

Forhidrol, a bioactive fraction of *Phaleria macrocarpa* (Scheff.) Boerl., increases reverse cholesterol transport pathway by down-regulation of cholesteryl ester transfer protein activity

Guntur Berlian, Olivia M. Tandrasasmita, Dwi A.S. Suciptan, Raymond R. Tjandrawinata

Section of Molecular Pharmacology, Department of Research Innovation and Invention, Dexa Laboratories of Biomolecular Sciences, PT Dexa Medica, Cikarang, Indonesia

Abstract

Phaleria macrocarpa (Scheff.) Boerl. is one of the most popular medicinal plants in Indonesia. This plant has traditionally been used to lower cholesterol and control hypertension. This study was performed to examine the effect of Forhidrol, a bioactive fraction of

P. macrocarpa (Scheff.) Boerl., to increase reverse cholesterol transport through the down-regulation of cholesteryl ester transfer protein (CETP) activity in hepatocellular carcinoma cell lines. Measurement of target genes including liver X receptor (LXR), sterol regulatory element-binding protein 1 (SREBP-1), scavenger receptor class B type 1, low-density lipoprotein (LDL) receptor, apolipoprotein B, CYP11B1, CYP11B2, peroxisome proliferator-activated receptor α (PPAR α), peroxisome proliferator-activated receptor δ (PPAR δ) and peroxisome proliferator-activated receptor γ (PPAR γ) were done using real-time polymerase chain reaction assay. Secreted CETP and apolipoprotein A-1 were analyzed using western blot. CETP activity *in vitro* was measured using CETP inhibitor drug screening kit and triglyceride synthesis was measured using enzyme-linked immunosorbent assay. Forhidrol was found to significantly down-regulate CETP mRNA expression, as well as total CETP and cholesteryl esters transfer activity ($P < 0.05$). It specifically reduced transcriptional level of regulatory genes of CETP promoter including SREBP-1 and LXR. Forhidrol also significantly increased PPAR δ and PPAR α expression ($P < 0.05$) and slightly repressed triglycerides synthesis. *In vivo* study showed elevated high-density lipoprotein (HDL) levels in rabbits after 4-week treatment of Forhidrol at a dose of 37.5 mg/1.5 kg body weight compared to placebo. Conversely, LDL, triglyceride and CETP activity were decreased. Forhidrol increased HDL levels by reducing CETP-dependent transfer of cholesterol from HDL to LDL particles. Looking at possible side effects, Forhidrol apparently acted as a safe agent without negative effect towards blood pressure. These findings suggested that Forhidrol may be further developed as a potential anti-atherogenic drug.

Correspondence: Raymond R. Tjandrawinata, Dexa Laboratories of Biomolecular Sciences (DLBS), PT Dexa Medica, Industri Selatan V Block PP No. 7, Jababeka Industrial Estate II, Cikarang, West Java 17550, Indonesia.
Tel: +62.21.89841901 - Fax: +62.21.89841905.
E-mail: raymond@dexa-medica.com

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Introduction

Cholesteryl ester transfer protein (CETP) is a 74-kDa hydrophobic glycoprotein which is mainly synthesized in liver and secreted to plasma.¹⁻⁴ It is also produced in lymph, adipose tissue, heart, kidney and small intestine, although in a lower level.^{2,5} CETP is a major determinant of plasma levels of high-density lipoprotein (HDL) cholesterol in human by mediating the exchange of cholesteryl esters (CE) in HDL particles for triglycerides in low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) particles through reverse cholesterol transport (RCT) pathway.^{2,4,6-8}

In epidemiological studies, subjects with CETP gene deficiency possessed higher HDL cholesterol and lower LDL cholesterol levels.^{3,9} Decreased HDL levels constitute a major risk factor for

coronary heart disease and atherosclerotic cardiovascular disease, however, there was no therapy reported that substantially raise HDL levels. Inhibition of CETP has been proposed as a strategy to raise HDL levels, as human CETP deficiency resulted in markedly elevated HDL levels. It has previously been reported that elevating HDL levels by decreasing CETP mass or activity is beneficial in the inhibition of atherosclerosis progression.^{4,10-13}

In addition to LDL protection from oxidation,¹⁴ the atheroprotective effect of HDL has also been seen on its role in RCT pathway, where excessive cholesterol in peripheral cells is distributed to the liver for secretion.³ Although there was an alternative that focused on direct administration of synthetic HDL (sHDL),¹⁵ therapies aimed to induce the production of HDL in the body may become other promising medical approaches.¹⁶

Phaleria macrocarpa (Scheff.) Boerl is a medicinal plant originated from Indonesia that is locally known as Mahkota Dewa, which has traditionally been used to lower cholesterol, as well as control hypertension and diabetes mellitus.¹⁷⁻¹⁹ Studies have proven its effect in the management of chronic diseases, such as hyperglycemia, dyslipidemia and kidney disorders.²⁰⁻²³ Earlier studies have reported that *P. macrocarpa* (Scheff.) Boerl. has the potential anti-cancer activity in breast^{24,25} and esophageal cancer cells,²⁶ as well as antidiabetic,^{27,28} anti-cholesterol,^{21,22} antiatherosclerosis²⁹ and antioxidant activities.²⁷ Previous study exhibited potential properties of *P. macrocarpa* (Scheff.) Boerl. to improve HDL levels and its related gene.²¹ Other study also showed improved cholesterol profile,²⁹ thus supporting the potential effect of *P. macrocarpa* (Scheff.) Boerl as anti-atherosclerosis. In this study, we examined the effect of Forhidrol, a bioactive fraction of *P. macrocarpa* (Scheff.) Boerl., on CETP mRNA expression and its activity on human hepatoblastoma cells (HepG2). Additional preliminary *in vivo* study in rabbit was performed to examine whether the rise in HDL observed clinically with Forhidrol treatment could be a result of decreased CETP activity.

Materials and Methods

Preparation and phytochemical characterization of Forhidrol

P. macrocarpa (Scheff.) Boerl. was collected from Central Java, Indonesia. This plant has been identified in Herbarium Bogoriense, Research Center of Biology, Indonesian Institute of Sciences with certificate No. 261/IPH.1.02/If.8/XII/2009. Dried fruits of *P. macrocarpa* (Scheff.) Boerl. were macerated with ethanol 96% at 25-50°C for 2-6 h, followed by filtration and evaporation. The extract was further dried in vacuum oven for 2-4 h to obtain dry material and analyzed by thin layer chromatography (TLC). The dried material was referred as Forhidrol bioactive fraction. The concentration of Forhidrol used in this experiment was based on its dry weight ($\mu\text{g}/\text{mL}$).

Identification of active compound in Forhidrol was carried out qualitatively using TLC method. The sample was spotted on silica gel plate and eluted with a mixture of chloroform and methanol (10:1). Observation was done under UV light at 254 and 366 nm. Forhidrol was also characterized using fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy. FTIR analysis was carried out using FTIR-6100 (Jasco, Japan) at a wavelength range of 4000-700 cm^{-1} , while NMR analysis was carried out using chloroform-D as the solvent (¹H at 500 MHz and ¹³C at 125 MHz) and NMR JEOL 500 MHz (JEOL, Japan).

