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A new multifunctional hydroxytyrosol-clofibrate with hypolipidemic, antioxidant, and hepatoprotective effects

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ABSTRACT

Oxidative stress has been regarded as the leading mechanism of the hepatotoxicity of clofibrate (**CF**). To achieve multifunctional novel hypolipidemic agents with hypolipidemia, antioxidant, and ameliorating liver injury, clofibric acid derivative hydroxytyrosol-clofibrate (**CF-HT**) was synthesized by molecular hybridization. **CF-HT** exhibited significant hypolipidemia, reducing serum triglyceride (TG), total cholesterol (TC), and malonaldehyde (MDA) by 30%, 33%, and 29% in hyperlipidemic mice induced by Triton W*R* 1339. **CF-HT** also shown hepatoprotective effect, a significant decrease in hepatic indices toxicity was observed, i.e. aspartate and lactate transaminases (AST and ALT) activities, alkalines phosphatases (ALP), and total bilirubin (TBIL) levels. The liver weight and liver coefficient were also ameliorated. Serum superoxide dismutase (SOD) was significantly elevated, and serum catalase (CAT) and malondialdehyde (MDA) content were remarkably restored. The hepatic glutathione (GSH) content was obviously increased and hepatic oxidized glutathione (GSSG) content was reduced dramatically by **CF-HT**, as compared to the **CF** treated mice ($p < 0.05$). Moreover, the histopathological damage that hepatocyte hyperplasia and hypertrophy was also significantly ameliorated by treatment with **CF-HT**. Therefore, the results indicated that **CF-HT** exerted more potent hypolipidemic activity and definite hepatoprotective effect which may mainly be associated with its antioxidative property in mice.

Hyperlipidemia is the major risk factor of cardio-cerebrovascular diseases and metabolic syndromes such as atherosclerosis, coronary heart disease, myocardial infarction, and cerebral apoplexy. Morbidity of hypertriglyceridemia is higher than hypercholesterolemia, which presents a rising tendency.[1] It has been attracted more and more attentions on hypertriglyceridemia, the contributor to cardio-cerebrovascular and other diseases. Therefore, it is important to ameliorate and control hypertriglyceridemia. Clofibrate (**CF**), a synthetic agonist of peroxisome proliferator activated receptor-α (PPAR-α), characterized by decreasing triglyceride, plays an important role in reducing blood lipids and ameliorating the related diseases. The previous related literature reported that a disturbance of metabolic function in the liver were observed after long-term administration **CF**, which included a distinction increase in liver weight (due to hepatocyte hyperplasia and hypertrophy), midzonal mitoses, centrilobular hypertrophy, and changes in clinical pathology.[2,3] Moreover, **CF** increased hepatic mitochondrial oxidative DNA and protein damage in mice.[4] These results indicated that **CF** can induce hepatotoxicity in body after treatment. Liver damage following the use of fibrates agents has been reported as a cellular damage (increases in AST or ALT enzymes, bilirubin and or alkaline phosphatase increases).[5] Moreover, the superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2) were marked increase as well as superoxide dismutase (SOD) and glutathione peroxidase (GPX) were significantly decreased in rat liver during treatment with **CF**.[6] Oxidative

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, , aspartate transaminase; CA, clofibric acid; CAT, catalase; **CF**, clofibrate; **CF-HT**, hydroxytyrosol-clofibrate ^{13}C NMR, ^{13}C-nuclear magnetic resonance; DMAP, 4-dimethylaminopyridine; DMF, *N,N*-dimethylformamide; EDCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride; GPX, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione; ^{1}H NMR, ^{1}H-nuclear magnetic resonance; **HT**, hydroxytyrosol; MDA, malondialdehyde; SOD, superoxide dismutase; TC, totol cholesterol; TG, triglyceride; TBIL, total bilirubin

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stress has been regarded as the major mechanism of the hepatotoxicity. Thus, ameliorating oxidative stress is good for **CF** to treat hyperlipidemia.

