Clinics in Surgery

Research Article
Published: 10 May, 2019

The Dynamic Effect of Non-CYP3A4-Metabolized and CYP3A-Metabolized Statins on Clopidogrel Resistance in Patients with Cerebral Infarction

Bo Rong Zhou[1#], Hong Ting Shi[2#*], Wan Li Gao[1#], Yong Yuan Chen[3#], Xiao Ying Li[4#], Jian Hua Luo[5#], Guang Hong Zhong[6#], Jia Jia Hu[1], Hai Tao Guan[1], Yu Ming Liu[1] and Min Zhang[7]*

[1]Department of Neurology, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China

[2]Department of Neurology, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, China

[3]Department of Neurology, The Fifth Affiliated Hospital of Guangzhou Medical University, Guangzhou, China

[4]Department of Neurology, Tung Wah Affiliated Hospital of Sun Yat-sen University, Dongguan, China

[5]Department of Neurology, Yang Chun People Hospital, Yang Chun, China

[6]Department of Neurology, He Yuan People Hospital, He Yuan, China

[7]Department of Epidemiology and Health Statistics, Guangdong Pharmaceutical University, Guangzhou, China

[#]Co-first authors: Zhou BR, Shi HT, Gao WL, Li XY, Luo JH, Chen YY and Zhong GH

Abstract

Objective: To evaluate dynamic effect of statins in combination with clopidogrel in patients with Cerebral Infarction (CI).

Methods: The 130 of none Clopidogrel Resistance (CR) patients were divided into dynamic CR (DCR) and Continuous NCR (CNCR) groups. A total of 98 cases were completed all observations. They were randomly divided into AC group (atorvastatin 40 mg/d + clopidogrel, 51 cases) and RC group (rosuvastatin 20 mg/d + clopidogrel, 47 cases). Patient Platelet Aggregation Rate (PAR) was tested by PL series platelet function analyzer (PL-11) at visit 0 (baseline), visit 1 (one week after clopidogrel alone treatment) and visit 2 to 4 (1, 3 and 6 months after clopidogrel plus statins treatment). Platelet Reactivity Index (PRI) was assessed by flow cytometry at visit 0, 2 and 4, and Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) was used to monitor clopidogrel thiol metabolite (H4) levels at visit 2 and 4.DNA sequencing was used to determine *CYP3A4*, *CYP2C9* and *CYP2C19* genotypes in all patients.

Results: PAR, PRI and H4 were similar between AC and RC groups (P>0.05). Compared with the AC group, the DCR ratio of the RC group and the genotype frequencies of *CYP2C9*3εC*, *CYP2C19*2εA* and *CYP2C19*3εA* were not significantly different (P>0.05). *CYP2C19εA* (*2 and *3) were independent risk factors for DCR (P<0.05).

Conclusion: The incidence of DCR in Chinese population was high, which was related to *CYP2C19εA*. Clopidogrel combined with atorvastatin (CYP3A4-metabolized) did not affect platelet inhibition by clopidogrel and did not increase the incidence of DCR.

Keywords: Dynamic; Clopidogrel resistance; Statins; Medicine metabolism; Medicine interactions

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Correspondence:
Bo Rong Zhou, Department of Neurology, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou 510150, China, E-mail: m13660486148_1@163.com
Hong Ting Shi, Department of Neurology, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou 510150, China, E-mail: 317564816@qq.com

Received Date: *21 Mar 2019*
Accepted Date: *02 May 2019*
Published Date: *10 May 2019*

Citation:
Zhou BR, Shi HT, Gao WL, Chen YY, Li XY, Luo JH, et al. The Dynamic Effect of Non-CYP3A4-Metabolized and CYP3A-Metabolized Statins on Clopidogrel Resistance in Patients with Cerebral Infarction. Clin Surg. 2019; 4: 2423.

Introduction

Clopidogrel is widely used for the prevention of cardiovascular and cerebrovascular events [1-3]. Clinical research has demonstrated the effectiveness of clopidogrel in preventing Major Adverse Cardiovascular and Cerebrovascular Events (MACCE) in stroke victims [4,5]. However, despite this form of antiplatelet therapy, recurrent cerebrovascular ischemic events still occur in some patients. A number of studies have clearly shown that there is marked variability in the responsiveness to clopidogrel, and those with poor responders have a higher risk of short- and long-term MACCE [3-6]. Poor response is associated with Clopidogrel Resistance (CR). Previous studies estimate that the overall incidence of CR varies from 16.8% to 21.0%, and may be as high as 44% in victims of Cerebral Infarction (CI) [6,7].

