Bioorganic & Medicinal Chemistry Letters xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and biological evaluation of the codrug of Leonurine and Aspirin as cardioprotective agents

Huan Gao [a,b], Xiaohong Yang [a], Xianfeng Gu [b,*], Yi-Zhun Zhu [c,*]

[a] Department of Medicinal Chemistry, School of Pharmacy, Jilin University, Changchun 130021, China
[b] Department of Medicinal Chemistry, School of Pharmacy, Fudan University, Shanghai 201203, China
[c] Department of Pharmacology, School of Pharmacy and Institute of Biomedical Sciences, Fudan University, Shanghai 201203, China

ARTICLE INFO

Article history:
Received 6 May 2016
Revised 3 August 2016
Accepted 19 August 2016
Available online xxxx

Keywords:
Leonruine
Aspirin
Cardioprotective agents

ABSTRACT

The novel codrugs of Leonurine and Aspirin, compounds **545** and **503** have been synthesized and evaluated on their cardioprotective effects. Preliminary pharmacological studies showed that both compounds **545** and **503** were able to increase cell viability of hypoxia-induced H9c2 cells, and compound **545** exhibited at least ten fold potency than **503** and their parent drugs (Leonurine and Aspirin). Further mechanisms studies indicated that the cardioprotective effect of **545** due to its (1) anti-oxidative ability by increasing SOD and CAT enzymes activity and decreasing MDA content and LDH leakage rate, (2) anti-apoptosis activity by regulating apoptosis-associated proteins expression during hypoxia, (3) anti-inflammatory effect by suppression of pro-inflammatory mediators. All of these results demonstrate that compound **545** as a new class of Leonurine analogue could be a drug candidate in our further drug development studies.

The concept of codrug or mutual prodrug refers to two or more compounds with similar or different therapeutic effects bonded via a covalent chemical linkage. It has to be hydrolyzed to provide two (or more) different drugs in cells or organs. So the ester group and amide group are the prefer linkage in the codrug designing. As it well known that codrugs could elicit synergistic action or help the parent drugs to target specific site/organ/cells respectively. Therefore, the drug design basing on codrug strategy has become an efficient approach for drug optimization.[1]

Because of the high mortality, acute myocardial ischemia (AMI), has been one of the most serious diseases threatening human health. Sustained ischemia causes several types of damage to cardiac tissues. The causes of AMI are complicated and varied. As we know, apoptosis, oxidative stress and inflammation are tightly related with the development and progression of AMI. Therefore, preservation of cardiac tissues and cells from the deleterious effects of apoptosis, oxidative stress and inflammation would be an effective approach to treat AMI.

Leonurine (**LEO**) is an effective ingredient derived from *Leonurus artemisia* which has long been used in Chinese traditional medicine. The pharmaceutical studies on **LEO** show that it has cardioprotective effects both in vitro and in vivo due to its anti-oxidation and anti-apoptosis properties.[6–9] Moreover, the animal studies of **LEO** display cardioprotective effects on myocardial ischemia (MI) disease in rats after intraperitoneal injection.[10] However, several issues, such as low content in natural resource, relatively short half-life and moderate biological activity,[11,12] have blocked the progress on the therapeutic development of **LEO**. To improve pharmacological efficacy of **LEO**, structure modification might be a practicable way to gain more potent drug candidates.

Aspirin (ASA), one of the nonsteroidal anti-inflammatory drugs (NSAIDs), is the most versatile pharmaceutical agent. It is used most commonly in the treatment of pain, fever, and inflammation. It also has an antiplatelet effect. So long-term use of Aspirin at low doses could help prevent heart attacks, strokes, and blood clot formation in people at high risk of developing blood clots. Otherwise, some evidences show that Aspirin may be effective at preventing certain types of cancer, particularly colorectal cancer. Due to its significant and versatile pharmaceutical effects, Aspirin has become a good choice for developing new drugs by the strategy of codrug designing.[2–5] Several codrugs of Aspirin have been reported and even used in clinics, such as Benorilate.

As inspired by the studies of codrugs, we were intrigued to assume whether combining **LEO** with another cardioprotective compound such as Aspirin to form new compound will have a dual mode of cardioprotective action and be able to improve cardioprotective efficiency of the precursor. Based on these considerations, compound **545** and **503**, two codrugs of **LEO** and Aspirin as well as **LEO** and salicylic acid, have been designed and synthesized.