Cell culture and *in vitro* experiments

HepG2 lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were maintained in minimum essential-alpha (Gibco, CA, USA) medium supplemented with 10% fetal bovine serum (Gibco, CA, USA), 100 $\mu\text{g}/\text{mL}$ penicillin-streptomycin (Gibco, CA, USA) and 1 mM sodium pyruvate (Gibco, CA, USA) at temperature of 37°C in 5% CO₂ atmosphere. Eighty percent of confluent cells were maintained in serum-free medium for 4 h before treatment. The cells were then treated with 0-100 $\mu\text{g}/\text{mL}$ Forhidrol and 30 μM linoleic (Sigma, MO, USA) or arachidonic acids (MP Biomedical, OH, USA) for 24 h.

Real-time polymerase chain reaction analysis for mRNA expression

Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. The RNA concentration was quantified using NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). The expressions of several genes including liver X receptor (LXR), sterol regulatory element-binding protein 1 (SREBP-1), scavenger receptor class B type 1 (SR-B1), LDL receptor (LDL-R), apolipoprotein B (ApoB), CYP11B1, CYP11B2, peroxisome proliferator-activated receptor α , δ and γ (PPAR α , PPAR δ and PPAR γ), were determined using real-time polymerase chain reaction (PCR). Primers for each gene (Table 1) were synthesized by First Base Laboratories (Singapore). The PCR amplification was performed in a total volume of 25 μL reaction

Table 1. Sequences of the primers used in the polymerase chain reaction measurements.

| Gene | Sequence (5' → 3') |
|----------------|--|
| CETP | Forward: GTGCTTGCCCTTCTGCTACAAG Reverse: CGAGTCTTCCACTCGCTGGC |
| LXR | Forward: CGGGCTTCCACTACAATGTT Reverse: TCAGCGGATCTGTTCTTCT |
| SREBP-1 | Forward: TGCATTTTCTGACACGCTTC Reverse: CCAAGCTGTACAGGCTCTCC |
| SR-B1 | Forward: CTGTGGGTGAGATCATGTGG Reverse: GCCAGAAGTCAACCTTGCTC |
| ApoB | Forward: TTTGCCCTCAACCTACCAAC Reverse: TCCGATCTTGTGGCTACTG |
| LDL-R | Forward: TCTGTCGTGTGTGTTGGGAT Reverse: ACGACAAGATTGGGGAAGTG |
| PPAR α | Forward: GCTGGTGCAGATCATCAAGA Reverse: GCCGGTTACCTACAGCTCAG |
| PPAR δ | Forward: ACTGAGTTCGCCAGGAGCAT Reverse: GCGTTGAACCTTGACAGCAAA |
| PPAR γ | Forward: ACTGAGTTCGCCAAGAGCAT Reverse: GCGTTGAACCTTGACAGCAAA |
| CYP11B1 | Forward: CTCTACCCTGTGGGTCTGTTTTC Reverse: GGGTTATAGCGCTCAGGCC |
| CYP11B2 | Forward: CTCTACCCTGTGGGTCTGTTTTC Reverse: GGATTATACCGCTCAGGC |
| β -actin | Forward: ACCCACACTGTGCCCATCTA Reverse: CGGAACCGCTCATTGCC |

CETP, cholesteryl ester transfer protein; LXR, liver X receptor; SRBP-1, sterol regulatory element-binding protein 1; SR-B1, scavenger receptor class B type 1; ApoB, apolipoprotein B; LDL-R, low-density lipoprotein receptor; PPAR α , peroxisome proliferator-activated receptor α ; PPAR δ , peroxisome proliferator-activated receptor δ ; PPAR γ , peroxisome proliferator-activated receptor γ .

mixture consisting of 12.5 μL iQTM SYBR[®] Green Supermix (Biorad, CA, USA), 0.8 μM of each targeted primer and 0.1 μM of each targeted primer and 0.1 μM of each β -actin primer as the internal control. The real-time PCR was performed using Mini Opticon MJ MiniTM (Biorad, CA, USA) at optimum conditions for each primer.

Western blot

Secreted CETP and Apolipoprotein A-I (ApoA-I) proteins in the medium were analyzed using western blot (BioRad, CA, USA). The medium was concentrated up to 10 fold using filter with 10 kDa-molecular weight cut-off membrane (Millipore, MA, USA), and measured using Bradford method. The protein was further separated by SDS-PAGE using 10% acrylamide gel at 100 V for 150 min. The protein was then transferred to polyvinylidene difluoride (PVDF) membrane by blotting system at 500 mA for 75 min. The blots were incubated with CETP, ApoA-I and β -actin primary as well as secondary antibodies, with dilution of 1:500 and 1:10000, respectively. The protein band was visualized using luminol chemiluminescence reagent and exposed to a film (FUJIFILM, Tokyo, Japan) in the dark room. Antibodies (both primary and secondary), PVDF membranes and luminol reagent were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cholesteryl ester transfer protein activity assay

Effect of Forhidrol on *in vitro* CETP activity was assayed using CETP inhibitor drug screening kit (BioVision, CA, USA) according to the manufacturer's protocol. The results were evaluated based on the fluorescence intensity of neutral lipid transferred to the acceptor molecule mediated by CETP. The concentrations of Forhidrol used in this experiment were ranged from 0-100 $\mu\text{g}/\text{mL}$.

Triglycerides synthesis assay *in vitro*

Effect of Forhidrol on *in vitro* triglycerides synthesis was assayed using triglycerides synthesis enzyme-linked immunosorbent assay (ELISA) kit. (Abcam, Cambridge, UK) according to the manufacturer's protocol. Oleic acid (Sigma, MO, USA) at a concentration of 0.5 mM was pre-treated to HepG2 cells to induce triglycerides synthesis and used as the negative control. The concentrations of Forhidrol used in this experiment were ranged from 0-100 $\mu\text{g}/\text{mL}$.

In vivo study

Kits for the measurement of HDL, LDL, triglycerides and CETP activity levels were purchased from BioVision (CA, USA). Ten male New Zealand white rabbits (2.3-2.5 kg) were maintained at animal laboratory of DEXA Laboratories of Biomolecular Sciences (Cikarang, Indonesia). The animal study was conducted after research ethics approval from ethical institutional advisory committee as suggested by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The rabbits were individually housed according to AAALAC guidelines and given free access to water and fed a normal diet. Before experiment, blood plasma of each rabbit was collected for the measurement of normal baseline levels of HDL, LDL and triglyceride, as well as CETP activity. They were further divided into two groups consisting of control (placebo) and Forhidrol treatment group. Rabbits in the control group were fed a normal diet, while the treatment groups were fed the same diet supplemented with 37.5 mg Forhidrol/1.5 kg body weight. The level of plasma lipids and CETP activity were measured every week within 4-week treatment. All measurements were done using blood samples from 12-h fasted rabbits which were withdrawn from ear veins.

Statistical analysis

Statistical differences between test and control groups were determined by t-test analysis using the StatView package (Abacus Concepts, Inc., Berkeley, CA). Values are expressed as mean \pm standard deviation (SD) for 3 independent experiments.

