

# Iristectorigenin B isolated from *Belamcanda chinensis* is a liver X receptor modulator that increases ABCA1 and ABCG1 expression in macrophage RAW 264.7 cells

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**Abstract** A novel liver X receptor (LXR) modulator, iristectorigenin B isolated from *Belamcanda chinensis*, stimulated the transcriptional activity of both LXR- $\alpha$  and LXR- $\beta$ . In macrophages, iristectorigenin B suppressed cholesterol accumulation in a dose-dependent manner and induced the transcriptional activation of LXR- $\alpha$ / $\beta$ -responsive genes, ATP-binding cassette transporters A1 and G1. It did not induce hepatic lipid accumulation nor the expression of the lipogenesis genes sterol regulatory element-binding protein-1c, fatty acid synthase, and stearoyl-CoA desaturase-1. Iristectorigenin B thus is a dual-LXR agonist that regulates the expression of key genes in cholesterol homeostasis in macrophage cells without inducing hepatic lipid accumulation.

**Keywords** *Belamcanda chinensis* · Cholesterol · Iristectorigenin B · LXR modulator

## Introduction

Coronary heart disease (CHD) is the leading cause of mortality in the Western world, accounting for nearly 50 % of all deaths (Bulliyya 2000). Major risk factors for the development of CHD are hypercholesterolemia and dyslipoproteinemia. Many studies have identified decreased high-density lipoprotein (HDL) and increased low-density lipoprotein (LDL) cholesterol as major contributors to CHD. Consequently, current therapies for the treatment of CHD are aimed at lowering LDL or increasing HDL-cholesterol.

Liver X receptors (LXRs) are ligand-activated transcription factors of the nuclear receptor superfamily. They are characterized as key transcriptional regulators of lipid and carbohydrate metabolism (Geyeregger et al. 2006). LXRs function as cholesterol

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sensors and regulators of a set of genes associated with cholesterol absorption, transport, efflux, and excretion, and as such, control whole body cholesterol homeostasis. The activation of LXRs results in improved reverse cholesterol transport and increased circulating levels of HDL. Treatment of atherosclerotic mice with synthetic LXR ligands, such as GW3965 and T0901317, inhibits the progression and promotes regression of atherosclerotic plaques. Furthermore, transplantation of macrophages that lack LXR- $\alpha$  and - $\beta$  into a host that is predisposed to atherogenesis results in increased foam cell differentiation and arterial plaque formation, even after treatment with LXR agonists (Joseph et al. 2002; Levin et al. 2005). These findings led to the identification of LXR agonists as potent antiatherogenic agents in rodent models of atherosclerosis.

Of importance, however, synthetic LXR agonists may induce lipogenesis, leading to increased plasma triacylglycerol concentrations and hepatic steatosis (Schultz et al. 2000). Thus, specific LXR ligands that do not induce fatty acid synthesis in the liver are of interest. Several groups have described such agents that have beneficial effects on lipid metabolism. Quinet et al. (2009) and Kratzer et al. (2009) identified two novel LXR agonists, WAY-252623 and *N,N*-dimethyl-3 $\beta$ -hydroxy-cholena-mide, that reduce atherosclerosis without activating SREBP1c or increasing hepatic lipogenesis. This finding raised the possibility that some of the anti-atherosclerotic effects of LXR agonists may be independent of systemic lipid metabolism in hepatocytes and may be attributable to direct actions on the vascular wall that activate reverse cholesterol transport. Hence, LXR is an attractive target for novel pharmaceutical agents.

*Belamcanda chinensis* is a perennial shrub that grows in East Asia including the Korean peninsula. It has been used as Asian traditional medicine for the treatment of inflammation and asthma as well as throat disorders such as cough, tonsillitis, and pharyngitis (Yamaki et al. 1990). In addition, isoflavonoids from this plant have physiological benefits including anti-inflammatory, anti-angiogenic, anti-cancer, anti-mutagenic, and hypoglycemic properties (Jung et al. 2003; Wozniak et al. 2006; Wu et al. 2011). In a previous study, we found that a methanol extract of *B. chinensis* roots had potent LXR agonist activity. In this study, we report that iristectorigenin B, isolated from the methanol extract of BC, has hypocholesterolemic activities that may reduce the risk of CHD.

