho inhibits TGF-β1 signalling and suppresses renal fibrosis in mice (Doi *et al.*, 2011); BMP-7 markedly inhibits silica-induced PF by suppressing the activation of the BMP-7/Smad and TGF-β/Smad pathways (Yang *et al.*, 2013).

The findings mentioned above allowed us to postulate that madecassoside promotes the generation of endogenous anti-fibrotic factors from the intestinal tract, and these factors enter the blood circulation and lung tissues and display anti-PF effects. To verify this hypothesis, we investigated the effect of madecassoside on anti-fibrotic factors in the intestinal tract, blood and lung tissues of mice. The results showed that madecassoside did not affect the mRNA expressions of IL-10, Klotho and BMP-7 in small intestines, colons and lungs, and mRNA expressions of HGF and IFN-γ in small intestines and lungs, but markedly increased the expressions of HGF and IFN-γ mRNA in colon tissues of mice. In contrast, madecassoside significantly up-regulated the protein levels of HGF and IFN-γ in sera, lungs and colon tissues of mice. These seemingly incompatible alterations in mRNA and protein levels of HGF and IFN-γ in the sera and lung tissues of PF mice treated with madecassoside merited special attention. In combination with the pharmacokinetic features of madecassoside, we speculated that the increased HGF and IFN-γ in the mouse circulation and lung tissues evoked by madecassoside were originating from colon tissues.

Spearman bivariate correlation analysis was performed to assess the relationship between the increases in the two anti-fibrotic factors HGF and IFN-γ and anti-PF efficacy of madecassoside. As expected, the levels of HGF in lung tissues of mice treated with madecassoside were significantly negatively correlated with PF extent, and levels of IFN-γ were moderately negatively correlated with PF extent. Additionally, the increased HGF level induced by madecassoside was closely related to that of the IFN-γ level. There are reports indicating that HGF regulates the secretion of IFN-γ and is involved in the immunomodulatory effect of the latter (Wada *et al.*, 2009; Benkhoucha *et al.*, 2010). In agreement with this, our present study demonstrated that the specific HGF receptor antagonists (SU11274 and PHA-665752) markedly decreased IFN-γ levels in the colons, sera and lungs of mice with BLM-induced PF. These findings indicate that the increase in IFN-γ in the colon tissues of PF mice induced by madecassoside was secondary to that of HGF. Madecassoside might ameliorate mouse PF by promoting the generation of endogenous anti-fibrotic factors, in particular HGF, in the colon tissues.

The effect of HGF is mediated by its specific receptor c-METa TK receptor (Naldini *et al.*, 1991). Active HGF binding to c-MET results in the activation of the HGF/c-MET signalling pathway and displays numerous bioactivities including an anti-PF action (Crestani *et al.*, 2012). *In vitro*, HGF inhibits TGF-β1-induced differentiation of fibroblasts to the myofibroblast phenotype and enhances apoptosis of myofibroblasts (Mizuno *et al.*, 2005; Lee *et al.*, 2008). It also inhibits TGF-β-induced epithelial-mesenchymal transition of rat alveolar epithelial cells (Shukla

et al., 2009). Exogenous HGF can promote lung repair and reduce fibrosis in models of lung injury. Administration of HGF protein or sustained expression of HGF using an adenovirus markedly attenuates the fibrotic remodelling in both rat and mouse PF models (Yaekashiwa *et al.*, 1997; Adamson and Bakowska, 1999; Dohi *et al.*, 2000; Ebina *et al.*, 2002). An electrotransfer of HGF-encoding plasmid into muscle tissue can suppress BLM-induced fibrotic remodelling in mice (Umeda *et al.*, 2004). Furthermore, it was found that a defect in the generation of HGF in fibroblasts isolated from idiopathic PF patients resulted in a level of HGF insufficient to prevent the occurrence of PF, and the addition of exogenous HGF was found to reverse fibrosis and promote lung repair (Marchand-Adam *et al.*, 2003). These findings indicate that HGF is deeply involved in the pathogenesis of PF and has the potential to be of therapeutic value for PF. In the present study, to ascertain whether p.o. administration of madecassoside exerted its anti-PF effect by up-regulating HGF, two different specific HGF receptor antagonists were employed in mice with PF induced by BLM. The data show that both SU11274 and PHA-665752 almost completely reversed the inhibitory effect of madecassoside on PF, further demonstrating that the anti-PF effect of madecassoside was dependent on HGF.