Results

Phytochemical characterization of Forhidrol

Observation under UV light at 254 and 366 nm showed that Forhidrol contains a group of compounds with Rf value between 0.16-0.35 by which it conferred its specific biological activity. FTIR analysis at a wavelength range of 4000-700 cm^{-1} showed a functional group of OH, aliphatic and carbonyl (C=O). Moreover, NMR analysis revealed that Forhidrol may contain unsaturated fatty acid esters (C25). This evidence concludes that Forhidrol is a multicomponent bioactive fraction containing several hydrocarbon compounds as well as fatty acids.

Forhidrol decreases the expression and activity of cholesteryl ester transfer protein

Dose response and time course studies of Forhidrol in decreasing CETP activity were evaluated in HepG2 cells. As shown in Figure 1A, mRNA levels of CETP were decreased in the presence of Forhidrol in a dose-dependent manner (Figure 1A). At the highest concentration (100 $\mu\text{g}/\text{mL}$), CETP mRNA level was decreased by 82%. Time course study showed that Forhidrol reduced CETP mRNA level by almost 50% after 4 h of incubation. Within 16 h, lower CETP expression was clearly found as the result of increasing incubation time. Although Forhidrol activity was slightly decreased after 24 and 32 h of incubation, overall data revealed that Forhidrol decreased CETP expression in a time-dependent manner. Moreover, inhibitory activity of Forhidrol was also found at the protein level of CETP which was revealed by western blot. CETP protein was significantly declined in the cells treated with 100 $\mu\text{g}/\text{mL}$ Forhidrol (Figure 1C). These results indicated that Forhidrol contributed to the repression of CETP in transcription and translation levels.

We also investigated the effect of Forhidrol on CETP activity *in vitro*. Administration of Forhidrol resulted in a significant inhibition of CETP activity which occurred in a dose-dependent manner (Figure 1D). This finding suggests that Forhidrol also served as a CETP activity inhibitor in mediating cholesterol transport. The maximum inhibition of CETP activity by Forhidrol was 30%, which was found at a concentration of 100 $\mu\text{g}/\text{mL}$.

Forhidrol regulates cholesteryl ester transfer protein transcription regulatory genes

CETP transcription is regulated by several regulatory genes including SREBP1, LXR, LRP1 and LXR. In this study, the genes were investigated for their response towards Forhidrol administration. mRNA level analysis revealed that LXR and SREBP1 genes were progressively down-regulated following Forhidrol administration (0-75 $\mu\text{g}/\text{mL}$) (Figure 2). Forhidrol at a concentration of 75 $\mu\text{g}/\text{mL}$ exhibited a down-regulation of LXR expression by up to 75%, while higher level was observed on SREBP1 gene which exhibited 95% inhibition. In other hands, we did not find any alteration in other CETP transcriptional regulatory genes (data not shown). Based on the result, we suggested that inhibition of CETP transcription may occur due to the down-regulation of LXR and SREBP1.

Effect of Forhidrol on apolipoprotein A-I protein level

The effect of Forhidrol on ApoA-I, a major protein component of HDL, was also evaluated. Our *in vitro* study in HepG2 cells using western blot revealed that Forhidrol slightly elevated ApoA-I protein level in a dose-dependent manner, with 30% increment of ApoA-I by the highest Forhidrol concentration (100 $\mu\text{g}/\text{mL}$) (Figure 3A). This result indicated that CETP inhibition might induce the cells to express more ApoA-I in order to produce new HDL particles.

Effect of Forhidrol on scavenger receptor class B type 1, low-density lipoprotein receptor, and apolipoprotein B mRNA level

The potential effect of Forhidrol towards genes related to HDL and LDL levels was further studied in order to evaluate the effect of Forhidrol in cholesterol transport pathway. As seen in Figure 3B, the expression of SR-B1 gene, which encodes HDL receptor, was decreased by Forhidrol treatment in a dose-dependent manner with inhibition level of up to 70% at Forhidrol concentration of 100 $\mu\text{g}/\text{mL}$. Forhidrol also altered the expression of LDL-R by increasing its expression in a dose-dependent manner in which the optimum elevation (200%) was observed at a concentration of 50

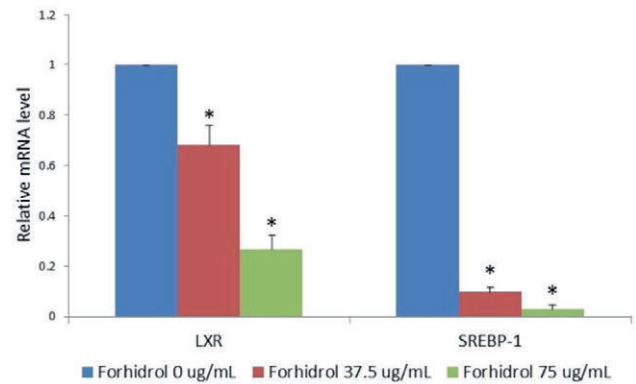


Figure 2. Forhidrol down-regulates regulatory genes of cholesteryl ester transfer protein promoter. Hepatocellular carcinoma cells were incubated with Forhidrol (75 $\mu\text{g}/\text{mL}$) for 24 h and total RNA was extracted. The mRNA levels of liver X receptor (LXR) α and sterol regulatory element-binding protein 1 (SREBP-1) were analyzed by real-time polymerase chain reaction and normalized to β -actin. Results are expressed as mean \pm SD of two independent experiments. *P<0.05 indicates a significant difference compared to control.