In the recent years, antioxidant compounds have received more and more attention to overcome the harmful effects caused by many toxic chemicals mainly through their scavenging ability of free radicals.[7] Virgin olive oil is the major component of Mediterranean diet, and many researches showed that phenolic compounds are a beneficial factor to the health Mediterranean diet.[8–10] Accumulating evidence indicates that its health benefits include cardioprotection, chemoprotection against several types of cancer, modification of inflammatory responses, etc. Hydroxytyrosol (**HT**, 3,4-dihydroxy phenylethyl alcohol), the simple phenolic compound found in olive oil, was also shown to exhibit various pharmacological health benefits such as antithrombotic, antiatherogenic, anti-inflammatory, and hypolipidemic activities.[11] It has been reported to inhibit oxidative stress induced by various toxic chemicals and improvising the antioxidant defense system by modulating several signaling pathways.[12]

CF has been widely used in the treatment of hypertriglyceridemia and mixed hyperlipidemia in clinical practice. It was hydrolyzed by esterase to ethanol and **CA** which modulates the expression of related genes involved in lipid metabolism through the activation of PPAR-α in the body after its absorption.[13,14]

Based on these, we designed compound **CF-HT** containing **CA** and **HT** pharmacophore. (Chart 1) We replaced the oxethyl of **CF** with 3,4-dihydroxy phenylethyl to obtained compound **CF-HT** and expected that the new compound can be hydrolyzed by esterase to **CA** and **HT**, which not only remains hypolipidemic activity, but also exerts antioxidant activity.

The preparation of **CF-HT** was demonstrated in Scheme 1. The catechol of **HT** was firstly prevented to the **1a** using benzyl bromide and potassium carbonate in acetone. Then **CA** was esterified with **1a** to give **2b**, which was deprotected to **CF-HT** in hydrogen and Pd/C.[15,16] The product was purified by column chromatography and was identified by ^{1}H NMR, ^{13}C NMR, and EI-MS.

Antioxidant activities of compound **CF-HT** *in vitro* were determined through DPPH radical scavenging, the Fenton reaction, and anti-lipid peroxidation.[17–19] As showed in Fig. 1, **CF-HT** exhibited excellent

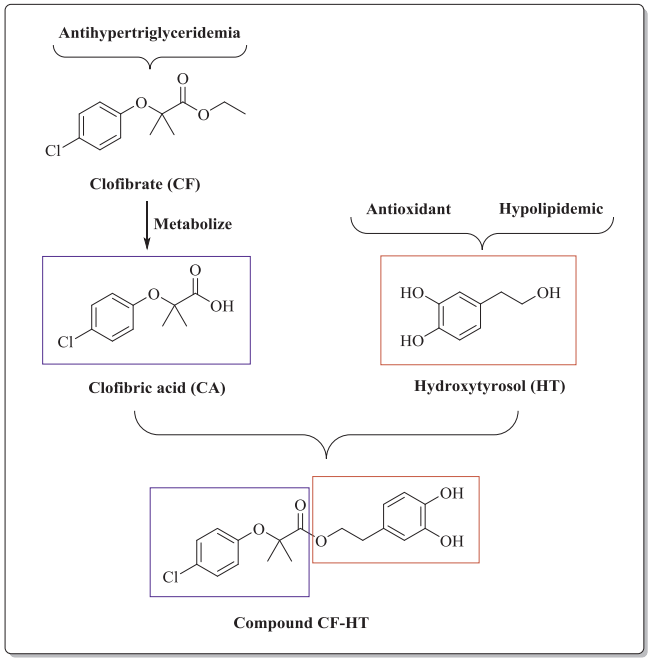
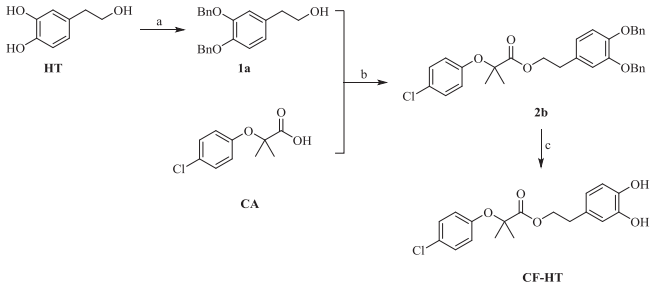


Chart 1. Design of compound **CF-HT** and structures of Clofibrate (**CF**), Clofibric acid (**CA**), and Hydroxytyrosol (**HT**).