* Corresponding authors. Tel.: +86 21 51980112; fax: +86 51980008.
E-mail addresses: xfgu@fudan.edu.cn (X. Gu), zhuyz@fudan.edu.cn (Y.-Z. Zhu).

http://dx.doi.org/10.1016/j.bmcl.2016.08.058
0960-894X/

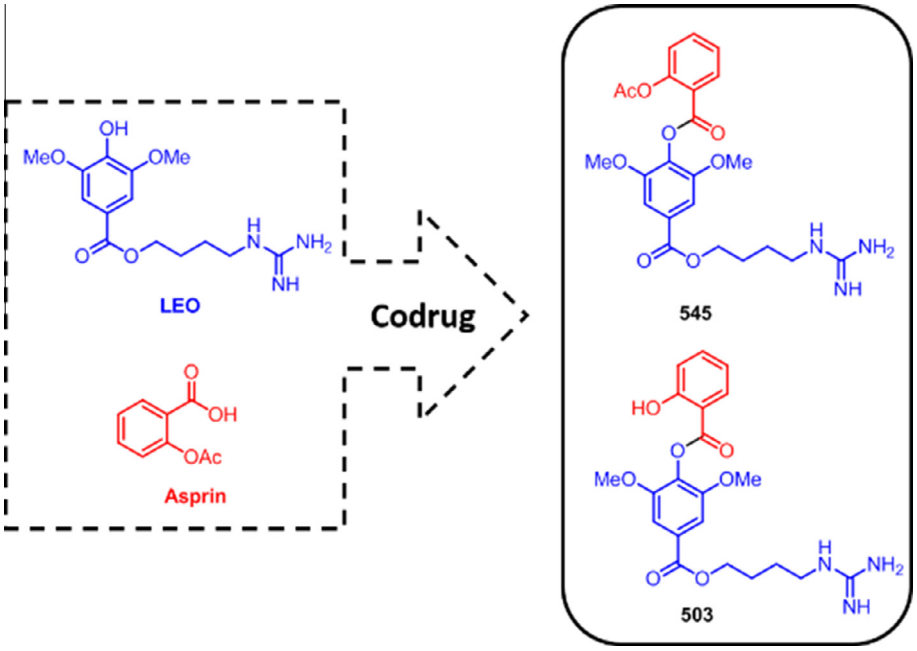
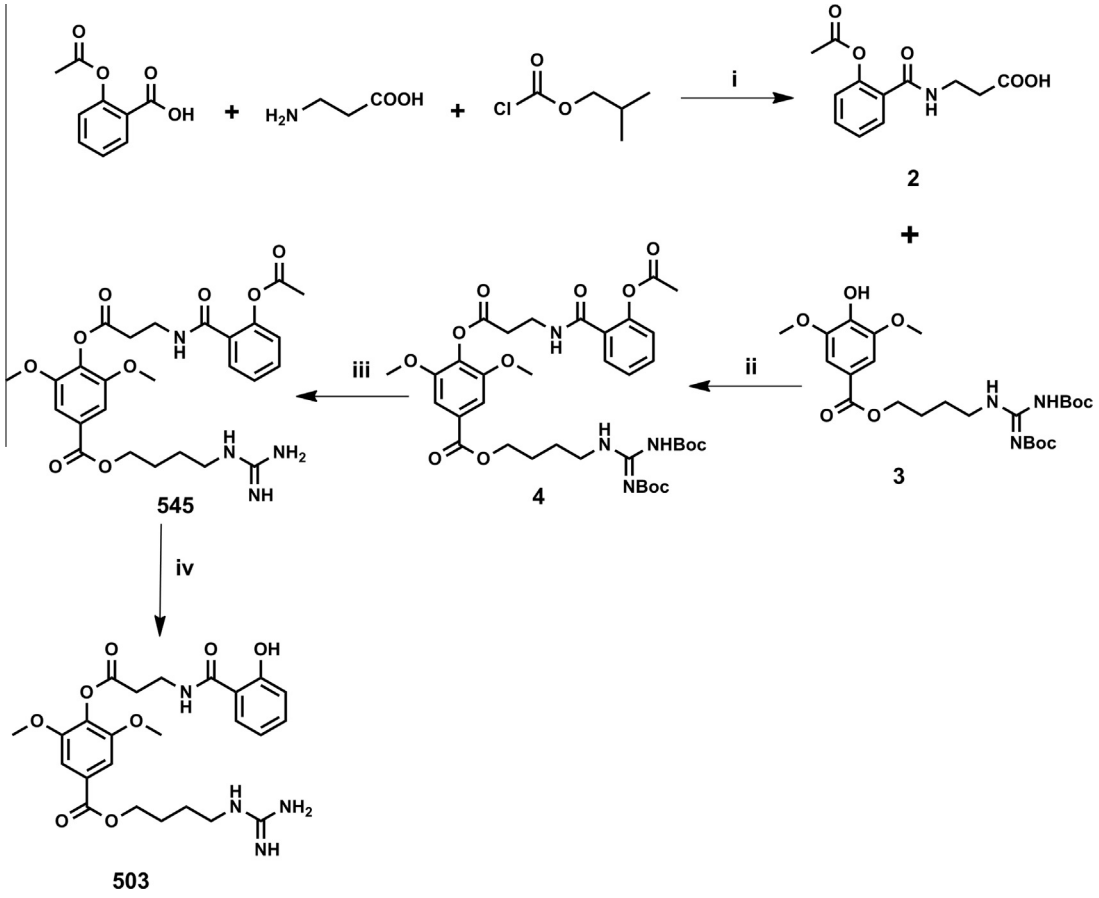