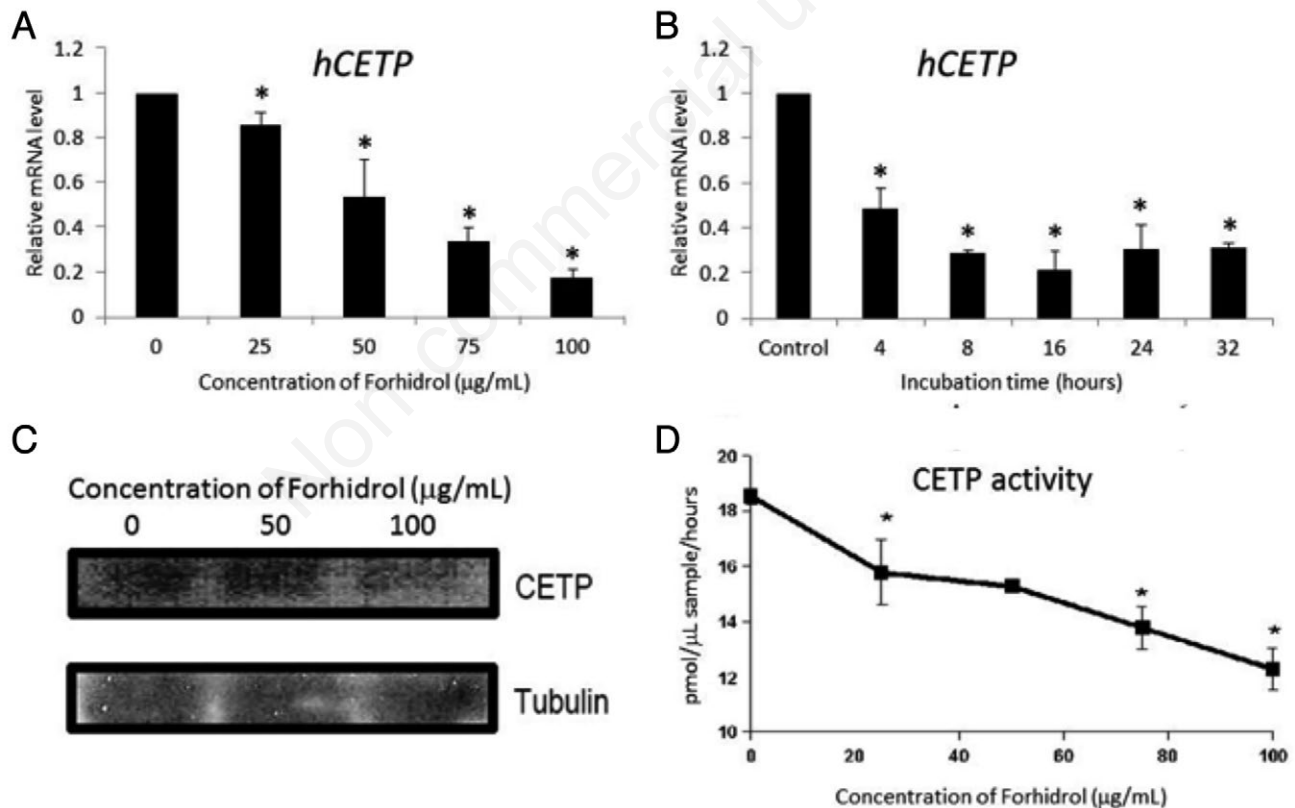


Figure 1. Forhidrol decreases cholesteryl ester transfer protein (CETP) at transcription level in dose-dependent (A) and time-dependent manner (B). Forhidrol also affects CETP expression at protein level (C), as well as CETP activity (D). For dose-response study, Hepatocellular carcinoma (HepG2) cells were treated with Forhidrol (0-100 $\mu\text{g}/\text{mL}$) for 24 h and total RNA and protein were extracted. The expression levels of CETP mRNA were analyzed by real-time polymerase chain reaction (PCR) and normalized to β -actin. CETP protein levels were analyzed by western blot method while CETP activity was analyzed using CETP inhibitor drug screening kit. For time-course study, HepG2 cells were treated with Forhidrol (75 $\mu\text{g}/\text{mL}$) for 4, 8, 16, 24 and 32 h, then total RNA were extracted. The expression levels of CETP mRNA were analyzed by real-time PCR and normalized to β -actin. Results are expressed as mean \pm SD of two independent experiments. *P<0.05 indicates a significant difference compared to control.

$\mu\text{g/mL}$. Although LDL-R expression observed at 50 $\mu\text{g/mL}$ was higher than that at higher Forhidrol concentration, its administration still produced a significant increment of LDL-R expression compared to that of the control. Alterations of gene expression by Forhidrol were also observed in apolipoprotein B (ApoB), which is the main component of LDL. Forhidrol conferred down-regulation of ApoB gene at mRNA level in a dose-dependent manner. The down-regulation was started to be significant at a concentration 50 $\mu\text{g/mL}$ and progressively decreased at higher concentrations.

Effect of Forhidrol and polyunsaturated fatty acid on cholesteryl ester transfer protein mRNA level

Polyunsaturated fatty acids (PUFAs), *e.g.* arachidonic acid (AA) and linoleic acid (LA), were used as the control for observation of CETP inhibition at mRNA level. As seen in Figure 4, we found that administration of AA and LA at a concentration of 30 μM decreased the expression of CETP. Moreover, addition of Forhidrol (75 $\mu\text{g/mL}$) in PUFA-treated sample showed a higher decrement of CETP expression compared to PUFAs alone.

Effect of Forhidrol in regulating proliferator-activated receptors expression at mRNA level

mRNA level of transcription factor PPAR family was evaluated for their response towards Forhidrol administration. Compared to the control, administration of Forhidrol was able to significantly increase PPAR δ expression in a dose-dependent manner, with the highest increment of up to 4.1 folds, while significant increase of PPAR α expression by 3 folds only occurred at a concentration of 100 mg/mL , and there was no significant effect found on PPAR γ (Figure 5). These results indicate that Forhidrol also modulated

lipoprotein metabolism by up-regulating the transcription levels of PPARs, including PPAR δ and PPAR α .

Effect of Forhidrol on triglycerides synthesis *in vitro*

Triglycerides, as far as is currently known, are not directly atherogenic but represent an important biomarker of cardiovascular diseases risk due to their association with proatherogenic proteins found on all classes of the plasma lipoproteins. The effect of Forhidrol on triglycerides synthesis was evaluated. *In vitro* study in HepG2 cells revealed that triglyceride levels was increased by 83% after the addition of oleic acid compared to control, while Forhidrol administration was found to reduce triglycerides by up to 31% compared to oleic acid-treated group (Figure 6).

Forhidrol administration increased beneficial effect on the lipoprotein profile *in vivo*

Since rabbit is a species with natural high levels of CETP, *in vivo* study was conducted in male New Zealand rabbits to assure the effect of CETP inhibition by Forhidrol on HDL, LDL and triglycerides levels. The treated rabbits were fed a normal diet and supplemented with 37.5 mg Forhidrol/1.5 kg body weight/day, while non-treated rabbits were fed a normal diet without Forhidrol. However, we found that the variation of HDL, LDL, triglycerides and CETP values between individuals in each group were high, which consequently increased the deviation values.

The correlation of HDL, LDL, triglycerides and plasma CETP activity changes after 30 days between control and treatment groups are presented in Table 2. The changes were calculated based on the ratio to each baseline. After 4 weeks of treatment, HDL levels was reduced by $9.46\pm 7.40\%$ in placebo group and elevated by $19.59\pm 33.51\%$ in the treatment groups. Conversely, LDL and

