Interaction Between Clopidogrel and Panax Notoginseng Saponins

Yunzhen Hu (✉ 1504039@zju.edu.cn)
Department of Pharmacy, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China

Xi Yang
Zhejiang University

Research

Keywords: panax notoginseng saponins, clopidogrel, CYP2C19, CYP3A4, CES1

DOI: https://doi.org/10.21203/rs.3.rs-59584/v1

License: ©  This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

Background: Panax notoginseng saponins (PNS) is commonly used in combination with clopidogrel in clinics. This study was to investigate the effect of PNS on the pharmacokinetics of clopidogrel active metabolite CAMD and the effect on the activities of clopidogrel metabolic enzymes in rats.

Methods: In pharmacokinetics studies, the rats were divided into clopidogrel and combination groups, and continuously administered for seven days. The concentration of CAMD was determined by LC-MS/MS. In enzyme studies, the rats were divided into control and PNS groups. After administration for seven days, CYP2C19 and CYP3A4 activities were measured by the metabolic rate of the specific substrate in rat liver microsomes. The activity of CES1 enzyme was determined by double antibody sandwich method.

Results: PNS significantly increased $AUC_{0-\infty}$ of CAMD from 43.1±11.6 to 72.0±25.1 h·ng/mL ($p<0.05$). Combination group had lower CL/F and Vd/F than clopidogrel group ($p<0.05$). PNS significantly decreased the activity of CYP3A4 and CES1 ($p<0.01$), but no significant effect on CYP2C19.

Conclusions: The combination use of PNS and clopidogrel produced a significant increase in the AUC of clopidogrel active metabolite CAMD. PNS could inhibit the activity of CYP3A4 and CES1 enzyme in rat.

Background

Panax notoginseng saponins (PNS) is a class of saponins derived from the flowers, fruits, leaves, roots and aloe of Panax notoginsenoseng (Burk) F.H. Chen. It contains about sixty kinds of saponin components, of which notoginsenoside R1, ginsenoside Rb1, Rg1, Rd and Re are its main components, accounting for about 80% of the total saponin content. It has extensive effects, such as inhibition of platelet aggregation, increasing the blood flow in the coronary arteries and protective effects against cerebral ischemia reperfusion injury, has been used for coronary heart disease, cardiac angina, stroke, and so on. Based on the pharmacological action of PNS, the combination of PNS and cardio-cerebrovascular medicine is commonly in clinic, clopidogrel is one of them.

Clopidogrel is an antiplatelet drug, is widely used due to its rapid onset, strong effect and good tolerance. Its efficacy has been affirmed by many high-quality large-scale clinical trials [1,2]. It is a prodrug, about 85% is hydrolyzed into inactive carboxy acid derivatives by carboxylesterase-1 (CES1), and only 15% is metabolized to the 2-bromo-3’-methoxyacetophenone- (MPB-) derivatized clopidogrel active thiol metabolite (CAMD) by CYP450 enzymes, mainly CYP2C19 and CYP3A4. Although clopidogrel has significant clinical benefit in most patients, there are still some patients with low or no response to clopidogrel, which inevitably leads to thromboembolic endpoint events. Among the related causes, drug-drug interactions are considered to be one of the main reasons. With the widespread use of PNS in clinic, the interaction of PNS with clopidogrel should be emphasized.
Meng reported that in patients undergoing percutaneous coronary intervention (PCI), the ADP-induced platelet aggregation inhibition rate in the PNS-clopidogrel group was significantly higher than that in the clopidogrel group [3]. Zhao reported PNS Injection could reduce platelet aggregation in acute cerebral infarction patients with clopidogrel resistance [4]. These studies indicated that PNS and clopidogrel had significant interactions, however the exact mechanism was unclear. This paper was designed to find some evidence on synergistic effect mechanism of PNS and clopidogrel, whether PNS can increase the exposure of active metabolite of clopidogrel, whether PNS may affect metabolic enzymes activity of clopidogrel? Taking these factors into consideration, we performed the study on the effect of PNS on the pharmacokinetics of clopidogrel active metabolite and the effect of PNS on metabolic enzymes. According to the metabolic characteristics of clopidogrel, the metabolic enzymes mainly aimed at CYP2C19, CYP3A4 and CES1.