Figure 1. Chemical structure of codrugs and their parent drugs.



Scheme 1. The synthetic route of Leonurine-(β-alanine)-Aspirin conjugates (**545** and **503**). Conditions: (i) TEA, THF, −5 °C, 3 h, (ii) DPTs/DIC, CH_2Cl_2, rt, 12 h (iii) TFA, CH_2Cl_2, rt, 2 h, (iv) hydrochloric acid–methanol solutions, CH_2Cl_2, rt, 2 h.

The preliminary studies indicated both compounds exhibited cardioprotective effects, and compound **545** even possessed higher biological activities than its parent drugs (at less 10-fold than its parent drugs, **LEO** and Aspirin). These results encouraged us to further explore the pharmaceutical mechanisms of compound **545**. Therefore, the effects of compound **545** on anti-oxidant, anti-apoptosis and anti-inflammatory were evaluated. The results suggest compound **545** is able to prevent cellular apoptosis by up-regulating the expression of Bcl-2 and down-regulating Bax even under a lower concentration (10-fold less than its parent drugs, **LEO** and

Please cite this article in press as: Gao, H.; et al. *Bioorg. Med. Chem. Lett.* (2016), http://dx.doi.org/10.1016/j.bmcl.2016.08.058

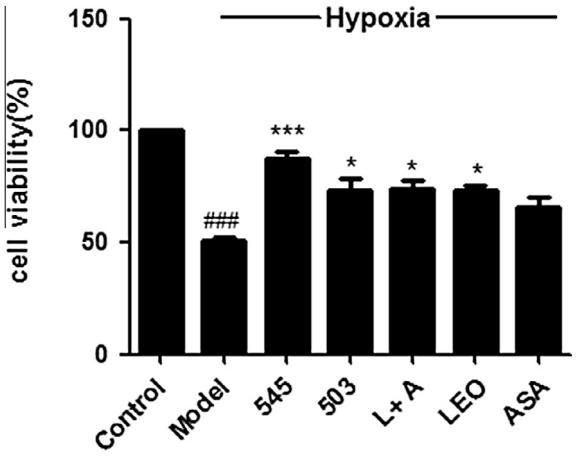


Figure 2. Effects of all compounds on cell viability. Concentration: ASA (1 μM), LEO (1 μM), A + L (ASA1 μM + LEO1 μM), Compound **545** (0.1 μM). Data represent mean ± SEM of more than three independent experiments. $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001 versus model group. $^{\#}P$ <0.05, $^{\#\#}P$ <0.01, $^{\#\#\#}P$ <0.001 versus control group.

Aspirin). Moreover, it can increase the activity of the anti-oxidant enzymes Superoxide Dismutase (SOD) and Catalase (CAT), as well as reduce the inflammatory cytokines, which finally display effective protection against oxidative stress injury (Fig. 1).