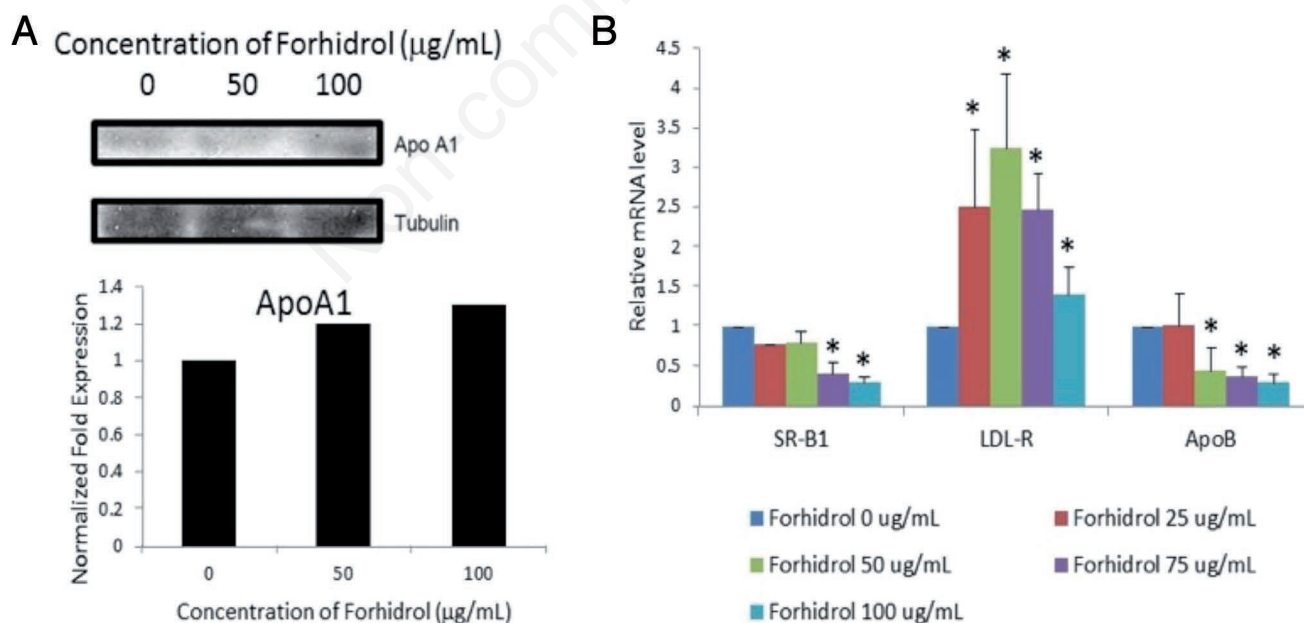


Figure 3. Forhidrol slightly increases ApoA-I protein level (A). Forhidrol down-regulates scavenger receptor class B type 1 (SR-B1) and apolipoprotein B (ApoB) genes expressions and up-regulates low-density lipoprotein receptor (LDL-R) gene expression at mRNA level (B). Hepatocellular carcinoma cells were incubated with Forhidrol (75 $\mu\text{g/mL}$) for 24 h and total RNA was extracted. Protein level was analyzed by western blot. The mRNA levels of SR-B1, LDL-R and ApoB genes were analyzed by real-time polymerase chain reaction and normalized to β -actin. Results are expressed as mean \pm SD of two independent experiments. * $P < 0.05$ indicates a significant difference compared to control.

triglyceride levels were reduced after 4 weeks of Forhidrol treatment. The increase of LDL, triglyceride levels and CETP activity in the control group were $3.89 \pm 20.2\%$, $23.48 \pm 32.53\%$ and $1.73 \pm 4.1\%$, respectively, while those levels were reduced in the treatment groups by $0.94 \pm 46.01\%$, $0.34 \pm 10.94\%$ and $1.23 \pm 1.09\%$, respectively (Table 2). In Forhidrol treatment group, the change in HDL levels was negatively correlated to the change in CETP activity, while the change in LDL levels was positively correlated to the change in CETP activity. It is well established that elevated triglyceride and LDL levels are the major risk factors for CVD.

Effect of Forhidrol on blood pressure level

CYP11B1 and CYP11B2 have been reported to be correlated with blood pressure in human and animal models. In this study, we also investigated the effect of Forhidrol on those genes. Forhidrol was shown to down-regulate the expression of CYP11B1 and CYP11B2 by 80-90% and 40-50%, respectively (Figure 7). Although there was a higher level of CYP11B1 expression observed at 100 $\mu\text{g/mL}$ of Forhidrol, the expression of this gene was still significantly lower compared to control. Therefore, it appeared that Forhidrol may not elevate blood pressure.

Discussion

Decreased HDL level has been well-known as one of the major risk factors of coronary heart disease. However, there are no

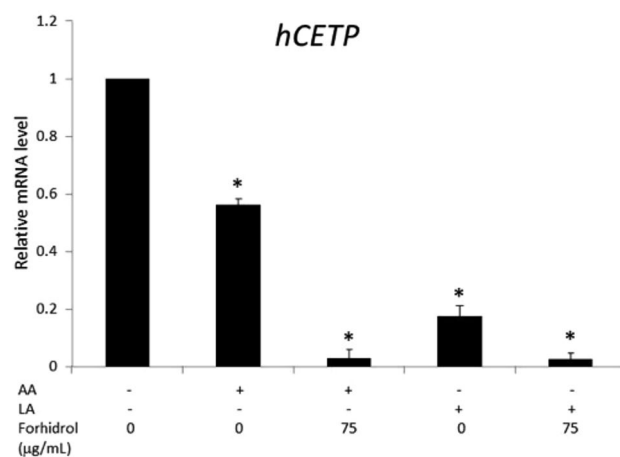


Figure 4. Forhidrol and Polyunsaturated fatty acids synergistically reduce cholesteryl ester transfer protein (CETP) at transcriptional levels. Hepatocellular carcinoma cells were treated with Forhidrol (75 $\mu\text{g/mL}$) and/or arachidonic acid (AA) or linoleic acid (LA) (30 μM) and total RNA was extracted after 24 h of incubation. The expression of CETP mRNA was analyzed by real-time polymerase chain reaction and normalized to β -actin. Results are expressed as mean \pm SD of two independent experiments. * $P < 0.05$ indicates a significant difference compared to control.