Scheme 1. Synthesis route to compound **CF-HT**. Reagents and conditions: (a) BnBr, K_2CO_3, acetone, reflux; (b) EDCI, DMAP, DMF; (c) H_2, Pd/C, ethanol.

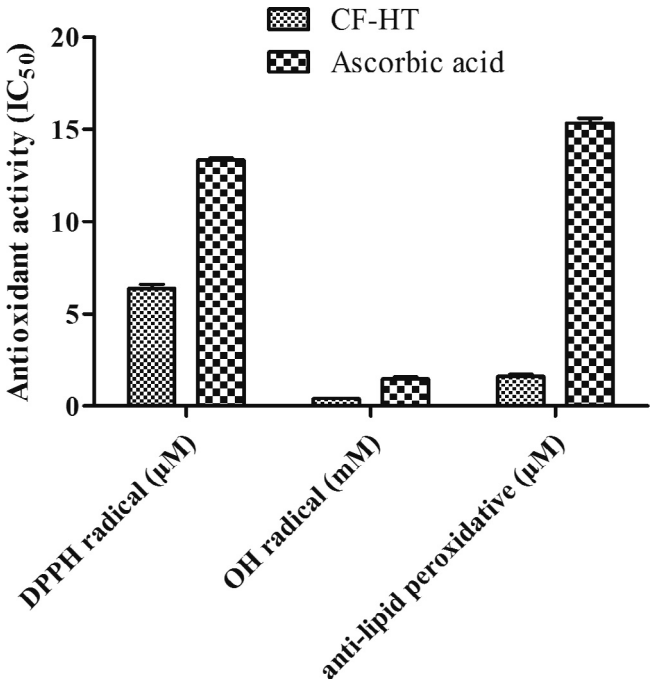


Fig. 1. The antioxidant effects of **CF-HT** were evaluated *in vitro* towards scavenging free radicals (DPPH· and OH·) and inhibiting lipid peroxidation. Data are presented as the mean ± SEM, n = 3.

antioxidant abilities, while **CF** did not show antioxidant properties. The antioxidant abilities of **CF-HT** were improved *in vitro*, compared to **HT**. Both **CF-HT** and **HT** have the structure of catechol which was favorable for scavenging reactive free radicals. Furthermore, the antioxidant activities may be associated with the lipid solubility.

The hypolipidemic activity of the **CF-HT** *in vivo* was evaluated in acute hyperlipidemic mice induced by Triton WR 1339.[20,21] **CF-HT** was found able to decrease plasma TG by 30% and TC by 33%. While **CF** reduced plasma TG by 24% and TC by 23%. (Fig. 2) Therefore, **CF-HT** showed to be more significant effects on antihyperlipedemia compared to that of **CF**. We inferred that compound **CF-HT** that incorporate **CF** with **HT** contributes to a significantly synergistic hypolipidemic effects. Moreover, **CF-HT** and **CF** reduced plasma MDA levels by 29% and 14%. (Fig. 3) **CF-HT** reduced MDA could be explained by antioxidant and hypolipidemia. **CF** reduced MDA might be explained by anti-hypolipidemia. Thus, the increased antioxidant activity *in vitro* of **CF-HT** was also confirmed *in vivo*.

Finally, the hepatoprotective effect of **CF-HT** *in vivo* was evaluated in normal mice. Male ICR mice (20.0 ± 2.0 g) were distributed into the following groups: (1) normal (the same volume of vehicle), (2) **CF** (240 μmol/kg/day), (3) **CF-HT** (240 μmol/kg/day). To assess liver function, liver coefficient and hepatic histopathology were researched, as well as hepatic biochemical indexes (AST, ALT, AKP and TBIL) and