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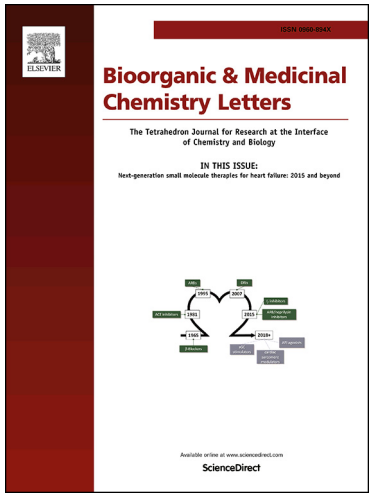

A new multifunctional hydroxytyrosol-clofibrate with hypolipidemic, antioxidant, and hepatoprotective effects

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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 WR 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.

Keywords: hydroxytyrosol-clofibrate; hypolipidemic; antioxidant; hepatoprotective

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.

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

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.

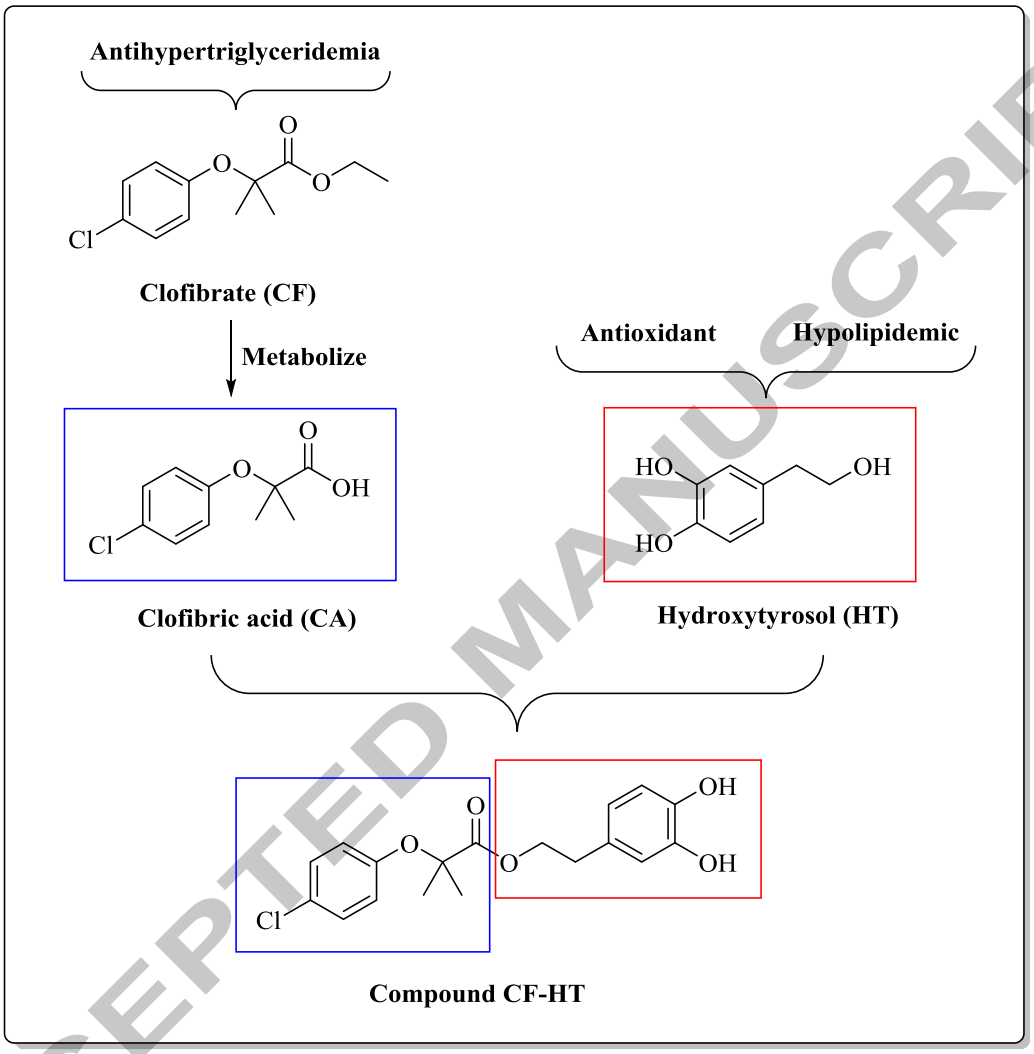
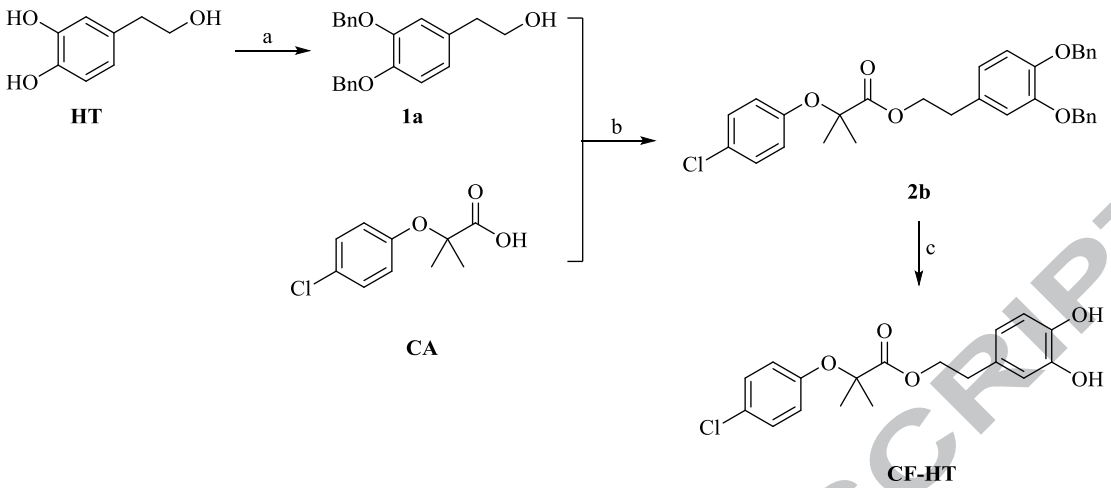