Methods

Materials and chemicals

PNS was purchased from Chengdu DeSiTe Biological Technology Co., Ltd. Clopidogrel bisulfate, testosterone, (S)-mephenytoin, buspirone, loratadine, 6b-hydroxytestosterone, 4-hydroxymephenytoin, trinatric isocitric acid, isocitric dehydrogenase, b-nicotinamide adenine dinucleotide phosphate (NADP) and b-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) were purchased from Sigma (St Louis, Mo, USA). The 2-bromo-3'-methoxyacetophenone (MPB), CAMD were purchased from the Toronto Research Chemicals.

LC-MS/MS Analysis

The concentrations of CAMD, 4-hydroxymephenytoin and 6b-hydroxytestosterone were determined by modified LC-MS/MS methods. Separations were performed on a UPLC BEH C18 column (1.7 µm, 2.1 mm i.d.×50 mm). The flow rate of the mobile phase was 0.3 ml/min, with a gradient ranging from 10 to 95% methanol containing 0.1% formic acid in a 3-min run. The mass spectrometric analysis was carried out on an electrospray ionization (ESI) source in positive ion mode, and the quantification was performed using multiple reaction monitoring (MRM) Mode (the ion pair of CAMD at 504.0→354.1, 4-hydroxymephenytoin at 235.1→150.1, 6b-hydroxytestosterone at 305.4→269.3, loratadine (IS) at 383.2→267.1.

Animals

Male Sprague-Dawley (SD) rats were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Science (Shanghai, China).

Effect of PNS on the pharmacokinetics of clopidogrel active metabolite

Ten rats were randomly divided into two groups: the clopidogrel group and the combination group. For oral dosing, clopidogrel bisulfate and PNS dissolved in a 0.1% CMC-Na solution were continuously
administered to the rats for seven days by gavage at doses of 30 mg/kg and 40 mg/kg, respectively. The combination group received PNS 15 min prior to clopidogrel administration. On the seventh day, blood samples (150mL) were collected into 1.5-mL pretreated EDTA centrifuge tubes from the fossa orbitalis vein before (0 hour) and after clopidogrel bisulfate administration at 0.083, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 hours. Immediately after collection, 2μL of 500 mM MPB in acetonitrile was added to each blood sample to derivatize the active metabolite of clopidogrel [5]. The blood samples were gently mixed. Then, the samples were centrifuged at 4000 rpm for 10 min at 4 °C, and the separated plasma samples were stored at −80°C until assay. All frozen standards and samples were thawed on wet ice before homogenization. A 50 μL aliquot of each plasma sample and 150 μL of loratadine methanol solution (IS) were transferred into a 1.5 mL centrifuge tube. The mixture was vortex-mixed for 2 min and centrifuged at 20000 rpm for 15min. Then the supernatant was transferred to the LC-MS/MS for analysis. The concentration of clopidogrel active metabolite CAMD was determined. The research ethics committee of our hospital approved the protocol followed in this study. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies [6].

**Effect of PNS on rat liver enzymes**

The rats were divided into control and PNS (40 mg/kg/d) groups. The rats were administrated orally for seven days. Liver microsomes were prepared by calcium precipitation method [7]. The microsomes preparations were stored at -80 °C until used. Protein concentrations were determined by the Lowry method [8], with bovine serum albumin as the standard.

**CYP2C19 and CYP3A4 Enzyme activity assay**

(S)-mephenytoin and testosterone were chosen as the typical substrates for CYP2C19 and CYP3A4. The incubation was performed in 0.1 mL of incubation mixture containing 50mmol (s)-mephenytoin or 40 mmol testosterone, the microsomes protein was 0.5 mg/mL. The metabolic reaction was stopped by adding 0.3 mL of methanol (contain IS loratadine) to the incubation mixture at 30 min (for mephenytoin) and 10min (for testosterone), respectively. The contents were vortex-mixed and centrifuged. An aliquot of 5µL of supernatant was injected into the LC-MS/MS system. The concentrations of 4-hydroxymephenytion and 6β-hydroxytestosterone were determined.