Compound **3**, a key intermediate for preparing compound **545** and **503**, was synthesized according to literature method (Fig. 1).[13] Another important intermediate **2** was obtained by the reaction between Aspirin and a linker (β-alanine)[14,15] in the presence of $ClCO_2Et$ under basic condition. Subsequently, the carboxylic acid **2** condensed with **3** in the presence of *N,N′*-diisopropylcarbodiimide (DIC) and 4-(dimethylamino)-pyridinium-4-toluene sulfonate (DPTs), to afford intermediate **4**. Finally, the Boc groups of **4** were removed by TFA to give **545** as the product with good yield. Alternatively, if HCl was utilized to remove Boc groups, the resulting product is compound **503**, in which the acetyl group was also removed at same time (Scheme 1).

Compound **545**, **503** and their parent drugs Aspirin and **LEO** were evaluated on H9c2 (rat myocardial cell line) in vitro. MTT assay was used to investigate their myocardial protection effects (Fig. 2).[16] Four concentrations (0.1 μM, 1 μM, 10 μM, and 100 μM) were tested in MTT assay. H9c2 cells were pretreated with all compounds for 8 hour, followed by 2-h H_2O_2 (40 mM) incubation in the cell culture medium. The cell survival rate showed that all drug-treated groups increased cell viability in varying degrees. Compared with the model group, compound **545** could significantly increase cell viability at 0.1 μM (P <0.001), which is even more potent than the groups treated by 1 μM of **LEO** or Aspirin (P <0.05) or 1 μM of **LEO** + 1 μM of Aspirin group, which indicated that compound **545** has more potent cardioprotective effects than its parent drugs.

Lactate dehydrogenase (LDH) and Lipid Peroxidation (MDA), the biomarker for oxidative stress, reflect the degree of damage of cell membrane function and integrity.[17] At 0.1 μM, compound **545** is able to reduce the LDH leakage in the supernatant of cell culture, and decrease the MDA level, finally protected the cell membrane from oxidative stress injury. MDA contents of compound **545**