Iristectorigenin B reduces macrophage cholesterol levels in vitro by activating LXR target genes, without inducing hepatic steatosis.

## Materials and methods

### Extraction, purification, and identification of iristectorigenin B from *B. chinensis*

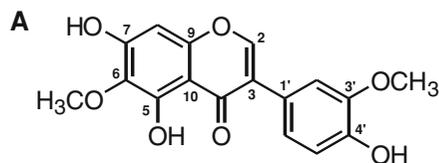
Powdered roots of *B. chinensis* were extracted three times with methanol at room temperature. After filtration and evaporation of the solvent under reduced pressure, the combined methanol extract (510 g) was suspended in water (1.5 l), and extracted into dichloromethane (DCM). The extract (150.5 g) was separated by silica gel column chromatography and eluted with DCM/methanol (1:0 to 0:1), to give 16 fractions (BCC1–BCC16). Fraction BCC3 was further purified by semi-preparative HPLC using a YMC ODS H-80 column, (150  $\times$  20 mm i.d.) and eluting with acetonitrile/water (70:30 v/v to 50:50 v/v) to obtain compound 1 (21.5 mg). The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker AMX 500 MHz nuclear magnetic resonance spectrometer using dimethyl sulfoxide (DMSO)- $d_6$  as a solvent. The melting point was measured on a Buchi model B-540 without correction. ESI-MS analysis was performed on an LCQ Fleet mass spectrometer.

### Methylthiazol tetrazolium (MTT) assay

The viability of cultured cells was determined based by the amount of MTT reduced to formazan (Lee et al. 2012). After treatment with various concentrations of iristectorigenin B, culture medium containing MTT (0.5 mg ml $^{-1}$ ) was added to each well, and the cells were incubated at 37 °C for 3 h before mixing with DMSO to dissolve the formazan crystals. Then the absorbance at 570 nm was measured. Values were normalized to the samples' protein concentration.

### Transfection and luciferase assays

The vectors used in luciferase assays included pGL4.35[luc2P/9XGAL4UAS/Hygro] (Promega, Madison, WI, USA), pSV- $\beta$ -galactosidase (kindly provided by Dr. Soo-Jong Um, Sejong University, Seoul, Korea), pFN26AhLXR $\alpha$ , and pFN26AhLXR $\beta$ . The