Chart 1. Design of compound **CF-HT** and structures of Clofibrate (**CF**), Clofibric acid (**CA**), and Hydroxytyrosol (**HT**).

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.



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.

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 Figure 1, **CF-HT** exhibited excellent 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.

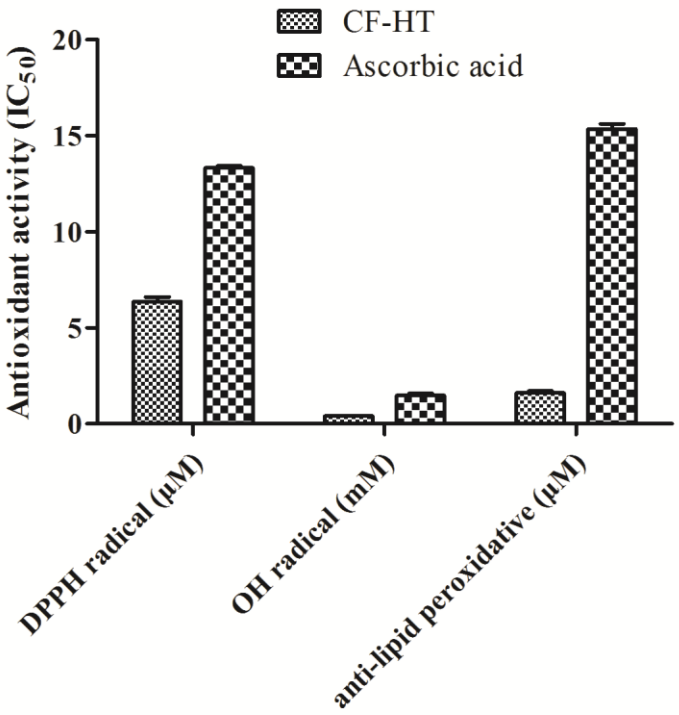


Figure 1. The antioxidant effects of **CF-HT** were evaluated *in vitro* towards scavenging free radicals (DPPH• and OH•) and inhibiting lipid peroxidation. Data are presented as the mean±SEM, n = 3.