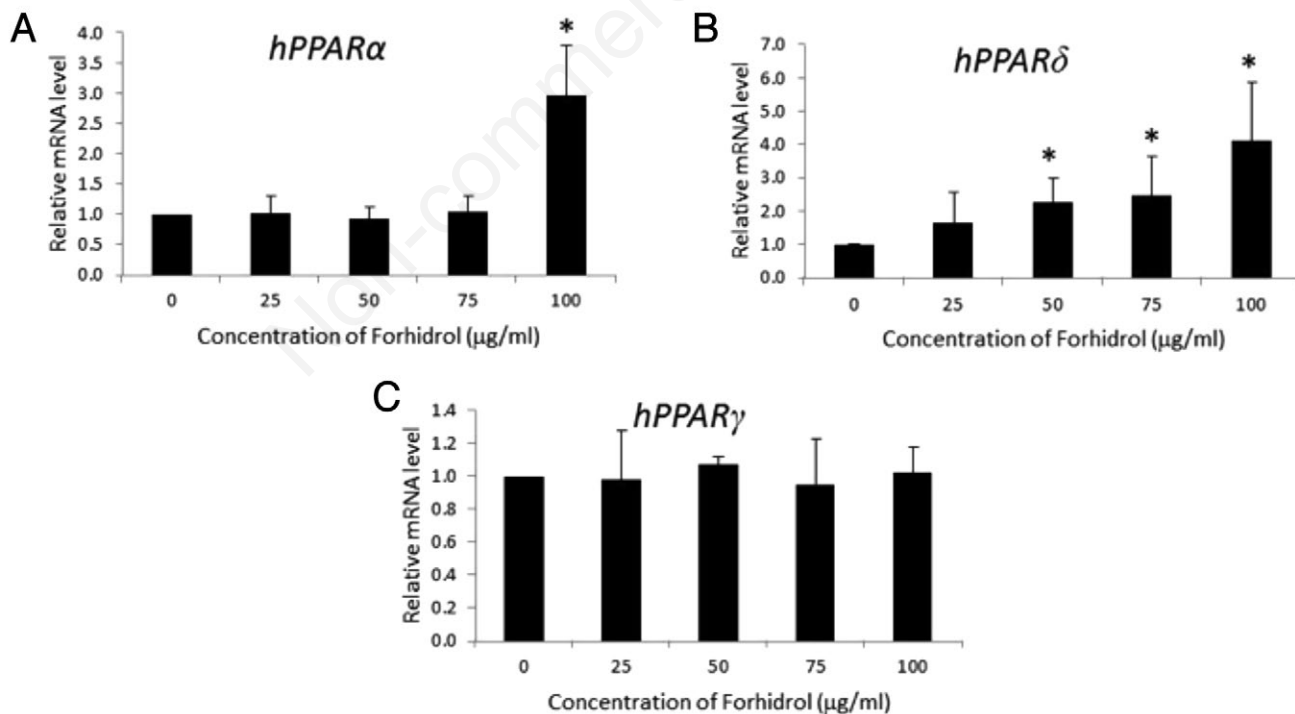


Figure 5. Forhidrol significantly increases the expression of peroxisome proliferator-activated receptor α (PPAR α) at a concentration of 100 $\mu\text{g/mL}$ (A) and peroxisome proliferator-activated receptor δ (PPAR δ) in a dose-dependent manner (B). Forhidrol did not affect peroxisome proliferator-activated receptor γ (PPAR γ) expression (C). Hepatocellular carcinoma cells were treated with Forhidrol (0-100 $\mu\text{g/mL}$) for 24 h. Total RNA was isolated and mRNA levels of PPAR α , PPAR δ and PPAR γ were analyzed by real-time polymerase chain reaction. Glyceraldehyde 3-phosphate dehydrogenase was used as the internal loading control. Results are expressed as mean \pm SD of three independent experiments. * $P < 0.05$ indicates a significant difference compared to the control.

therapies that substantially raise HDL levels. Recently, inhibition of CETP has been proposed as a promising strategy to raise HDL levels.^{3,4} CETP is a major determinant of plasma levels of HDL in humans by mediating the exchange of cholesteryl esters in HDL particles for triglycerides in LDL and VLDL particles through RCT, a pathway by which peripheral cell cholesterol can be returned to the liver for further catabolism.^{12,13}

Since the expression and activity of CETP is one of the determinants of plasma HDL levels in human, in the present study we focused on investigating the potential activity of Forhidrol on affecting CETP. Forhidrol reduced CETP mRNA level in dose- and time-dependent manners. The same trend was also found at the protein level, however, Forhidrol activity on reducing CETP mRNA level was much higher than that in the protein level. These data suggested that Forhidrol is more likely to affect CETP at its transcriptional level, which is in line with other reports that found mRNA level as the major determinant of secreted CETP protein.³⁰⁻³² Moreover, Forhidrol also dose-dependently reduced CETP activity in mediating lipid transfer.

Some regulatory elements were known to control CETP transcription by modulating its promoter, which include LXR,^{5,33,34} SREBP,⁶ LRH-1,⁵ ARP-1, CCAAT/enhancer-binding protein (C/EBP),³⁵ trans retinoic acid,³⁶ and Sp1 and Sp3 transcription factors.^{37,38} However, experiment on the specific regulatory pathways that directly affect CETP transcription is still limited.² In this study, we investigated several regulatory elements of CETP at mRNA level. We found alterations in SREBP-1 and LXR at mRNA level isolated from Forhidrol-treated HepG2 cells, but not in LRH-1 and ARP-1 at mRNA level. LXRs are nuclear receptors that form heterodimers with RXRs.³² It is highly expressed in the liver and plays a central role in maintaining cholesterol homeostasis in the liver.³⁴ We found that the transcription level of LXR α was declined after Forhidrol administration. Reduced expression of this gene may subsequently affect the transcription of SREBP-1 since LXR/RXR complex was identified as the activator of SREBP-1 promoter.^{5,32} With regards to the previous study, we found a similar circumstance where SREBP-1 expression at mRNA level was seen to be declined due to Forhidrol administration, confirming the correlation of LXR and SREBP-1 genes.

SREBPs are synthesized in endoplasmic-reticulum membrane, proteolytically cleaved to the active form and released to the nucleus to activate the transcription of some related genes, including CETP. Chouinard *et al.*⁶ found that SREBP-1 contributes to CETP expression in liver and adipose tissue. LXRs bind to a site of CETP promoter and activate the transcription process.^{5,39} Honzumi *et al.*³⁹

reported that LXR agonist enhanced plasma CETP activity and resulted in decreased HDL *in vitro* and *in vivo*. Therefore, the mechanism of CETP transcription is complex and requires interaction between LXR and SREBP transcription factors.^{40,41} Our findings indicated that down-regulation of LXR and SREBP by Forhidrol subsequently reduced CETP mRNA level.

Forhidrol was further found to influence lipoprotein receptors and apolipoprotein expressions. Previous studies have shown the association between CETP deficiency or mutation with changes in lipoprotein receptors or apolipoprotein expressions.^{39,42}

ApoA-I presents in majority of HDL particles by constituting approximately 70% of apolipoprotein content in HDL protein.^{43,44} Immunoblotting study revealed that Forhidrol up-regulated ApoA-I protein in HepG2-treated cells. ApoA-I mediates cholesterol removal in RCT pathway where cholesteryl esters (CEs) bind to the apolipoproteins. Through several steps which were catalyzed by several related enzymes, ApoA-I particles are converted to HDL particles, while carrying cholesteryl esters.⁴⁵ As a consequence,

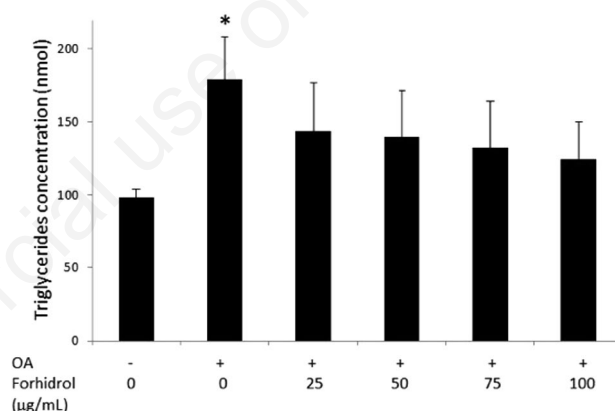


Figure 6. Forhidrol slightly decreases triglyceride synthesis in hepatocellular carcinoma (HepG2) cells. HepG2 cells were incubated with Forhidrol (0-100 µg/mL) for 24 h and triglycerides were extracted. Cells were pre-treated with 0.5 mM oleic acid to induce triglyceride synthesis and used as negative control. Human triglycerides abbexa enzyme-linked immunosorbent assay kit was used to analyze triglyceride level *in vitro*. Results are expressed as mean±SD of three independent experiments. *P<0.05 indicates a significant difference compared to the control.