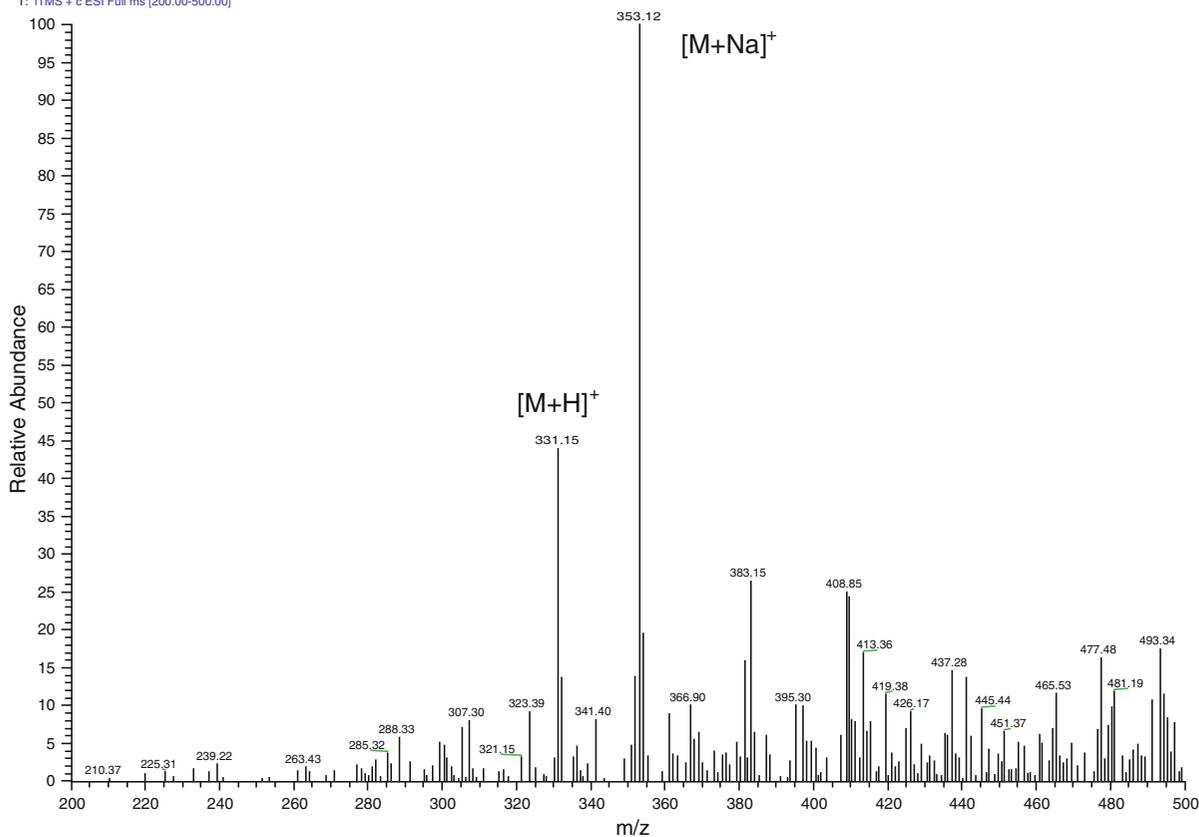
Iristectorigenin B

Chemical Formula:  $C_{17}H_{14}O_7$ 

Exact Mass: 330.07

**B**

bcc3cry\_100831164553 #342 RT: 1.21 AV: 1 NL: 6.69E4  
T: ITMS + c ESI Full ms [200.00-500.00]



ESI-MS spectrum of iristectorigenin B

**Fig. 1** Chemical structure (a), ESI-MS (b),  $^1\text{H}$  NMR (c), and  $^{13}\text{C}$  NMR (d) spectrum of iristectorigenin B isolated from *Belamcanda chinensis*

pFN26AhLXR $\alpha$  and pFN26AhLXR $\beta$  clones were constructed by subcloning the ligand-binding domains (LBDs) of hLXR- $\alpha$  (amino acids 137–102) and hLXR- $\beta$  (amino acids 209–468), respectively, into pFN26A(BIND)hRluc-neo Flexi (Promega), digested with *SgfI* and *PmeI*.

Transfection and reporter gene assays were performed using HEK 293 cells. Cells ( $2 \times 10^5$

cells  $\text{ml}^{-1}$ ) were plated in 24-well culture plates and then incubated in Dulbecco's Modified Eagle Medium (DMEM) without antibiotics. On the following day, cells were cotransfected with pGL4.35[luc2P/9XGA-L4UAS/Hygro], pSV- $\beta$ -galactosidase, and either pFN26AhLXR $\alpha$  or pFN26AhLXR $\beta$  using HilyMax transfection reagent (Dojindo Molecular Technologies, Gaithersburg, MD, USA) according to the