With regard to the underlying mechanism by which madecassoside promotes HGF expression in the colon tissues of mice with PF, we focused on PPAR-γ, for the following reasons: (i) PPAR-γ functions mainly through ligand-dependent translocation from the cytosol into the nucleus binding as a heterodimer with the retinoid X receptor to specific DNA response elements (PPREs) within promoters, inducing the expression of several PPAR-γ-responsive genes, such as CD36 and aP2 (Ricote and Glass, 2007; Han *et al.*, 2002). PPRE is present in the promoter region of the HGF gene, which can increase local HGF gene expression (Jiang *et al.*, 2001); (ii) the PPAR-γ level in the colon of normal mice is strikingly higher than in other tissues. In the present study we further demonstrated that the expression of PPAR-γ mRNA was up-regulated exclusively in the colon of BLM-instilled mice, but not in small intestine and lung; (iii) PPAR-γ agonists telmisartan and irbesartan have been shown to exert anti-renal fibrosis actions via the PPAR-γ/HGF pathway (Kusunoki *et al.*, 2012; Kusunoki *et al.*, 2013).

In vitro studies confirmed that madecassoside concentration- and time-dependently facilitated the expression of HGF in HT-29 cells. It also up-regulated the mRNA expression, nuclear translocation and DNA-binding activity of PPAR-γ, as well as the expressions of PPAR-γ target genes CD36 and aP2. More importantly, both the PPAR-γ antagonist GW9662 and siRNA interference attenuated the madecassoside-induced increased expression of HGF in HT-29 cells. In BLM-induced PF mice, madecassoside was shown to facilitate the mRNA expressions of PPAR-γ, CD36 and aP2, and GW9662 treatment almost completely reversed the inhibitory effect of madecassoside on PF and interfered with the promotion of HGF expression. These findings strongly suggest that PPAR-γ might be the target protein of madecassoside for its promotion of HGF generation in colon tissues and consequent anti-PF effect.

In conclusion, the potent anti-PF effects induced by madecassoside in mice are not mediated by its metabolites or itself after absorption into blood, but through activation of PPAR-γ and the subsequent generation of HGF in the colon. The up-regulated HGF then probably enters the circulation and lung to impede PF. To our knowledge, this is the first time that the anti-PF mode of action of a compound with a pharmacokinetic-pharmacodynamic disconnection has been shown to be mediated by its effects on intestine-derived endogenous inhibitory factors. The present study provides a paradigm for the mechanistic studies of compounds with similar characteristics to madecassoside.

Acknowledgements

This work was supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions and partially supported by the National Natural Science Foundation of China (no. 81374038).

Author contributions

Y.X. and Z.F.W. performed all experiments and analysed the data. Q.L., M.F.Y., S.M.Q. and Y.Y. wrote the manuscript. Y.D. and Y.F.X. conceived of project and designed experiments. All authors read and approved the paper.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

References

Adamson Y, Bakowska J (1999). Relationship of keratinocyte growth factor an hepatocyte growth factor levels in rat lung lavage fluid to epithelial cell regeneration after bleomycin. Am J Pathol 155: 949–954.

Alexander SPH, Kelly E, Marrion N, Peters JA, Benson HE, Faccenda E, *et al.* (2015a). The Concise Guide to PHARMACOLOGY 2015/16: Overview. Br J Pharmacol 172: 5729–5143.

Alexander SPH, Cidlowski JA, Kelly E, Marrion N, Peters JA, Benson HE, *et al.* (2015b). The Concise Guide to PHARMACOLOGY 2015/16: Nuclear hormone receptors. Br J Pharmacol 172: 5956–5978.