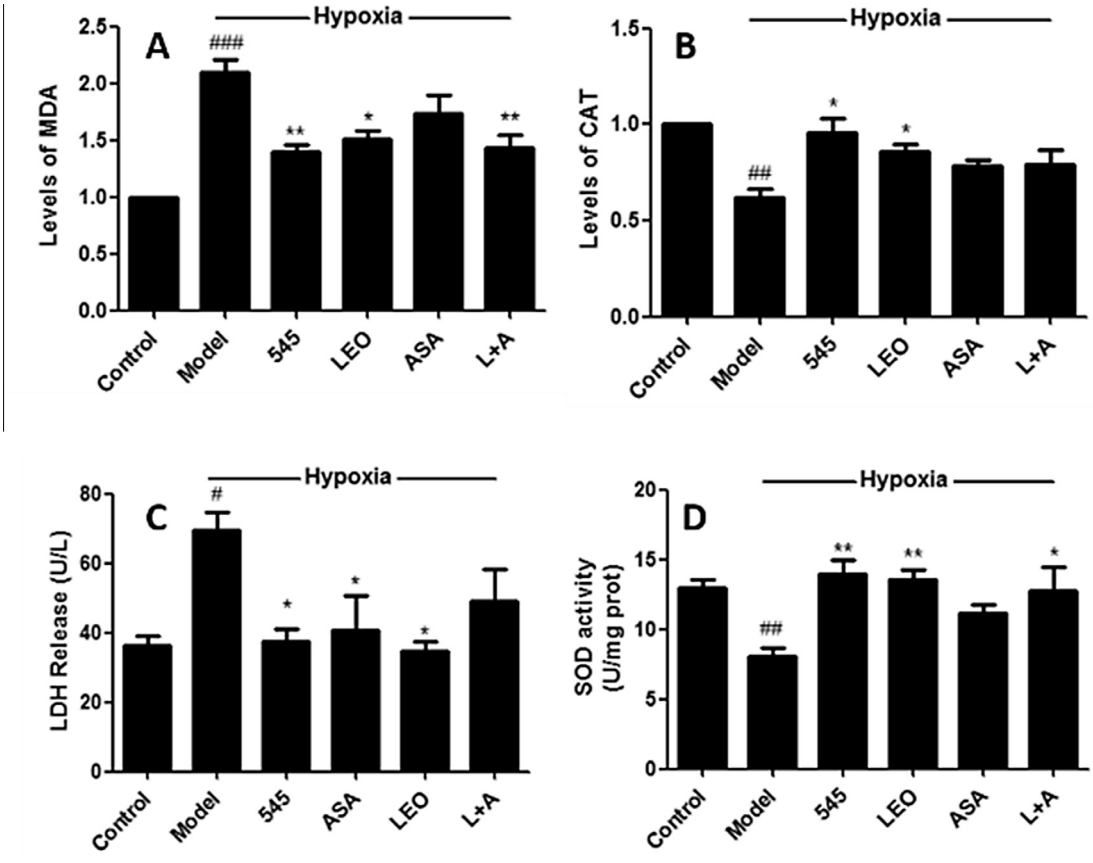


Figure 3. Effect of compound **545** on hypoxia-induced H9c2 cells. (A) Effects on MDA content. (B) Effects on CAT activity. (C) Effects on LDH leakage. (D) Effects on SOD activity. Concentration: ASA (1 μM), LEO (1 μM), L + A (ASA 1 μM + LEO 1 μM), Compound **545** (0.1 μM). Data represent mean ± SEM of more than three independent experiments. $^{*}P$ < 0.05, $^{**}P$ <0.01, $^{***}P$ <0.001 versus model group. $^{\#}P$ <0.05, $^{\#\#}P$ <0.01, $^{\#\#\#}P$ <0.001 versus control group.

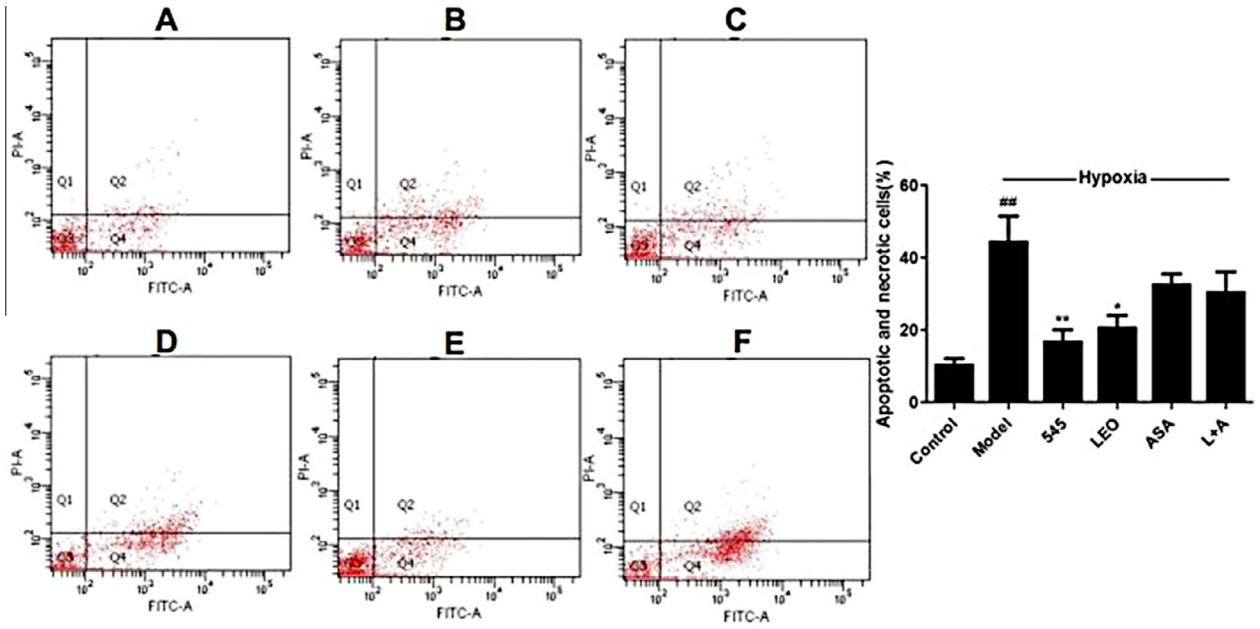


Figure 4. Effect of Compound 545 on anti-apoptosis by FCM with Annexin V FITC/PI. (A) ASA (1 μM). (B) LEO (1 μM). (C) L + A (ASA 1 μM + LEO 1 μM). (D) Compound **545** (0.1 μM). (E) Model. (F) Control. Data represent mean ± SEM of more than three independent experiments. $^{*}P$ <0.05, $^{**}P$ <0.01versus model group. $^{\#\#}P$ <0.01 versus control group.