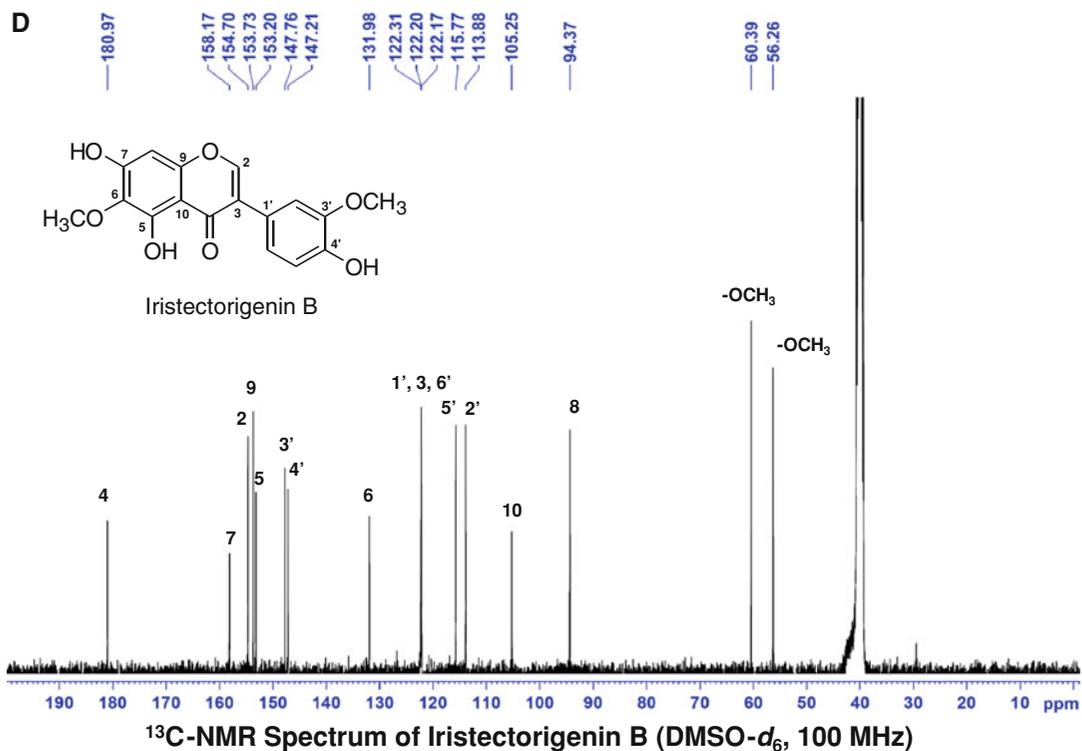
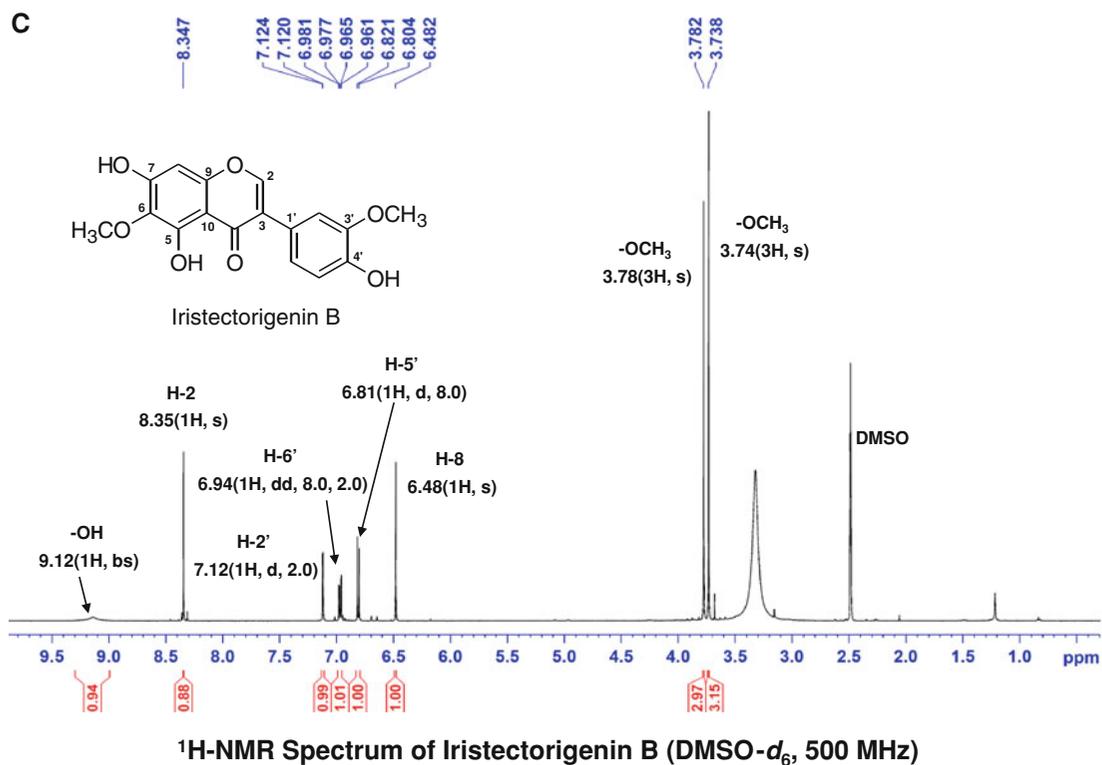
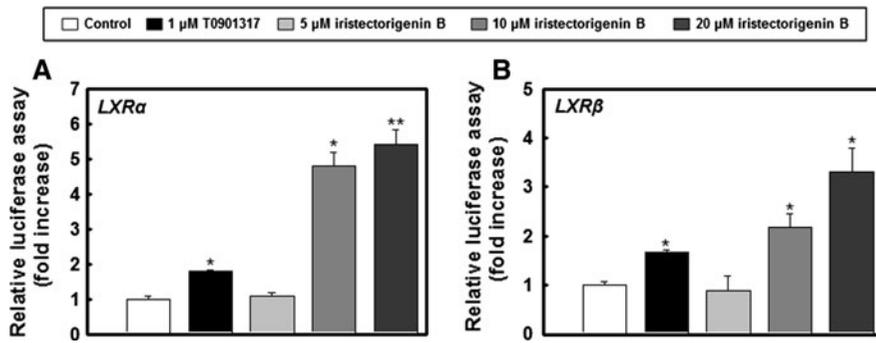