Alexander SPH, Fabbro D, Kelly E, Marrion N, Peters JA, Benson HE, *et al.* (2015c). The Concise Guide to PHARMACOLOGY 2015/16: Catalytic receptors. Br J Pharmacol 172: 5979–6023.

Atamas SP, White B (2003). Cytokine regulation of pulmonary fibrosis in scleroderma. Cytokine Growth Factor Rev 14: 537–550.

Benkhoucha M, Santiago-Raber ML, Schneiter G, Chofflon M, Funakoshi H, Nakamura T, *et al.* (2010). Hepatocyte growth factor inhibits CNS autoimmunity by inducing tolerogenic dendritic cells and CD25 + Foxp3+ regulatory T cells. Proc Natl Acad Sci U S A 107: 6424–6429.

Bian D, Zhang J, Wu X, Dou Y, Yang Y, Tan Q, *et al.* (2013). Asiatic acid isolated from *Centella asiatica* inhibits TGF-β1-induced collagen expression in human keloid fibroblasts via PPAR-γ activation. Int J Biol Sci 9: 1032–1042.

Border WA, Noble NA (1994). Transforming growth factor beta in tissue fibrosis. N Engl J Med 331: 1286–1292.

Bouros D, Antoniou KM, Tzouvelekis A, Siafakas NM (2006). Interferon-gamma 1b for the treatment of idiopathic pulmonary fibrosis. Expert Opin Biol Ther 6: 1051–1060.

Chakraborty S, Chopra P, Hak A, Dastidar SG, Ray A (2013). Hepatocyte growth factor is an attractive target for the treatment of pulmonary fibrosis. Expert Opin Investig Drugs 22: 499–515.

Crestani B, Marchand-Adam S, Quesnel C, Plantier L, Borensztajn K, Marchal J, *et al.* (2012). Hepatocyte growth factor and lung fibrosis. Proc Am Thorac Soc 9: 158–163.

Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SPA, Giembycz MA, *et al.* (2015). Experimental design and analysis and their reporting: new guidance for publication in BJP. Br J Pharmacol 172: 3461–3471.

Dignass AU, Sturm A (2001). Peptide growth factors in the intestine. Eur J Gastroenterol Hepatol 13: 763–770.

Dohi M, Hasegawa T, Yamamoto K, Marshall BC (2000). Hepatocyte growth factor attenuates collagen accumulation in a murine model of pulmonary fibrosis. Am J Respir Crit Care Med 162: 2302–2307.

Doi S, Zou Y, Togao O, Pastor JV, John GB, Wang L, *et al.* (2011). Klotho inhibits transforming growth factor-beta1 (TGF-beta1) signaling and suppresses renal fibrosis and cancer metastasis in mice. J Biol Chem 286: 8655–8665.

Ebina M, Shimizukawa M, Narumi K, Miki M, Koinuma D, Watabe M, *et al.* (2002). Towards an effective gene therapy for idiopathic pulmonary fibrosis. Chest 121: 32S–33S.

Feng F, Xi-Yu X, Fu-Lei L, Wen-Yuan L, Ning X (2014). Triterpenoid saponins from *Patrinia scabra*. Chin J Nat Med 12: 43–46.

Gao S, Basu S, Yang Z, Deb A, Hu M (2012). Bioavailability challenges associated with development of saponins as therapeutic and chemopreventive agents. Curr Drug Targets 13: 1885–1899.

Gao W, Bing X, Li M, Yang Z, Li Y, Chen H (2013a). Study of critical role of c-Met and its inhibitor SU11274 in colorectal carcinoma. Med Oncol 30: 546.

Gao Y, Lu J, Zhang Y, Chen Y, Gu Z, Jiang X (2013b). Baicalein attenuates bleomycin-induced pulmonary fibrosis in rats through inhibition of miR-21. Pulm Pharmacol Ther 26: 649–654.

Han J, Hajjar DP, Zhou X, Gotto AM Jr, Nicholson AC (2002). Regulation of peroxisome proliferator-activated receptor-gamma-mediated gene expression. A new mechanism of action for high density lipoprotein. J Biol Chem 277: 23 582–23 586.