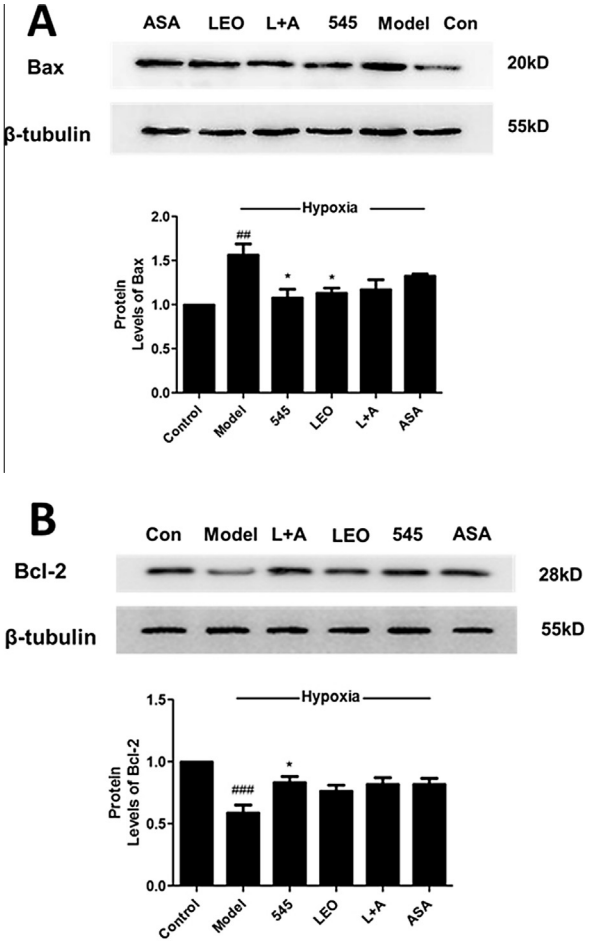


Figure 5. Effect of Compound **545** on apoptosis-relative protein. (A) Effects on expression of Bax. (B) Effects on expression of Bcl-2. Concentration: ASA (1 μM), LEO (1 μM), L + A (ASA 1 μM + LEO 1 μM), Compound **545** (0.1 μM). Data represent mean ± SEM of more than three independent experiments. *P <0.05, **P <0.01, $^{***}P$ <0.001 versus model group. $^{\#}P$ <0.05, $^{\#\#}P$ <0.01, $^{\#\#\#}P$ <0.001 versus control group.

group (0.1 μM, P <0.01) were observably lower than the groups treated by 1 μM of its parent drugs (**LEO** or Asprin or **LEO** + Asprin). As we all know, superoxide dismutase (SOD) and catalase (CAT) are important anti-oxidative enzymes, which can remove excess oxygen-free radical (ROS). Compared with model group, 0.1 μM of compound **545** significantly elevated SOD activity (P <0.01) and CAT activity (P <0.05), which even more potent than the treatments by 1 μM of **LEO** or Aspirin or **LEO** + Aspirin. Thus, the results above revealed compound **545** exhibited significant, even more potent anti-oxidative effect than its parent drugs on hypoxia-induced H9c2 cells (Fig. 3).

Several genes have been reported to be related to programmed cell apoptosis,[18] and the ratio of Bcl-2/Bax has been suggested as a marker for determining survival or death after an apoptotic stimulus.[5,6] The results on the expression of Bcl-2 and Bax protein after treated by drugs in hypoxia-induced H9c2 cells showed compound **545** could reduce the cell death rate with a significant decrease of Bax (P <0.05), and an obvious increase of Bcl-2 (P <0.05) (Fig. 5), which consistent with MTT results.

The anti-apoptosis effect of compound **545** was further confirmed by AnnexinV-FITC/PI with Flow Cytometry Method (FCM). FCM is a method which could exactly screen the apoptotic cell and necrotic cell respectively from total cells. The results showed that compound **545** remarkably decreased the number of total apoptotic cells (include necrotic cells, P <0.01) (Fig. 4).