**CES1 Enzyme activity assay**

The concentration of CES1 in rat hepatic microsomes was determined by double antibody sandwich method. Purified rat CES1 antibody was used to coat microtiter plate wells and make solid-phase antibody. Rat CES1 was successively added to the wells, then combined with horseradish peroxidase (HRP) labeled antibody to form the antibody-antigen-enzyme-antibody complex. After thorough washing, tetramethylbenzidine (TMB) substrate was added for color development, then TMB substrate was converted to blue by HRP enzyme, and to the final yellow by sulphuric acid solution. The color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of CES1 in the samples was then calculated by the standard curve.
Results

Effect of PNS on the pharmacokinetics of CAMD

The effect of PNS on the pharmacokinetics of CAMD following oral administration was investigated in rats. As shown in Table 1 and Figure 2, when coadministered with PNS for seven days, the \( C_{\text{max}} \) and \( AUC_{0-\infty} \) of CAMD increased from 15.8±5.5 to 34.7±28.9 ng/mL (p>0.05) and 43.1±11.6 to 72.0±25.1 h·ng/mL (p<0.05), respectively. Combination group had lower CL/F and Vd/F than clopidogrel group (p<0.05).

**Table 1** Pharmacokinetic parameters of CAMD after oral administration of clopidogrel or coadministration of clopidogrel and PNS for seven days in rats.

| Parameter       | Clopidogrel group | Combination group |
|-----------------|-------------------|-------------------|
| \( T_{1/2} \) (h) | 2.2±0.7           | 2.1±0.7           |
| \( T_{\text{max}} \) (h) | 0.4±0.1          | 0.5±0.0           |
| \( C_{\text{max}} \) (ng/mL) | 15.8±5.5         | 34.7±28.9         |
| \( AUC_{0-\text{t}} \) (h·ng/mL) | 37.0±9.9         | 61.1±18.0         |
| \( AUC_{0-\infty} \) (h·ng/mL) | 43.1±11.6        | 72.0±25.1         |
| \( \text{CL/F(L/h/kg)} \) | 790.3±163.4*     | 461.5±179.8      |
| \( \text{Vd/F(L/kg)} \) | 2428.5±923.5*    | 1347.0±477.3     |

*p < 0.05, compared with those combination group

Effect of PNS on CYP2C19 and CYP3A4 enzyme activity

As shown in Figure 2, compared to the control group (15.8±1.8 pmol/mg protein/min), there was no significant decrease in the CYP2C19 activity (the formation of 4-hydroxymephenytion) in rat hepatic microsomes pretreated with PNS (13.9±0.2 pmol/mg protein/min) (p>0.05).

As shown in Figure 3, compared to the control group (4.0±0.1 nmol/mg protein/min), there was a significant decrease in the CYP3A4 activity (the formation of 6β-hydroxytestosterone) in rat hepatic microsomes pretreated PNS (3.5±0.1 nmol/mg protein/min) (p<0.01).

Effect of PNS on CES1 Enzyme activity
As shown in Figure 4, compared to the control group (34.3±0.6 pg/mg protein), there was a significant decrease in the CES1 activity in rat hepatic microsomes pretreated PNS (30.4±1.3 pg/mg protein) (p<0.01).