Table 2. Comparison of cholesteryl ester transfer protein (CETP)-lowering effect on plasma lipoprotein levels at baseline (0 day) and after 30 days of treatment with Forhidrol in New Zealand white rabbits. Animals in control group fed a normal diet, while those in treated groups fed the same diet supplemented with Forhidrol at a dose of 37.5 mg/1.5 kg of body weight/day. The amount of plasma lipids and CETP activity were measured using blood samples of 12-h fasted rabbits which were withdrawn from ear veins. Values are expressed as µg/100 µL for high-density lipoprotein (HDL) and low-density lipoprotein (LDL)-cholesterol, nmol/100 µL for triglycerides (TG), and pmol/µL/h for CETP activity.

| Group | Time | HDL-C | LDL-C | TG | CETP activity |
|---------------------|------------------|-------------|------------|--------------|---------------|
| Control | Baseline (Day 0) | 0.83±0.69 | 1.52±0.36 | 23.28±16.65 | 138.13 ±12.38 |
| | Day 30 | 0.81±0.64 | 1.58±0.50 | 29.57±24.34 | 140.23±9.30 |
| | % change | ↓9.46±7.49 | ↑3.89±20.2 | ↑23.48±32.53 | 1.73±4.11 |
| Forhidrol treatment | Baseline (Day 0) | 1.26±0.24 | 2.04±0.87 | 48.06±10.09 | 144.43±4.04 |
| | Day-30 | 1.54±0.63 | 1.75±0.42 | 48.08±11.93 | 142.65±4.80 |
| | % change | 19.59±33.51 | 0.94±46.01 | 0.34±10.94 | 1.23±1.09 |

The mean of individual percent (%) changes (±SD) relative to baseline. Results are expressed as mean values±SD of five subjects in each group.

ApoA-I concentration correlates positively to HDL level.^{43,45} HDL has been proven to possess protective properties against atherosclerosis by delivering CEs to the liver for disposal as bile acids.^{3,45} In addition, study in transgenic mice showed that ApoA-I overexpression was able to elevate HDL level and protect against atherosclerosis.⁴⁶ This reveals other property of Forhidrol in controlling cholesterol level in the body and, therefore, reducing the occurrence of atherosclerosis.

Effect of Forhidrol on SR-B1, a cell surface glycoprotein that acts as HDL receptor and mediates selective uptake of HDL-CE, were also investigated. Expression of SR-B1 gene isolated from HepG2 cells was down-regulated by Forhidrol administration. Transcriptional regulation of SR-B1 appears to be due to SR-B1 promoter binding sites for a number of transcription factors, including C/EBP, SF-1 and SREBP.⁴⁷ Since SREBP is a sterol transcription factor that induces SR-B1 gene expression,⁴⁸ the reduced expressions of SR-B1 showed in this study may also be associated with the down-regulation of SREBP-1 during CETP inhibition by Forhidrol. The reduction of SR-B1 was also reported in Fibrates, a normolipid drug class used in atherogenic dyslipidemia that raise HDL and decrease triglyceride levels.⁴⁹ In their study, Mardones *et al.*⁴⁹ described a novel effect of Fibrates as an alternative mechanism in modulating HDL metabolism through the suppression of SR-B1 expression. *In vivo* study in rats indicated that SR-B1 expression is modulated by feedback mechanism in response to changes in cellular cholesterol stores.⁵⁰ Huang *et al.*⁴⁵ also reported that although acute suppression of CETP expression lead to an elevation in cellular cholesterol stores, the return of HDL-CE to hepatocytes via SR-B1 pathway was inhibited, which further manifested the dual effects of CETP inhibition in HDL metabolism and further increase the formation of HDL and suppression HDL-CE catabolism via SR-B1 pathway.^{45,51} Furthermore, decreased SR-B1 by Forhidrol might be a beneficial mechanism to suppress HDL catabolism. In another hand, SR-B1 is required to uptake cholesterol of HDL-CE to the liver.⁵¹ Collet *et al.* stated that HDL-CE from plasma to the liver can be transported by either indirect pathway (by transferring to other lipoproteins followed by hepatic receptor-mediated endocytosis) or direct pathway (via SR-B1 and selective uptake).⁵¹ It seemed that Forhidrol transported HDL-CE to the liver via the indirect pathway.

Other important changes were also observed in LDL-R and

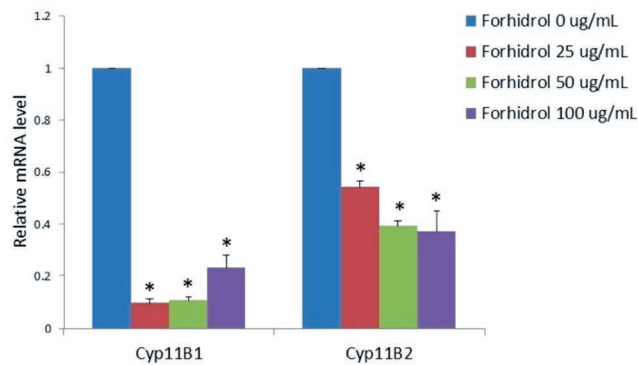


Figure 7. Forhidrol possibly does not increase blood pressure. Hepatocellular carcinoma cells were treated with Forhidrol (0-100 µg/mL) for 24 h. Total RNA was isolated and mRNA levels of CYP11B1 and CYP11B2 were analyzed by real-time polymerase chain reaction. β -actin was used as the internal loading control.

apolipoprotein B (ApoB), in which their expressions were up-regulated and down-regulated, respectively, after treatment with Forhidrol in HepG2 cells (Figure 3B). Deficiency of CETP and SREBP-1 may result in qualitative changes of ApoB, which is the major component of LDL particles, as well as LDL-R.⁵² Evidence showed that SREBP-1 was independently identified as a transcription factor that activate LDL-R gene expression under sterol depletion condition in cell culture.⁶ Furthermore, metabolic turnover of LDL and ApoB may be substantially increased due to up-regulation of LDL-R pathway. Based on these findings, where Forhidrol up-regulated LDL-R and down-regulated ApoB genes, a more comprehensive atheroprotective mechanism of Forhidrol was further revealed.

Prior studies reported that CETP was affected by various fatty acids.³⁰ Here, we also investigated the effects of two PUFAs, *e.g.* AA and LA, on CETP mRNA level. The results exhibited that CETP expression in PUFA-treated HepG2 cells declined drastically (Figure 4). Surprisingly, when PUFAs treatment was combined with Forhidrol, CETP inhibition was higher than PUFA treatment alone. PUFA has been reported to override pro-lipogenic effects through the modulation of pro-lipogenic genes, such as PPARs, SREBP and LXR.⁵³ As Forhidrol also modulated the same pro-lipogenic genes, it might be indicated that Forhidrol and PUFAs acted synergistically to reduce CETP at transcriptional level.