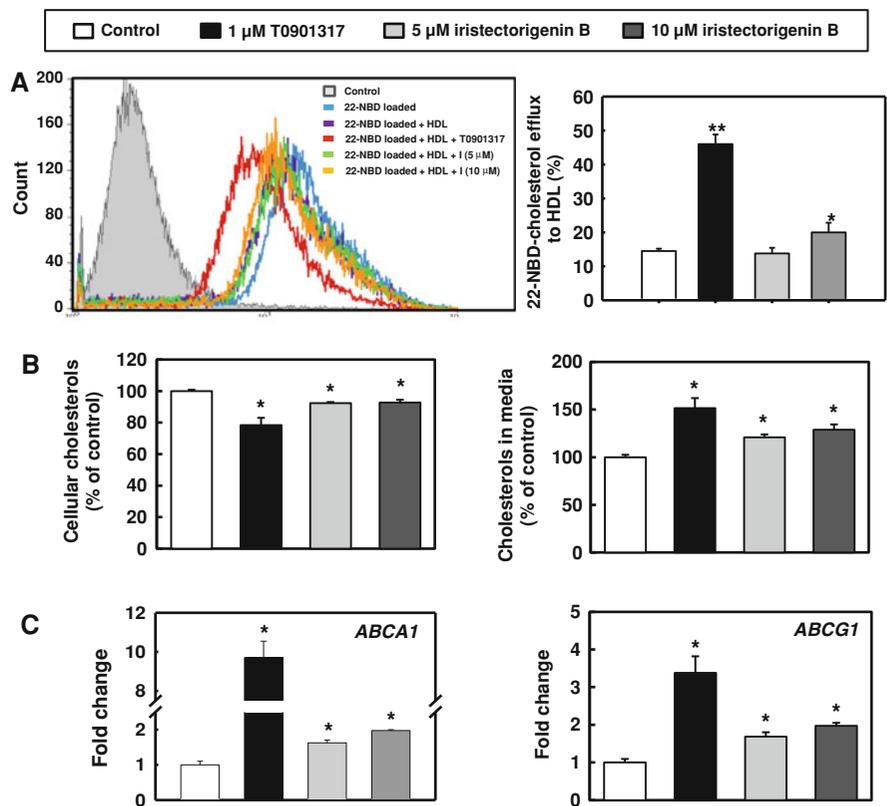
Fig. 1 continued



**Fig. 2** Iristectorigenin B stimulates liver X receptor ( $LXR$ )- $\alpha$  (a), and  $LXR$ - $\beta$  (b) transactivation activity. HEK 293 cells were co-transfected with the pGL4.35[luc2P/9XGAL4UAS/Hygro] vector and either pFN26Ah $LXR\alpha$  or pFN26Ah $LXR\beta$  and