It has been reported that oxidative stress injury could also cause inflammation.[19] The cell culture supernatant levels of the inflammatory cytokines TNF-α, IL-1β and IL-6 were measured using ELISA kits. As shown in Figure 5, the levels of all the inflammatory cytokines were increased in model group, while pretreatment of compound **545** could reverse this trend, and more effective than the groups treated by **LEO** or Aspirin or **LEO** and Aspirin. Thus the results suggested that compound **545** protect myocardial cell by suppression of pro-inflammatory mediators (Fig. 6). In addition, we also observed that compound **545** can increase the concentration of NO in the culture supernatant of the H_2O_2-induced H9c2 cells. This effect might be related to the guanidine group of compound **545**, which was similar with L-arginine, the NO donor in biological system.

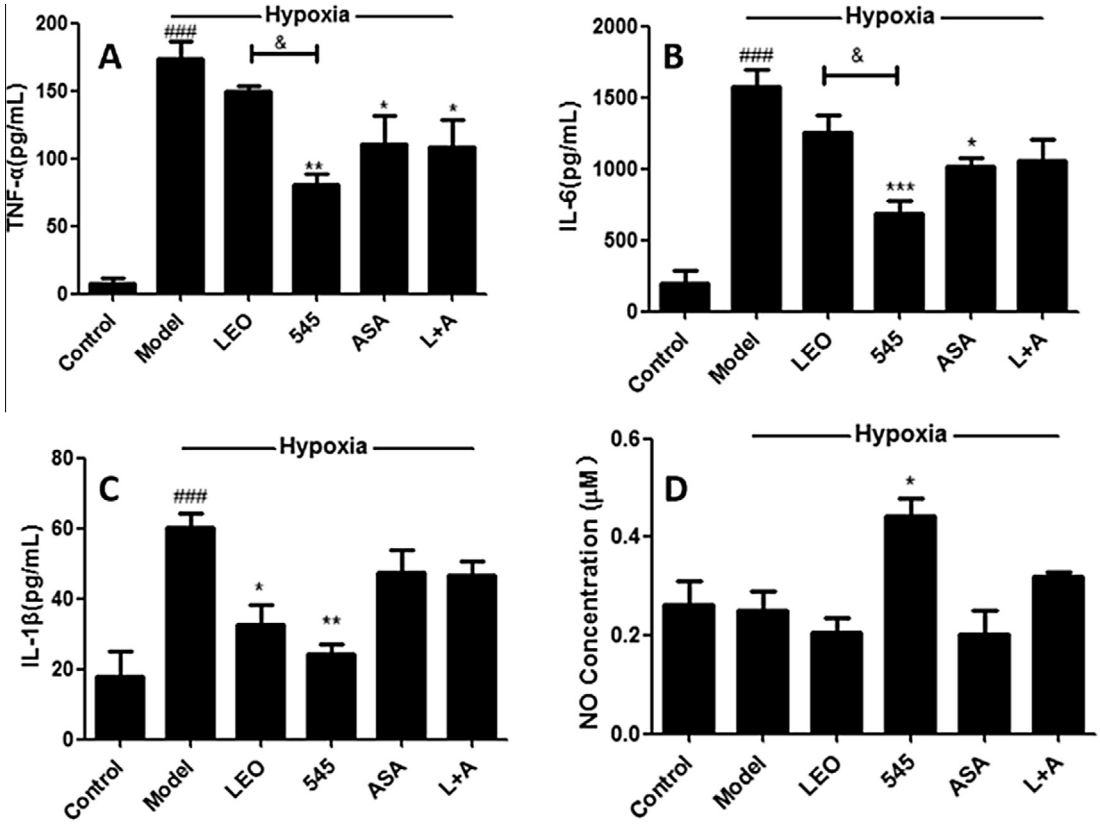


Figure 6. Effect of Compound **545** on the inflammatory cytokines. (A) TNF-α. (B) IL-6. (C) IL-1β. (D) NO concentration. Concentration: ASA (1 μM), LEO (1 μM), L + A (ASA 1 μM + LEO 1 μM), Compound **545** (0.1 μM). Data represent mean ± SEM of more than three independent experiments. $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001 versus model group. $^{\#}P$ <0.05, $^{\#\#\#}P$ <0.001 versus control group. $^{\&}P$ <0.05 versus Leonurine group.