Han M, Sha X, Wu Y, Fang X (2006). Oral absorption of ginsenoside Rb1 using *in vitro* and *in vivo* models. Planta Med 72: 398–404.

Hashim P, Sidek H, Helan MH, Sabery A, Palanisamy UD, Ilham M (2011). Triterpene composition and bioactivities of *Centella asiatica*. Molecules 16: 1310–1322.

Hattori M, Sakamoto T, Kobashi K, Namba T (1983). Metabolism of glycyrrhizin by human intestinal flora. Planta Med 48: 38–42.

Jiang JG, Johnson C, Zarnegar R (2001). Peroxisome proliferator-activated receptor gamma-mediated transcriptional up-regulation of the hepatocyte growth factor gene promoter via a novel composite cis-acting element. J Biol Chem 276: 25 049–25 056.

Ji Y, Wang T, Wei ZF, Lu GX, Jiang SD, Xia YF, *et al.* (2013). Paeoniflorin, the main active constituent of *Paeonia lactiflora* roots, attenuates bleomycin-induced pulmonary fibrosis in mice by suppressing the synthesis of type I collagen. J Ethnopharmacol 149: 825–832.

Kang A, Hao H, Zheng X, Liang Y, Xie Y, Xie T, *et al.* (2011). Peripheral anti-inflammatory effects explain the ginsenosides paradox between poor brain distribution and anti-depression efficacy. J Neuroinflammation 8: 100.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). NC3Rs Reporting Guidelines Working Group. Br J Pharmacol 160: 1577–1579.

King TE Jr, Pardo A, Selman M (2011). Idiopathic pulmonary fibrosis. Lancet 378: 1949–1961.

Kusunoki H, Taniyama Y, Azuma J, Iekushi K, Sanada F, Otsu R, *et al.* (2012). Telmisartan exerts renoprotective actions via peroxisome proliferator-activated receptor-γ/hepatocyte growth factor pathway independent of angiotensin II type 1 receptor blockade. Hypertension 59: 308–316.

Kusunoki H, Taniyama Y, Rakugi H, Morishita R (2013). Cardiac and renal protective effects of irbesartan via peroxisome proliferator-activated receptorγ-hepatocyte growth factor pathway independent of angiotensin II Type 1a receptor blockade in mouse model of salt-sensitive hypertension. J Am Heart Assoc 2: e000103.

Lee YH, Suzuki YJ, Griffin AJ, Day RM (2008). Hepatocyte growth factor regulates cyclooxygenase-2 expression via beta-catenin, Akt, and p42/p44 MAPK in human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 294: L778–L786.

Leng DD, Han WJ, Rui Y, Dai Y, Xia YF (2013). *In vivo* disposition and metabolism of madecassoside, a major bioactive constituent in *Centella asiatica* (L.) Urb. J Ethnopharmacol 150: 601–608.

Liang MJ, Zhang WD, Zhang C, Liu RH, Shen YH, Li HL, *et al.* (2007). Quantitative determination of the anticancer agent tubeimoside I in rat plasma by liquid chromatography coupled with mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 845: 84–89.

Li H, Gong X, Zhang L, Zhang Z, Luo F, Zhou Q, *et al.* (2009). Madecassoside attenuates inflammatory response on collagen-induced arthritis in DBA/1 mice. Phytomedicine 16: 538–546.

Li Y, Wen X, Spataro BC, Hu K, Dai C, Liu Y (2006). Hepatocyte growth factor is a downstream effector that mediates the antifibrotic action of peroxisome proliferator-activated receptor-gamma agonists. J Am Soc Nephrol 17: 54–65.

Liu M, Dai Y, Li Y, Luo Y, Huang F, Gong Z, *et al.* (2008b). Madecassoside isolated from *Centella asiatica* herbs facilitates burn wound healing in mice. Planta Med 74: 809–815.

Liu M, Dai Y, Yao X, Li Y, Luo Y, Xia Y, *et al.* (2008a). Anti-rheumatoid arthritic effect of madecassoside on type II collagen-induced arthritis in mice. Int Immunopharmacol 8: 1561–1566.