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%. (Figure 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%. (Figure 3) **CF-HT** reduced MDA could be explained by antioxidant and hypolipidemia. **CF** reduced MDA might be explained by antihypolipidemia. Thus, the

increased antioxidant activity *in vitro* of **CF-HT** was also confirmed *in vivo*.

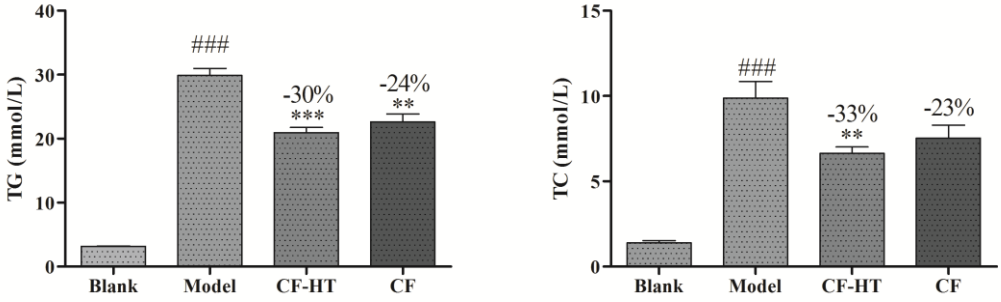


Figure 2. Percent reduction of triglyceride and total cholesterol plasma levels of Triton WR 1339 induced hyperlipidemic mice after ig administration of 240 μmol/kg **CF-HT** and **CF** for 7 days. mean±SEM, n=8, $^{###}P<0.001$ vs blank group; $^{**}P<0.01$, $^{***}P<0.001$ vs model group.

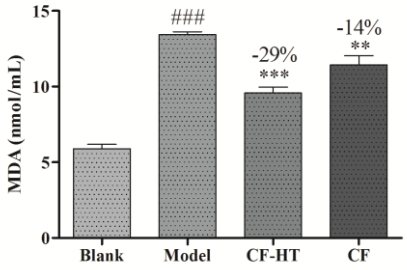


Figure 3. Plasma MDA (nmol/mL) levels of Triton WR 1339 induced hyperlipidemic mice in the presence of compounds **CF-HT** and **CF**. mean±SEM, n=8, $^{###}P<0.001$ vs blank group; $^{**}P<0.01$, $^{***}P<0.001$ vs model group.

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 antioxidant indexes (SOD, MDA, CAT, GSH, and GSSG) were measured using commercial kits (Jian Cheng, Nanjing, China).

As showed in Table 1, the body weight of **CF** group and **CF-HT** group mice was significantly lower than normal group mice, and the liver weight of **CF** mice were higher than normal mice, while the liver weight of **CF-HT** mice was closed to normal mice. Therefore, the liver coefficient of **CF** mice was clearly elevated (p<0.01) and the liver coefficient of **CF-HT** mice was also obviously lifted (p<0.05) compared to normal mice. Moreover, the liver coefficient of **CF-HT** mice was distinctly decreased (p<0.01) in comparison with **CF** mice. The results suggested that **CF-HT** possesses the hepatoprotective effect at the side of **CF**.

Table 1. Effects of **CF** and **CF-HT** on body weight, liver weight and liver coefficient in mice.[a]

Groups	Body weight (g)	Liver weight (g)	Liver Coefficient (mg/g)
Normal	36.8±0.70	1.49±0.42	40.50±0.71
CF	33.8±0.60$^{\#\#}$	1.77±0.08	52.12±1.60$^{\#\#}$
CF-HT	33.4±0.44$^{\#\#}$	1.48±0.04	44.17±1.23$^{\#**}$

[a] The results are summarized as mean±SEM (n=10) and analyzed by independent T-test.