In this study, two novel compound **545** and **503** have been designed and synthesized based on the strategy of codrug designing. The preliminary studies showed that compound **545** exhibited more effective cardioprotection than compound **503** and their parent drugs, **LEO** and Aspirin in hypoxia-induced H9c2 cells. Further mechanism studies suggested that the cardioprotective effects of compound **545** was related to improve anti-oxidative enzyme activity, attenuate MDA level, protect integrity of cell membrane and some cell organs, and regulate apoptosis-associated proteins expression during hypoxia. Moreover, compound **545** could also protect myocardial cell by suppression of pro-inflammatory mediators. All of these results demonstrated that compound **545** as a new class of multifunctional anti-myocardial ischemia agent, provided us important clues for the future design and modification of **LEO** analogues in our next study.

Acknowledgments

This study was supported by key laboratory program of the Education Commission of Shanghai Municipality (ZDSUS14005), and NSFC (No. 21002013).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.08.058.

References and notes

1. Das, N.; Dhanawat, M.; Dash, B.; Nagarwal, R. C.; Shrivastava, S. K. *Eur. J. Pharm. Sci.* **2010**, *41*, 571.
2. Fiorucci, S.; Orlandi, S.; Mencarelli, A.; Caliendo, G.; Santagada, V.; Distrutti, E.; Santucci, L.; Cirino, G.; Wallace, J. L. *Br. J. Pharmacol.* **2007**, *150*, 996.
3. Biljana, B.; Slavko, M.; Dragana, V.; Sasa, V.; Bela, B.; Miodrag, C. *Int. Immunopharmacol.* **2009**, *9*, 910.
4. Fiorucci, S.; Santucci, L.; Gresele, P.; Faccino, R. M.; Del Soldato, P.; Morelli, A. *Gastroenterology* **2003**, *124*, 600.
5. Neville, N. O.; Dan, J.; Aman, S. A. M.; Piero, D. S.; Anna, S. *Neurochem. Int.* **2012**, *60*, 365.
6. Liu, X. H.; Xin, H.; Zhu, Y. Z. *Sheng Li Xue Bao* **2007**, *59*, 578.
7. Liu, C.; Guo, W.; Shi, X. R.; Kaium, M. A.; Gu, X. F.; Zhu, Y. Z. *Eur. J. Med. Chem.* **2011**, *46*, 3996.
8. Liu, X. H.; Chen, P. F.; Pan, L. L.; Silva, R. D.; Zhu, Y. Z. *J. Cardiovasc. Pharmacol.* **2009**, *54*, 437.
9. Liu, H. C.; Zhang, X. J.; Du, Y. Y.; Ji, H.; Li, S. Y.; Li, L. T.; Xing, Y. X.; Zhang, X. L.; Dong, L. P.; Wang, C. H.; Zhao, K.; Ji, Y.; Cao, X. Y. *Brain Res.* **2012**, *1474*, 73.
10. Liu, X. H.; Pan, L. L.; Chen, P. F.; Zhu, Y. Z. *Phytomedicine* **2010**, *17*, 753.
11. Liu, X. H.; Pan, L. L.; Gong, Q. H.; Zhu, Y. Z. *Eur. J. Pharmacol.* **2010**, *649*, 236.
12. Liu, X. H.; Pan, L. L.; Chen, P. F.; Zhu, Y. Z. *Curr. Pharm. Biotechnol.* **2010**, *11*, 895.
13. Liu, C. H.; Gu, X. F.; Zhu, Y. Z. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 6942.
14. Ohsako, M.; Oka, Y.; Tsuzuki, O.; Matsumoto, Y. *Biol. Pharm. Bull.* **1995**, *18*, 310.
15. Dohno, C.; Uno, S.; Sakai, S.; Oku, M.; Nakatani, K. *Bioorg. Med. Chem.* **2009**, *17*, 2536.
16. Zhang, J.; Wang, H.; Zheng, Q. *Int. J. Biol. Macromol.* **2013**, *59*, 328.
17. Wang, Q.; Wang, X. L.; Liu, H. R.; Rose, P.; Zhu, Y. Z. *Antioxid. Redox Signal.* **2010**, *12*, 1155.
18. Liu, X. H.; Pan, L. L.; Deng, H. Y.; Xiong, Q. H.; Wu, D.; Gong, Q. H.; Zhu, Y. Z. *Free Radical Biol. Med.* **2013**, *4*, 93.
19. Zhao, Z. Q.; Nakamura, M.; Wang, N. P.; Wilcox, J. N.; Shearer, S.; Ronson, R. S.; Guyton, R. A.; Vinten-Johansen, J. *Cardiovasc. Res.* **2000**, *45*, 651.