Discussion

The use of traditional Chinese medicine has gained increasing acceptance all over the world due to its multi-target and multi-level function characteristic. PNS is a common used tradition Chinese medicine in clinic. The interaction between PNS and western medicine needs attention. Among the relevant causes of drug-drug interactions, the effect of drugs on metabolic enzymes have been identified as one of the main reasons. According to the literature reviewed, some studies have reported the effect of PNS on metabolic enzymes. Chen reported PNS could significantly induce CYP1A2 and CYP2E1 enzyme activity, mRNA expression and CYP2E1 protein expression leve1[9]. A vitro studies revealed that PNS could inhibit CYP3A activity [10]. Several studies had actually found that PNS could induce CYP1A2 activity [11,12]. In our study of the effect of PNS on the pharmacokinetics of clopidogrel active metabolite CAMD, coadministration with PNS for seven days significantly increased the exposure of CAMD approximately 1.7-fold, and CI/F was decreased to 58.4% of clopidogrel alone. In the study of the effect of PNS on metabolic enzymes, pretreated PNS could inhibit CYP3A4 enzyme activity in rat livers. This couldn't explain why PNS can increase CAMD exposure.

Qi reported PNS had a weak inhibition effect on CES, but concentraion -dependent inhibition on CES2 in vitro [13]. A study by Sun showed PNS were demonstrated to inhibit the CES activities responsible for aspirin hydrolysis in Caco-2 cells. PNS could also decrease the protein expression of CES1 and CES2, whereas exhibited minor effect on the mRNA expression [14]. A review showed that more than 50 natural inhibitors of CES1 or CES2, including phenolic compounds, triterpenoids and tanshinones, while inducers of CES1 and CES2 were less reported [15]. In our study, we found that pretreated 40mg/kg/d PNS could inhibit CES1 enzyme activity in rat livers, thereby reduce the inactivation of clopidogrel and indirectly increased the concentration of active metabolite CAMD.

We know that inhibition of CYP enzyme can reduce the activation of clopidogrel, while inhibition of CES1enzyme can reduce the production of inactive metabolites of clopidogrel. Therefore, the synergistic mechanism between PNS and clopidogrel is complex and multifaceted, the effect on CES1 enzymes probably be more significant. In subsequent studies, it is necessary to carry out experiments on the effects of notoginsenoside R1, ginsenoside Rb1, Rg1, Rd and Re, the main components of PNS on CES1 enzyme.

Conclusions

The combination use of PNS and clopidogrel produced a significant increase in the AUC of clopidogrel active metabolite CAMD. PNS could inhibit CYP3A4 and CES1 enzyme activity in rat liver. The synergistic mechanism of clopidogrel and PNS is probably related to the inhibition of CES1 enzyme.
Abbreviations

PNS: Panax notoginseng saponins; CAMD: clopidogrel active thiol metabolite; CES: carboxylesterase; HRP: horseradish peroxidase; TMB: tetramethylbenzidine; NADP: nicotinamide adenine dinucleotide phosphate; NADPH: nicotinamide adenine dinucleotide phosphate, reduced form

Declarations

Authors’ contributions

YZ Hu conceived and designed this study; YZ Hu and X Yang carried out the experiments; YZ Hu performed data analysis and drafted the manuscript; X Yang made critical contribution to the discussion. All authors revised the manuscript and approved the final manuscript.

Author details

The authors work at Department of Pharmacy, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China

Acknowledgements

Not applicable.

Competing interests

There are no competing interests to declare.

Availability of data and materials

Data available on request due to privacy/ethical restrictions.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The research ethics committee of the First Affiliated Hospital, College of Medicine, Zhejiang University approved the protocol followed in this study.

Funding

This work was supported by the National Natural Science Foundation of China (Grant No. 81703612).

References
1. Dewilde WJ, Oirbans T, Verheugt FW, Kelder JC, De Smet BJ, Herman JP, Adriaenssens T, Vrolix M, Heestermans AA, Vis MM, Tijsen JG, van ’t Hof AW, ten Berg JM. Use of clopidogrel with or without aspirin in patients taking oral anticoagulant therapy and undergoing percutaneous coronary intervention: An open-label, randomised, controlled trial. *Lancet*, 2013; 381(9872):1107-1115.

2. Chen ZM, Jiang LX, Chen YP, Xie JX, Pan HC, Peto R, Collins R, Liu LS. Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. *Lancet*. 2005; 366(9497):1607-1621.