$^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ vs Normal group

** $p<0.01$ vs **CF** group

Serum AST, ALT, ALP, and TBIL levels were analyzed as indications of hepatic function. As shown in Table 2, the levels of AST, ALT, ALP, and TBIL were

significantly higher in the **CF** group when compared to the normal group (p<0.05 or p<0.01). The administration of **CF-HT** significantly decreased AST, ALT, ALP, and TBIL levels (p<0.05 or p<0.01), as compared with **CF** group. The levels of ALT, ALP, and TBIL in **CF-HT** group did not show the significance compared to normal group, while the level of AST was apparently increased (p<0.05). In addition, as shown in Table 3, the serum SOD and CAT activities were significantly decreased, serum MDA, an index of lipid peroxidation, was obviously increased, and hepatic GSH content was clearly reduced and GSSG content was overtly elevated as well as the ratio of GSH/GSSG was visibly decreased after administration with **CF**, as compared with the normal group (p<0.01), suggesting that oxidative stress resulted from **CF** poisoning. **CF-HT** treatment was found able to increase SOD and CAT activities and decrease MDA concentrations as well as restore GSH contents and the ratio of GSH/GSSG effectively in contrast to the **CF** group (p<0.05 or p<0.01), but GSSG contents was not significantly decreased (p>0.05), thus ameliorating oxidative stress to some extent. Especially, the CAT activity and MDA concentrations in **CF-HT** group which were closed to the normal group showed no significance. Altogether, these findings suggested that the hepatoprotective activity of **CF-HT** was regarded as due to its antioxidant action.

Table 2. Effects of **CF** and **CF-HT** on serum ALT, AST, APK and TBIL levels in mice.[a]

Groups	AST (U/L)	ALT (U/L)	APK (U/100mL)	TBIL (μM)
Normal	107.25±2.77	36.25±1.08	20.02±0.61	0.63±0.09
CF	157.50±9.05$^{\#\#}$	63.13±11.52$^{\#}$	28.64±2.42$^{\#}$	13.09±0.94$^{\#}$
CF-HT	121.37±4.59$^{\#**}$	39.75±2.03*	17.57±0.76**	0.95±0.20*

[a] The results are summarized as mean±SEM (n=10) and analyzed by independent T-test.

$^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ vs Normal group

* $p<0.05$, ** $p<0.01$ vs CF group

Table 3. Effects of **CF** and **CF-HT** on serum SOD, MDA, and CAT levels, and hepatic GSH and GSSG levels in mice.[a]

Groups	SOD (U/mL)	MDA (μM)	CAT (U/mL)	GSH (μM)	GSSG (μM)	GSH/GSSG
Normal	337.64±4.82	6.00±0.20	2.83±0.22	43.38±4.59	2.91±0.31	16.4±2.34
CF	228.32±15.97$^{\#\#}$	7.79±0.34$^{\#\#}$	3.86±0.18$^{\#\#}$	11.60±1.86$^{\#\#}$	6.24±0.56$^{\#\#}$	2.1±0.41$^{\#\#}$
CF-HT	275.20±5.14$^{\#\#*}$	6.44±0.09**	2.04±0.29**	22.87±3.58$^{\#\#*}$	4.85±0.58$^{\#}$	4.1±0.70$^{\#\#*}$

[a] The results are summarized as mean±SEM (n=10) and analyzed by independent T-test.

$^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ vs Normal group

* $p<0.05$, ** $p<0.01$ vs CF group

Furthermore, the hepatoprotective effect was further confirmed by

histopathological analysis. As exhibited in Figure 4, the pathology slice examination showed the liver tissue organization of the mice in normal comparison group is normal and the hepatic lobules of them were well-arranged. In contrast, the hepatocyte hyperplasia and hypertrophy, midzonal mitoses, and centrilobular hypertrophy were observed in **CF** group. However, the histopathological changes were not recorded in **CF-HT** group. Histological observations support biochemical findings (hepatic and antioxidant indexes), as they visibly show morphological changes in connection with oxidative stress.