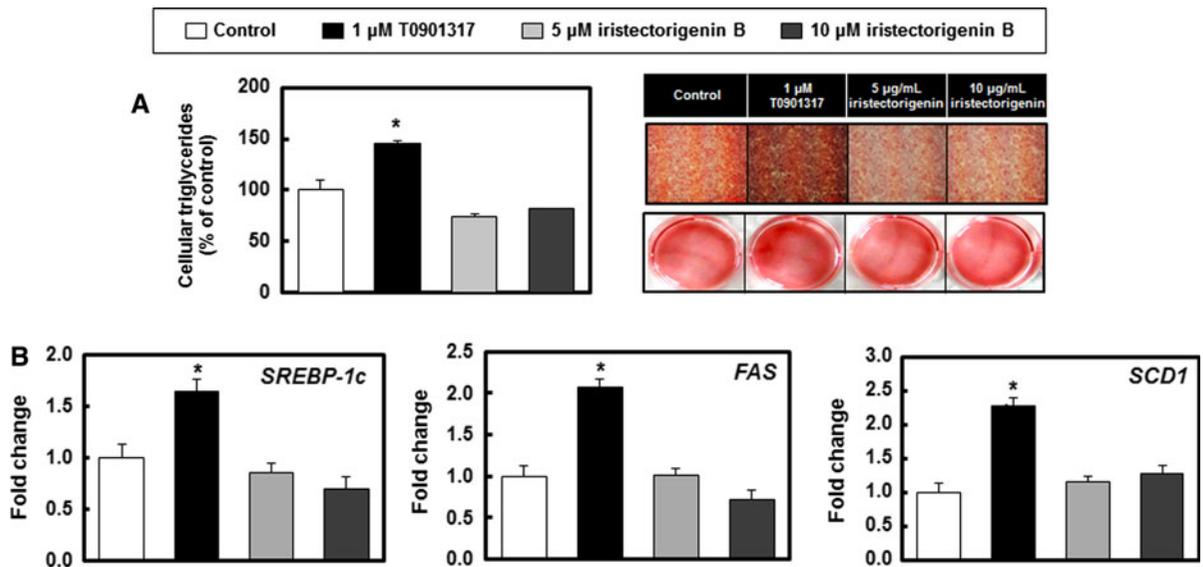
a  $\beta$ -galactosidase expression vector. Then luciferase activity was assayed and normalized to  $\beta$ -galactosidase activity. Data represent the mean  $\pm$  SE; \* $P$  < 0.05; \*\* $P$  < 0.05 vs. control ( $n$  = 3–5)

**Fig. 3** Effects of iristectorigenin B on cholesterol efflux (a), the cellular and medium lipid levels (b), and  $LXR$ -targeted gene transcription (c) in macrophage cells. Macrophage cells were treated with 5 and 10  $\mu$ M iristectorigenin B, 1  $\mu$ M T0901317 or vehicle control (1 % dimethyl sulfoxide (DMSO) for 24 h. Cholesterol efflux was quantified using 22-NBD-cholesterol and fluorescence-activated cell sorting analysis. Cellular and medium cholesterol in macrophage cells were measured by an enzymatic method. Total RNA was extracted and mRNA expression levels were measured by quantitative polymerase chain reaction (qPCR). \* $P$  < 0.05 and \*\* $P$  < 0.01 vs. control ( $n$  = 3–5)



manufacturer’s protocol. At 24 h post-transfection, the transfected cells were cultured in DMEM containing 1  $\mu$ M T0901317 (positive control for LXRs), iristectorigenin B (5–20  $\mu$ M), or solvent (1 % DMSO) for an additional 24 h. Next, the cells were lysed and

respectively assayed for luciferase and  $\beta$ -galactosidase activities using a firefly luciferase assay kit (Biotium, Hayward, CA, USA) and a  $\beta$ -galactosidase enzyme assay system (Promega) according to the manufacturers’ protocols. In each experiment,



**Fig. 4** Iristectorigenin B has modest effects on intracellular TAG levels in hepatocytes and the expression of hepatic lipogenesis genes. **a** Intracellular TAG levels and lipid staining of hepatocytes incubated with iristectorigenin B and T0901317.

**b** Expression of the genes encoding SREBP-1c, FAS, and SCD-1 in HepG2 cells incubated with iristectorigenin B and T0901317, as assessed by qPCR. \* $P < 0.05$  vs. control ( $n = 3-5$ )

luciferase assay results were normalized to  $\beta$ -galactosidase and expressed as relative luciferase activity.

#### Cell culture and treatments

HEK 293 cells, murine macrophage-like RAW 264.7, and HepG2 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). All cell lines were cultured in DMEM supplemented with 10 % (v/v) fetal bovine serum and 1 % penicillin/streptomycin before treatment. Cell lines were grown in 5 %  $\text{CO}_2$  at 37 °C. Prior to the experiments, RAW 264.7 macrophages and HepG2 cells were preincubated in serum-free medium for 24 h. The following day, the medium was removed and the cells were incubated an additional 24 h in 2 ml medium containing 1  $\mu$ M T0901317 (positive control), iristectorigenin B (5 or 10  $\mu$ M), or solvent (1 % DMSO). Each treatment was applied in triplicate.