Patients with primary CI or recurrent stroke have high rates of CR [7,8]. The mechanisms of CR have not been fully elucidated but are likely to be multifactorial, involving genetic polymorphisms, baseline disease (atherosclerosis, diabetes) and drug interactions. In addition, antiplatelet therapy combined with statins has become fixed combination of patients with MACCE. In China, clopidogrel is often used, but due to the lack of Chinese data, physicians are not paying enough attention to the drug-drug interaction between statins and clopidogrel. Therefore, reasonable and effective combination in patients with CI should be further explored.

Clopidogrel is inactive *in vitro* and requires *in vivo* oxidation by hepatic cytochrome P450 isoenzymes. In the two-step hepatic oxidation, cytochrome P450 (CYP) 3A4 is a major contributor to active metabolite generation [9,10]. Statins are frequently co-administered in patients with atherosclerosis, especially those who have undergone stent implantation. Furthermore, clopidogrel and several statins are predominantly metabolized by cytochrome P450 3A4 isoenzyme (CYP3A4) *in vivo* for activation and elimination, respectively. Thus, any drug–drug interactions that may adversely affect the efficacy of either drug could radically alter our standard treatment armamentarium. Most of the lipophilic statins, such as atorvastatin, simvastatin, and lovastatin, are predominantly metabolized by CYP3A4 [11,12]. In addition, these statins can also affect the bioavailability of clopidogrel by inhibiting the P-glycoprotein efflux transporter [13,14]. Therefore, drug-drug interactions influencing the function of CYP isoenzymes have been shown to affect the response to clopidogrel [15].

In our previous study [8], we reported that Dynamic CR (DCR) may occur after CI. CR probably due to the atorvastatin-clopidogrel interaction. However, that was only an observation study and did not compare the drug-drug interaction between different metabolized statins and clopidogrel. This study aimed to investigate the interaction of statins with CYP3A4 metabolism and statins with non-CYP3A4 metabolism with clopidogrel in patients with CI.

Subjects and Methods

Study design

This was a randomized, open-label, single-center study. Some research data [7,9,10] showed that static CR is mainly attributed to genetic polymorphisms, especially CYP2C19. Our previous research also [8] has demonstrated that DCR phenomenon occurs in addition to static CR. Therefore, static CR was excluded from this study to minimize the effects of genetic factors on CR in patients with CI. CI patients received clopidogrel 75 mg/daily (Xin Li Tai pharmaceutical co. LTD in China).

The CR individuals, identified by the Platelet Aggregation Rate (PAR) one week after clopidogrel treatment (PAR one week after clopidogrel treatment-PAR before clopidogrel treatment) was eliminated. Non-CR (NCR) was enrolled and randomly assigned into AC group (atorvastatin 40 mg/d, Pfizer + clopidogrel) and RC group rosuvastatin 20 mg/d AstraZeneca + clopidogrel) in 1:1 ratio. All patients were followed-up for 6 months. PAR was tested at visits 0, 1, 2, 3 and 4. In AC group, when CR occurred during the time treatment, then these patients were re-classified to RC group. In RC group, when CR occurred, they were withdrawn from the study. Patients were followed up by telephone or return visit to ensure compliance according to the secondary prevention of CI guidelines published by the AHA/ASA in 2014 [16].

Subjects

All subjects were recruited at the Third Affiliated Hospital of Guangzhou Medical University between March 2015 and September 2017. Initially, we recruited 160 patients with various types of CI who were stable after suffering an acute CI, and 30 of them (18.75%) occurred CR were eliminated after 1 weeks clopidogrel treatment. During the observation period, 98 cases out of the 130 (75%) were completed for final observation as 2 cases (1.5%) were withdrawn due to allergy, and 30 cases (23%) were withdrawn because of the financial burden and settlement abroad. Clinical diagnosis of CI was made in accordance with standards established by the World Health Organization (WHO) [17].

The patients in this study ranged in age from 45 to 85 with baseline platelet count between 150×10^9/L and 500×10^9/L. None of them received clopidogrel and statins the previous month. Neither patient in the two groups were allergic to clopidogrel, rosuvastatin and atorvastatin, and did not take aspirin, dipyridamole, thiamethoxam chloride, nor other drugs that affected platelet function in the past 2 weeks. Patients with severe renal impairment or bleeding, a history of bleeding diathesis, tumor, immune system, respiratory system diseases or atrial fibrillation were excluded from the study. Subjects who had undergone major surgery or sustained serious injury, National Institutes Health Stroke Scale (NIHSS) score >23, cannot outpatient visit regularly or have poor compliance were also excluded.