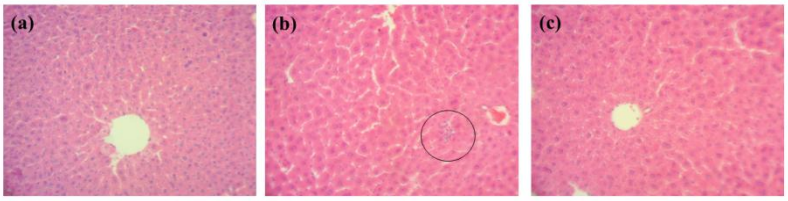


Figure 4. Histopathological studies of liver in mice (H&E 200×). (a) Normal group mouse liver. (b) **CF** group mouse liver. (c) **CF-HT** group mouse liver.

These findings reported in this research provide the first evidence that **CF-TH** is better than **CF** in preventing hyperlipidemia, oxidative stress, and hepatoprotective effectives in mice. Compared **CF-HT** with **CF** in the structure, we replaced oxethyl with **HT**. The action of **CF** in body involves the hydrolyzation of ester bond to **CA** and ethanol by esterase. The pharmacokinetics results of **CF-HT** in rats revealed that compound **CA** and **HT** were determined in serum, which did not describe in the paper. And the bioavailability of **CF-HT** was similar to **CF**. We deduced that **CF-HT** also can be hydrolyzed by esterase to **CA** and **HT**. **CA** was the active metabolite of **CF**

and activated PPAR-α to regulate lipid metabolism. It has been reported that **HT** showed marked hypolipidemic activities decreasing TG and TC. Treatment with **CF-HT** and **CF** showed the significant hypotriglyceridemic ability compared to the model group, furthermore, **CF-HT** exhibited obvious decrease in TC concentrations in Triton WR 1339 induced mice. The hypolipidemic results of **CF-HT** can be explained by the synergistic effects of **CA** and **HT** in mice. In liver protection experiment, **CF** exhibited a significant hepatotoxicity by elevating hepatic coefficient and biochemical findings (hepatic and antioxidant indexes) compared to normal group mice, while **CF-HT** shown obviously hepatoprotective effects by reducing hepatic coefficient and hepatic function indexes and ameliorating oxidative stress, as in contrast to **CF** group mice. Moreover, the hepatoprotective effects of **CF-HT** were also confirmed in histological observations. The possible interpretation may be considered to be via the action of **HT**. The previous research shown that treatment with **HT** significantly attenuated ROS-induced hepatotoxicity by inhibiting CYP1A1 expression, bolstering the tissue antioxidant defense system.[10]

In conclusion, a multi-target-directed approach in this study led us to design compound **CF-HT**, which was distinguished by the unique multimodal profile. **CF-HT** was able to significantly reduce TG, similar to that of **CF**. Moreover, **CF-HT** could potently decrease TC and ameliorate oxidative stress. Additionally, the results presented here demonstrated that treatment with **CF-HT** possesses hepatoprotective effects. The beneficiary effect of **CF-H**T against oxidative damages may be the most possible contributor to liver protection. All these properties of **CF-HT** were better

than **CF**, which could be conducive to amelioration of hyperlipidemia in patients. This approach may be significant for the secondary development and optimization of **CF**.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found in supplement material.

References and notes

1. Ong, C. S.; Pollock, C. A.; Caterson, R. J.; Mahony, J. F.; Waugh, D. A.; Ibels, L. S. *Medicine* **1994**, 73, 215.
2. Kim, S. C.; Hong, J. T.; Jang, S. J.; Kang, W. S.; Yoo, H. S.; Yun, Y. P. *The Journal of Toxicological Sciences* **1998**, 23, 113.
3. Baker, V. A.; Harries, H. M.; Waring, J. F.; Duggan, C. M.; Ni, H. A.; Jolly, R. A.; Yoon, L. W.; De Souza, A. T.; Schmid, J. E.; Brown, R. H. *Environ. Health Perspect.* **2004**, 112, 428.
4. Qu, B.; Li, Q.-T.; Wong, K. P.; Tan, T. M.; Halliwell, B. *Free Radic. Biol. Med.* **2001**, 31, 659.