#### Cholesterol efflux experiments

The cholesterol efflux measurements were performed according to a previously described method (Hoang et al. 2012).

#### Cellular triacylglycerols measurements

Cellular lipids were extracted as described previously (Hoang et al. 2012). The cellular content of triacylglycerols (TAG) was quantified enzymatically with a Cobas C111 automatic analyzer (Roche, Basel, Switzerland). The cholesterol levels were measured using an Amplex Red Cholesterol Assay Kit (Invitrogen) according to the manufacturer's instructions.

#### Quantitative PCR (qPCR)

Total RNA was extracted from macrophage RAW264.7 and HepG2 cells using an RNAiso Plus kit according to the manufacturer's protocol after 1 day of treatment with T0901317 and iristectorigenin B or vehicle control (1 % DMSO). To generate cDNA, 2  $\mu$ g total RNA was reverse-transcribed with oligo (dT)<sub>15</sub> using M-MLV reverse transcriptase (Mbiotech, Seoul, Korea) according to the manufacturer's protocol. Real-time qPCR was performed with Bio-Rad iQ SYBR Green Supermix reagent (Bio-Rad, Hercules, CA, USA) and the Bio-Rad iQ5 Cyclor System versions. The reaction conditions were: 95 °C for 3 min followed by 50 cycles of 95 °C for 10 s, 57 °C

for 15 s, and 72 °C for 20 s. A melting curve of 71 cycles, starting at 45 °C and increasing by 0.5 °C every 10 s was done to determine primer specificity. The primers are described in Hoang et al. (2012). Expression levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase with the normalized expression (CT) method according to the manufacturer's guidelines.

#### Statistical analysis

All data are expressed as the mean  $\pm$  standard error (SE). Two groups were compared using Student's *t* test. Differences were considered to be statistically significant at  $P < 0.05$ .

### Results and discussion

Through LXR agonist activity screening using a luciferase assay, we identified that compound 1 had strong LXR agonist activity (data not shown). Compound 1 was obtained as a white amorphous powder with physicochemical properties of ESI-MS  $m/z$ : 353  $[M + Na]^+$ ; m.p.: 186–187°;  $^1H$  NMR (500 MHz, DMSO- $d_6$ ) : 8.35 (1H, s, H-2), 7.12 (1H, d,  $J = 2.0$  Hz, H-2'), 6.94 (1H, dd,  $J = 2.0, 8.0$  Hz, H-6'), 6.81 (1H, d,  $J = 8.0$  Hz, H-5'), 6.48 (1H, s, H-8), 3.78 (3H, s,  $-OCH_3$ ), 3.74 (3H, s,  $-OCH_3$ );  $^{13}C$  NMR (100 MHz, DMSO- $d_6$ ) : 180.9 (C-4), 158.1 (C-7), 154.7 (C-2), 153.7 (C-9), 153.2 (C-5), 147.7 (C-3'), 147.2 (C-4'), 131.9 (C-6), 122.3 (C-1'), 122.2 (C-3), 122.1 (C-6'), 115.7 (C-5'), 113.8 (C-2'), 105.2 (C-10), 94.3 (C-8), 60.3 ( $-OCH_3$ ), 56.2 ( $-OCH_3$ ). The structure of compound 1 was identified as iristectorigenin B by comparison of its physicochemical and spectroscopic data to those reported previously (Eu et al. 1991).

Next, the cytotoxic effects of iristectorigenin B on cells were assessed in cell viability assays. Iristectorigenin B had no effect on cell viability (final viability  $>98\%$ ) at the tested concentrations (Supplementary Fig. 1). To confirm the LXR agonist activity of iristectorigenin B in vitro, HEK 293 cells were co-transfected with the pGL4.35[luc2P/9XGAL4UAS/Hygro] vector and either pFN26AhLXR $\alpha$  or pFN26AhLXR $\beta$ , and then incubated with iristectorigenin B. Iristectorigenin B significantly induced the transactivation of both LXR- $\alpha$  (+540 % at 20  $\mu$ M;

$P < 0.01$ ) and LXR- $\beta$  (+331 % at 20  $\mu$ M;  $P < 0.05$ ) in a dose-dependent manner (Figs. 1, 2a, b).