3. Meng K, Zhu HG, Song XT, Ge CJ, Zhou Y, Dai J, Lu SZ. Effects of Panax notoginseng combining with dual antiplatelet drugs on the major adverse cardiovascular events in patients undergoing percutaneous coronary intervention procedure. *Chinese medicine*. 2013; 8(4):445-447.

4. Zhao J, Geng WJ, Zhai BZ. Clinical observation on Xueshuantong Injection in treatment of acute cerebral infarction patients with clopidogrel resistance. *Chinese Traditional and Herbal Drugs*, 2005; 46(14):2122-2126.

5. Takahashi M, Pang H, Kawabata K, Farid NA, Kurihara A. Quantitative determination of clopidogrel active metabolite in human plasma by LC-MS/MS. *J Pharm Biomed Anal*. 2008; 48(4):1219-24.

6. Tveden-Nyborg P, Bergmann TK, Lykkesfeldt J. Basic & Clinical Pharmacology & Toxicology Policy for Experimental and Clinical studies. Basic Clin Pharmacol Toxicol. 2018;123(3):233-235.

7. Gibson GG, Skett P. Introduction drug metabolism. 2nd Ed. London: Blackie Academic & Professional; 1994; 217-219.

8. Lowry OH, Rosebrough NJ, Farr AL, Randall J. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951, 193: 265-275.

9. Chen YJ, Wang YG, Mang ZC, Xiao CR, Tan HL, Liang QD, Tang XL, Zhao YH, Wang DG, Gao Y. Effect of Panax notoginseng saponis on liver drug metabolic enzyme activity, mRNA and protein expressions in rats. *China journal of Chinese Materia Medica*. 2014, 39(19):3824-3828.

10. Yang ZM, Yang XF. Inhibitory effect of PNS on drug metabolism enzyme CYP3A in rat livers and its kinetic analysis. *China journal of Chinese Materia Medica*, 2012, 37(22):3486-3489.

11. Shi J, Chen AJ, Zhang F, Wang BJ. Effects of panaxnotoginoside on CYP450 subtype enzymes. Chinese journal of pharmacoepidemiology, 2008,17(5):281-284

12. Liu R, Qin M, Hang P, Liu Y, Zhang Z, Liu G. Effects of Panax notoginseng saponis on the activities of CYP1A2, CYP2C9, CYP2D6 and CYP3A4 in rats in vivo. *Phytother Res*. 2012; 26(8):1113-1118.

13. Qi Q, Wang Y, Mo YJ, Luo JY, Yu XL, Lu Y, Du SY. Effect of Panax notoginseng Saponins on Activity of Carboxylesterases in Vitro. *Modern Chinese Medicine*, 2019; 21(6):777-781.

14. Sun Z, Wu Y, Yang B, Zhu B, Hu S, Lu Y, Zhao B, Du S. Inhibitory Influence of Panax notoginseng Saponins on Aspirin Hydrolysis in Human Intestinal Caco-2 Cells. *Molecules*. 2018; 23(2). pii: E455.

15. Xu J, Qiu JC, Ji X, Guo HL, Wang X, Zhang B, Wang T, Chen F. Potential Pharmacokinetic Herb-Drug Interactions: Have we Overlooked the Importance of Human Carboxylesterases 1 and 2? *Curr Drug Metab*. 2019; 20(2):130-137.
Figures

Figure 1
Plasma concentration-time courses of CAMD in rats after oral administration of clopidogrel with or without PNS for seven days.
Figure 2

Effect of PNS on CYP2C19 enzyme activity in rat microsomes. As shown in Figure 3, compared to the control group (4.0±0.1 nmol/mg protein/min), there was a significant decrease in the CYP3A4 activity (the formation of 6β-hydroxytestosterone) in rat hepatic microsomes pretreated PNS (3.5±0.1 nmol/mg protein/min) (p<0.01).
Figure 3

Effect of PNS on CYP3A4 enzyme activity in rat microsomes
Figure 4

Effect of PNS on CES1 enzyme activity in rat microsomes