Lu GX, Bian DF, Ji Y, Guo JM, Wei ZF, Jiang SD, *et al.* (2014). Madecassoside ameliorates bleomycin-induced pulmonary fibrosis in mice by downregulating collagen deposition. Phytother Res 28: 1224–1231.

Lu SS, Yu YL, Zhu HJ, Liu XD, Liu L, Liu YW, *et al.* (2009). Berberine promotes glucagon-like peptide-1 (7-36) amide secretion in streptozotocin-induced diabetic rats. J Endocrinol 200: 159–165.

Luzina IG, Todd NW, Sundararajan S, Atamas SP (2014). The cytokines of pulmonary fibrosis: much learned, much more to learn. Cytokine. doi:10.1016/j.cyto.2014.11.008. [Epub ahead of print]

Marchand-Adam S, Marchal J, Cohen M, Soler P, Gerard B, Castier Y, *et al.* (2003). Defect of hepatocyte growth factor secretion by fibroblasts in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 168: 1156–1161.

McGrath JC, Lilley E (2015). Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. Br J Pharmacol 172: 3189–3193.

Mizuno S, Matsumoto K, Li MY, Nakamura T (2005). HGF reduces advancing lung fibrosis in mice: a potential role for MMP-dependent myofibroblast apoptosis. FASEB J 19: 580–582.

Nakagome K, Dohi M, Okunishi K, Tanaka R, Miyazaki J, Yamamoto K (2006). *In vivo* IL-10 gene delivery attenuates bleomycin induced pulmonary fibrosis by inhibiting the production and activation of TGF-beta in the lung. Thorax 61: 886–894.

Naldini L, Weidner KM, Vigna E, Gaudino G, Bardelli A, Ponzetto C, *et al.* (1991). Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. EMBO J 10: 2867–2878.

Niu CH, Wang Y, Liu JD, Wang JL, Xiao JH (2013). Protective effects of neferine on amiodarone-induced pulmonary fibrosis in mice. Eur J Pharmacol 714: 112–119.

Paek IB, Moon Y, Kim J, Ji HY, Kim SA, Sohn DH, *et al.* (2006). Pharmacokinetics of a ginseng saponin metabolite compound K in rats. Biopharm Drug Dispos 27: 39–45.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP, *et al.* (2014). The IUPHAR/BPS guide to PHARMACOLOGY: an expert-driven knowledge base of drug targets and their ligands. Nucleic Acids Res 42: D1098–D1106.

Porowski D, Niemczyk M, Ziółkowski J, Mucha K, Foroncewicz B, Nowak M, *et al.* (2009). Intestine as source of cytokines and growth factors. Transplant Proc 41: 2989–2991.

Puri N, Khramtsov A, Ahmed S, Nallasura V, Hetzel JT, Jagadeeswaran R, *et al.* (2007). A selective small molecule inhibitor of c-Met, PHA665752, inhibits tumorigenicity and angiogenesis in mouse lung cancer xenografts. Cancer Res 67: 3529–3534.

Raghu G, Brown KK, Bradford WZ, Starko K, Noble PW, Schwartz DA, *et al.* (2004). A placebo-controlled trial of interferon gamma-1b in patients with idiopathic pulmonary fibrosis. N Engl J Med 350: 125–133.

Ricote M, Glass CK (2007). PPARs and molecular mechanisms of transrepression. Biochim Biophys Acta 1771: 926–935.

Sampson JH, Raman A, Karlsen G, Navsaria H, Leigh IM (2001). *In vitro* keratinocyte antiproliferant effect of *Centella asiatica* extract and triterpenoid saponins. Phytomedicine 8: 230–235.

Shukla MN, Rose JL, Ray R, Lathrop KL, Ray A, Ray P (2009). Hepatocyte growth factor inhibits epithelial to myofibroblast transition in lung cells via Smad7. Am J Respir Cell Mol Biol 40: 643–653.

Song J, Xu H, Lu Q, Xu Z, Bian D, Xia Y, *et al.* (2012). Madecassoside suppresses migration of fibroblasts from keloids: involvement of p38 kinase and PI3K signaling pathways. Burns 38: 677–684.