LXR activation promotes cholesterol efflux, stimulates RCT in macrophages, and inhibits the accumulation of cholesterol in vitro and in vivo (Geyeregger et al. 2006). Therefore, we investigated the effect of iristectorigenin B treatment on cholesterol efflux and cellular cholesterol content in macrophage cells. Iristectorigenin B increased cholesterol efflux to HDL (Fig. 3a) and reduced cellular cholesterol concentration in a dose-dependent manner in macrophages (Fig. 3b). T0901317 showed similar results in agreement with Aravindhan et al. (2006).

LXRs regulate cholesterol homeostasis by promoting many of its target genes that are involved in the RCT pathway in macrophages (Geyeregger et al. 2006). Intracellular accumulation of cholesterol in macrophages leads to increased expression of ATP-binding cassette transporter A1 (ABCA1) which facilitates the transport of excess macrophage cholesterol to extracellular acceptors, such as apolipoprotein AI and HDL, for subsequent transport as HDL particles to the liver. Considerable evidence has established that LXRs are critical regulators of the ABCA1-dependent cholesterol efflux pathway (Costet et al. 2000). In addition, another cholesterol efflux transporter, ABCG1, is induced in macrophages in response to LXR ligands (Schmitz et al. 2001). In the present study, iristectorigenin B significantly and dose-dependently increased the expression of the LXR-responsive genes ABCA1 and ABCG1 in macrophages (Fig. 3c). At a concentration of 10  $\mu$ M, iristectorigenin B increased ABCA1 and ABCG1 mRNA expression 2.0- and 1.9-fold ( $P < 0.05$ ), respectively, compared to the control group. Similar but greater effects were observed in cells stimulated with T0901317. The effects of iristectorigenin B in macrophage cells were similar to those observed with LXR- $\alpha$  and LXR- $\beta$  agonists, which increase cholesterol efflux by inducing ABCA1 and ABCG1. Together, these findings suggest that iristectorigenin B as an LXR ligand suppresses cholesterol accumulation by promoting an efflux pathway in macrophages, which could lead to the elevation of circulating levels of HDL-cholesterol and prevention of hypercholesterolemia and atherosclerosis.

The role of LXR in the control of fatty acid metabolism has been implicated as a potential side effect of LXR therapy. The expression of fatty acid

synthesis genes, including sterol regulatory element-binding protein-1c (SREBP-1c), fatty acid synthase (FAS), and stearoyl-CoA desaturase-1 (SCD-1), is blunted in mice carrying a targeted disruption in the LXR- $\alpha$  gene (Peet et al. 1998). On the other hand, administration of synthetic LXR ligands in mice elevates plasma TAG levels, in part by inducing the hepatic lipogenic pathway (Schultz et al. 2000). In this study, stimulation of HepG2 cells with T0901317 increased cellular TAG levels, as previously reported (Fig. 4a), and also induced the expression of SREBP-1c, FAS, and SCD-1 (Fig. 4b). Interestingly, iristectorigenin B did not induce intracellular TAG concentrations in hepatocytes, as assessed by direct measurement as well as Oil Red O-lipid staining. In contrast, T0901317-treated cells showed significant accumulation of cellular lipid droplets (Fig. 4a). Induction of hypertriacylglycerolemia by LXR agonists is controversial. Increases in plasma TAGs by LXR agonists have been reported (Geyeregger et al. 2006). However, other studies have reported no change in plasma TAGs (Grefhorst et al. 2002) or only a transient increase. The reason for this inconsistency is unclear at present. Recently, Albers et al. (2006) reported that the selective LXR modulators GW3965 and 22R-HC differ from T0901317 in the induction of FAS or SCD-1 in the liver because of differences in the extent of co-activator recruitment. Therefore, this possibility should be examined in the future.

**In summary**, iristectorigenin B exhibits hypocholesterolemic effects by activating LXR. It also induced transactivation of LXR- $\alpha$  and LXR- $\beta$  and may stimulate cholesterol efflux in macrophages without inducing hepatic steatosis. These findings provide insights that may be useful in the development of pharmaceutical agents for treating hypercholesterolemia and atherosclerosis.

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