PPARs are transcription factors that play crucial roles in lipid and lipoprotein metabolism, which consists of three proteins including alpha, beta/delta and gamma.⁵⁴ Considerable evidences indicated that stimulation of PPARs in atherosclerosis condition has been reported to: i) inhibit hepatic triglyceride production; ii) increase lipoprotein lipase-mediated triglyceride lipolysis; iii) provide a higher affinity of remnants for the LDL-R; iv) enhance human ApoA-I and ApoA-II synthesis;⁵⁵ and v) reduce CETP expression.^{56,57} Several studies reported that PPARs agonism is beneficial due to their effect on improving different profiles of atherosclerosis. A dual PPAR α/γ agonist blocks the progression of pre-existing atherosclerosis by reducing triglyceride and LDL levels as well as increasing HDL level in transgenic mice.⁵⁸ Another study reported that PPAR γ suppressed CYP11B2 expression *in vitro*.⁵⁹ PPAR δ agonist serves as atheroprotective agent by decreasing monocyte chemoattractant protein 1 (MCP-1) and intracellular adhesion molecule 1 (ICAM-1) in LDL-R (-/-) mice.⁶⁰ Thus, regulations of these proteins remain an attractive therapeutic target for drug development in the treatment of chronic inflammatory diseases such as atherosclerosis.⁵⁴ Our findings revealed that Forhidrol treatment increased PPARs gene expressions in HepG2 cells, especially PPAR δ and PPAR α , which may be associated to the changes of LDL-R expression and LDL level, respectively. Although we did not evaluate the effect of Forhidrol on PPARs activities, there might be a direct correlation between PPARs expression and lipid metabolism, as higher PPARs expression may contribute to higher activation of PPARs. This statement was also supported by the activity of Forhidrol in the reduction of triglycerides levels. Triglycerides are reported as a substance that is not directly atherogenic, however, since they are also the component of LDL molecules which exert negative effect in atherosclerosis, high level of triglycerides represents an important biomarker of cardiovascular disease.⁶¹ The reduction of PPARs expression and triglycerides level by Forhidrol may support its beneficial effects in reducing LDL-CE.

In vivo study in New Zealand rabbits was also conducted to affirm the effects of Forhidrol in plasma lipid profiles and CETP activity. Inclusion of Forhidrol in the diet resulted in a decreased CETP activity which subsequently led to an elevation of HDL level

compared to the control group. CETP deficiency in the treated animal models exhibited a reduced exchange of CE from HDL particles to triglycerides in LDL particles. This condition was observed in our study wherein LDL and triglyceride levels were reduced in the treatment group. The present study confirmed that inclusion of Forhidrol in the diet resulted in an elevation of plasma HDL level by specific inhibition of plasma CETP activity in the experimental rabbits. Although low significance alterations were found in the Forhidrol-treated rabbits compared to the control groups, the data showed clear trends that Forhidrol treatment improved atherosclerosis condition by increasing HDL level and reducing LDL, triglycerides and CETP level.

In addition to the pharmacodynamic data, we also investigated the effect of Forhidrol as CETP inhibitor on other factors. One of CETP inhibitor that has been clinically tested is torcetrapib.

Brousseau *et al.*⁶² found that torcetrapib markedly increased HDL level and also decreased LDL level in patients with low HDL level. During the study, it was found that inhibition activity of torcetrapib was associated with an increase in blood pressure due to the stimulation of aldosterone and cortisol secretion through certain molecule-specific effects that are unrelated to CETP inhibition, which further led to a high mortality rate in torcetrapib-treated patients.⁶³ A study at mRNA level revealed that torcetrapib increased the expression of CYP11B1 and CYP11B2 which are the key enzymes in the last step of cortisol and aldosterone biosynthesis pathway, respectively.⁶⁴ Increased production and secretion of aldosterone and cortisol were found to further lead to blood pressure elevation.⁶⁵ Unlike torcetrapib, Forhidrol reduced CYP11B1 and CYP11B2 transcription (Figure 7) which suggested that Forhidrol did not result in the elevation of blood pressure. Based on the present result, Forhidrol may act as a safe preference of CETP inhibitor which does not affect the blood pressure.

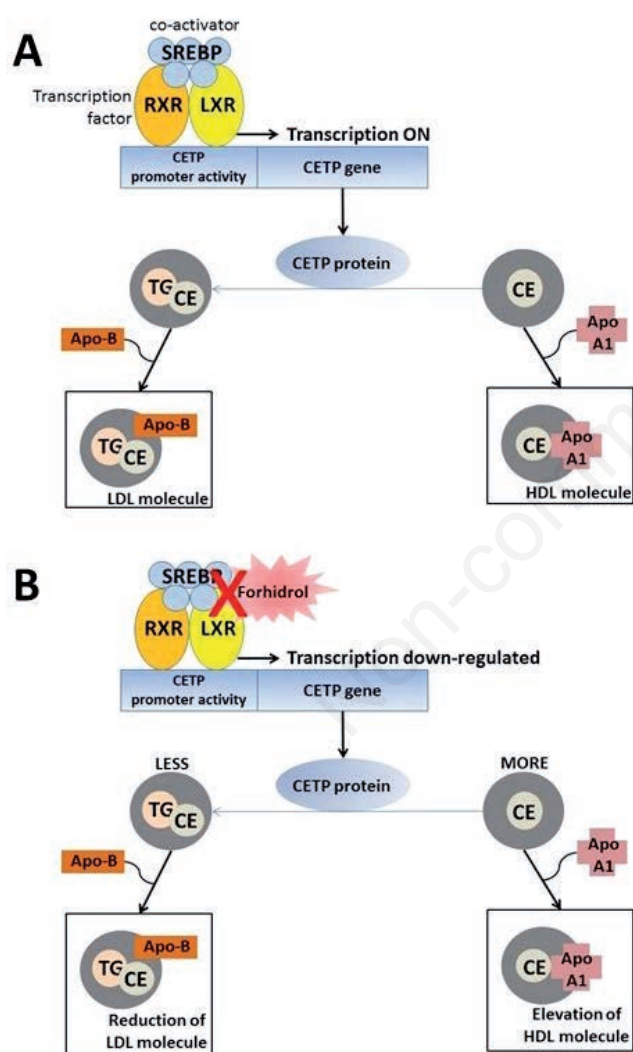


Figure 8. Forhidrol inhibits cholesteryl ester transfer protein (CETP) expression via down-regulation of liver X receptor (LXR) and sterol regulatory element-binding protein 1 (SREBP-1) and subsequently results in a higher level of HDL. (A) The normal transcription and role of CETP; (B) The Action of Forhidrol on CETP inhibition which results in the elevation of high-density lipoprotein (HDL) level.

Conclusions

In conclusion, our present study indicated that Forhidrol, a bioactive fraction derived from *P. macrocarpa* (Scheff.) Boerl., possesses several properties which increases RCT pathway by decreasing CETP expression and inhibiting its activity, as described in Figure 8. This bioactive fraction caused down-regulation of CETP transcriptional regulatory genes, including SREBP-1 and LXR α which resulted in an inhibition of CETP at mRNA and protein levels. Forhidrol also affected other important features of atherosclerosis such as increased PPARs and repressed triglyceride level, which further linked to the changes of atherosclerosis profiles such as the balance of LDL-HDL particles. Preliminary *in vivo* study in rabbit showed that Forhidrol administration has a beneficial effect on lipoprotein profile, which includes a decreased LDL level and an increased HDL level. These beneficial effects may be partially mediated via down-regulation of CETP. Additionally, our findings may be useful in the contribution of potential and safe therapy for atherosclerosis.

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