Spagnolo P, Wells AU, Collard HR (2015). Pharmacological treatment of idiopathic pulmonary fibrosis: an update. Drug Discov Today. doi: 10.1016/j.drudis.2015.01.001. [Epub ahead of print]

Umeda Y, Marui T, Matsuno Y, Shirahashi K, Iwata H, Takagi H, *et al.* (2004). Skeletal muscle targeting *in vivo* electroporation-mediated HGF gene therapy of bleomycin-induced pulmonary fibrosis in mice. Lab Invest 84: 836–844.

Wada N, Menicanin D, Shi S, Bartold PM, Gronthos S (2009). Immunomodulatory properties of human periodontal ligament stem cells. J Cell Physiol 219: 667–676.

Wang T, Leng DD, Gao FF, Jiang CJ, Xia YF, Dai Y (2014). A LC-ESI-MS method for the simultaneous determination of madecassoside and its metabolite madecassic acid in rat plasma: comparison pharmacokinetics in normal and collagen-induced arthritic rats. Chin J Nat Med 12: 943–951.

Wang T, Wei Z, Dou Y, Yang Y, Leng D, Kong L, *et al.* (2015). Intestinal interleukin-10 mobilization as a contributor to the anti-arthritis effect of orally administered madecassoside: a unique action mode of saponin compounds with poor bioavailability. Biochem Pharmacol 94: 30–38.

Warburton D, Shi W, Xu B (2013). TGF-β-Smad3 signaling in emphysema and pulmonary fibrosis: an epigenetic aberration of normal development? Am J Physiol Lung Cell Mol Physiol 304: L83–L85.

Yaekashiwa M, Nakayama S, Ohnuma K, Sakai T, Abe T, Satoh K, *et al.* (1997). Simultaneous or delayed administration of hepatocyte growth factor equally represses the fibrotic changes in murine lung injury induced by bleomycin. A morphologic study. Am J Respir Crit Care Med 156: 1937–1944.

Yang G, Zhu Z, Wang Y, Gao A, Niu P, Tian L (2013). Bone morphogenetic protein-7 inhibits silica-induced pulmonary fibrosis in rats. Toxicol Lett 220: 103–108.

Zhang LN, Zheng JJ, Zhang L, Gong X, Huang H, Wang CD, *et al.* (2011). Protective effects of asiaticoside on septic lung injury in mice. Exp Toxicol Pathol 63: 519–525.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13421

Figure S1 Effect of oral administration or intraperitoneal injection of madecassoside (Mad) on bleomycin (BLM)-induced pulmonary fibrosis (PF) in mice. (A) Body weight (g) of each mouse was recorded and lung wet weight (mg) was determined immediately after being removed. The lung index (mg/g) was calculated by dividing the wet lung weight by the body weight. (B) The content of hydroxyproline in the upper lobes of left lung tissues was measured by using kits according to manufacturer's instructions. (C) The mRNA expression of collagen type I in the right lung tissues was measured by using Q-PCR assay. The relative expression of transcription factors was normalized to that of GAPDH in each sample. (D, E) Histopathological changes of the lower lobes of left lung tissues were examined by hematoxylin and eosin (H&E) stain and Masson's trichrome stain (original magnification 200×), respectively. The histological scores of all groups were calculated. Data were expressed as means ± S.E.M., n = 6-8. $^{\#}p<0.05$ *vs*. normal; $^{*}p<0.05$ *vs*. model.

Figure S2 Spearman rank test. (A) Correlations between the fibrosis scores and HGF contents in lung tissues obtained from pulmonary fibrosis (PF) mice treated with madecassoside (Mad). (B) Correlations between the fibrosis scores and IFN-γ contents in lung tissues obtained from PF mice treated with Mad. (C) Correlations between the IFN-γ contents and HGF contents in colons and lung tissues obtained from PF mice treated with Mad. *Rs* = Spearman's rank correlation coefficient. $P < 0.05$ were considered as significant difference, $n = 5$.