5. Bertolami, M. C. *Arq. Bras. Cardiol.* **2005**, 85, 25.

6. Ciriolo, M. R.; Mavelli, I.; Rotilio, G.; Borzatta, V.; Cristofari, M.; Stanzani, L. *FEBS Lett.* **1982**, 144, 264.

7. Wu, Z. R.; Bai, Z. T.; Sun, Y.; Chen, P.; Yang, Z. G.; Zhi, D. J.; Li, Y.; Wang, X.; Du, J. J.; Yang, R. *Bioorg. Med. Chem. Lett.* **2015**, 25, 5424.

8. Yan, M. X.; Li, Y. Q.; Meng, M.; Ren, H. B., et al *Nutr. Metab. Cardiovasc. Dis.* **2010**, 20, 284.

9. Cicerale, S.; Lucas, L.; Keast, R. *Int. J. Mol. Sci.* **2010**, 11, 458.

10. Servili, M.; Sordini, B.; Esposto, S.; Urbani, S.; Veneziani, G.; Di Maio, I.; Selvaggini, R.; Taticchi, A. *Antioxidants* **2013**, 3, 1.

11. Koshy, A. S.; Anila, L.; Vijayalakshmi, N. *Food Chem.* **2001**, 72, 289.

12. Kalaiselvan, I.; Samuthirapandi, M.; Govindaraju, A.; Sheeja Malar, D.; Kasi, P. D. *Pharm. Biol.* **2016**, 54, 338.

13. Crouthamel, W. G.; Cenedella, R. J. *Pharmacology* **1975**, 13, 465.

14. Houin, G.; Thebault, J.; d'Athis, P.; Beaumont, J.L. *Eur. J. Clin. Pharmacol.* **1975**, 8, 433.

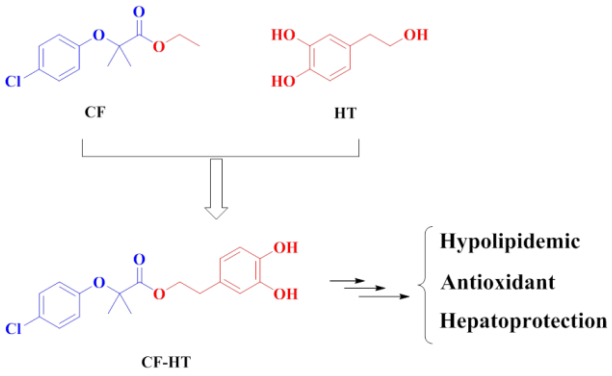
15. Bian, X.; Xie, Y.; Yang, G.; Li, Y.; Zhang, J.; Lin, R.; Lu, W.; Yang, Y.; Xu, Y.; Chen, J. CN 105693517A, **2016**, 26.

16. Xie, Y.; Xu, Y.; Chen, Z.; Lu, W.; Li, N.; Wang, Q.; Shao, L.; Li, Y.; Yang, G.; Bian, X. *Biomed. Pharmacother.* **2017**, 95, 1749.

17. Xie, Y.D.; Chen, Z.Z.; Li, N.; Lu, W.F.; Xu, Y.H.; Lin, Y.Y.; Shao, L.H.; Wang, Q.T.; Guo, L.Y.; Gao, Y.Q.; Yang, G.D.; Li, Y.P.; Bian, X.L. *Biomed.*

Pharmacother. **2018**, 99, 715.

18. Xie, Y.; Yang, Y.; Li, S.; Xu, Y.; Lu, W.; Chen, Z.; Yang, G.; Li, Y.; Cao, Y.; Bian, X. *Bioorg. Med. Chem.* **2017**, 25, 4407.
19. Jun, L.; Jianke, L.; Wei, Z.; Yingli, L. *Journal of Food Science and Biotechnology* **2012**, 31, 159.
20. Sena, V. L. M.; Srivastava, R. M.; Oliveira, S. P.; Lima, V. L. M. *Bioorg. Med. Chem. Lett.* **2001**, 11, 2671.
21. Ladopoulou, E.; Matralis, A. N.; Kourounakis, A. P. *J. Med. Chem.* **2013**, 56, 3330.



Highlights

1. Design of **CF-HT** with a significantly hypolipidemic activity.
2. **CF-HT** displayed potent antioxidant and hepatoprotective effects.
3. Study indicated that C**F-HT** was better than clofibrate toward antihypolipidemic action.