All patients underwent skull CT or MRI scans and routine blood tests (platelet count, INR, blood glucose and, lipid analysis). Coding of atrial fibrillation and carotid stenosis was undertaken following ECG or extracranial vascular imaging. Hypertension was defined as Systolic Blood Pressure (SBP) ≥ 140 mmHg and/or Diastolic Blood Pressure (DBP) ≥ 90 mmHg, and/or the use of antihypertensive medication. Diabetes mellitus was defined as fasting blood glucose (FBG) level ≥ 7.0 mmol/L, or HbA1c ≥ 6.0%. The High Total Cholesterol (HTC) was defined as total cholesterol ≥ 5.0 mmol/L and hyperlipidemia was defined as serum low-density lipoprotein ≥ 3.5 mmol/L.

Platelet function

PAR: Blood samples, in which the first 4 mL of blood was discarded to avoid spontaneous platelet activation, was tested within 2 h after sampling for platelet numbers and the platelet aggregation by PL-11 (SINNOWA Medical Science & Technology Co., Nanjing, China) and flow cytometer (BD Corporation, USA) [18]. The maximal PAR was calculated by the system using the following formula: PAR max (%) = (the baseline platelet count- the lowest platelet count)/the baseline platelet count × 100%. CR was defined as being present when the maximal PAR was ≥ 55%.

PRI: Flow cytometer [19] was use for the evaluation of platelet reactivity to agonists *in vitro* and the presence of circulating active platelets, platelet-leukocyte aggregates and platelet-derived micro particles. The method was: blood sample was first incubated with Prostaglandin E1 (BIOCYTEX Corporation, French) alone or PGE1 (Prostaglandin E1, PGE1) + Adenosine Aiphosphate (ADP). After a cellular permeabilization, Vasodilator Stimulated Phosphoprotein (VASP) under its phosphorylated state was labeled by indirect no wash immunofluorescence using a specific monoclonal antibody (clone 16C2). Dual color flow cytometry analysis with parallel control of each series allowed to compare the two tested conditions and to evaluate the capacity of ADP to inhibit VASP phosphorylation for each sample. Corrected Mean Fluorescence Intensities (MFIC) was calculated after subtraction of the MFI from the negative control.

A Platelet Reactivity Index (PRI) was calculated using MFIc in the presence of PGE1 alone (PGE1) or PGE1 + ADP according to the following calculation: PRI=[(MFIc PGE1-MFIc (PGE1+ADP)]/MFIc PGE1 × 100. CR was defined as: post therapy PRI > (pretherapy mean PRI - at 2 standard deviation).

Assessment of clinical characteristics

Demographic information including age, sex, and blood pressure were recorded, together with details of each patient's history of Coronary Vascular Disease (CVD), hypertension, Diabetes Mellitus (DM), stent implantation, Transient Ischemic Attack (TIA) or history of ischemic stroke. Personal history such as smoking was also recorded together with detailed usage of calcium ion antagonist and angiotensin inhibitor (ACEI/ARB) drugs. Family history such as a hereditary amyloid degeneration of the blood vessels and other genetically disease was documented.

Genetic analysis

All patients were genotyped for *CYP3A4*, *CYP2C9* and *CYP2C19*. The reference sequence was found in NCBI, and DNA was extracted from whole blood samples with TIANamp Blood DNA Kit (Sigma Corporation, USA) according to the manufacturer's instructions. The primers (synthesized by Huada Biotechnology Company, China) were designed by Primer Premier 3.0 software. PCR reactions were performed with 20.8 μL H_2O, 2 μL template, 0.2 μL Taq DNA polymerase, 3 μL 10 X buffer, 2 μL dNTP mixture, 1 μL each primer (10 pmol). The PCR conditions were as follows: 5 min at 96°C, followed by 30 cycles of 20 s at 96°C, 20 s at 55°C, and 30 s at 72°C. The final extension was 5 min at 72°C. Finally, single-base primer extension reactions were conducted using the following reaction mixture: 2 μL Mix (Bigdye 3.1, 5 X sequencing buffer, H_2O) 2 μL Purified PCR products, 1 μL each primer (5 mmol/L). The reaction was carried out as: 15 s at 95°C, followed by 35 cycles of 15 s at 95°C, 5 s at 50°C, and 90 s at 60°C, and final extension at 72°C for 3 min. Genotypes of *CYP3A4*, *CYP2C9* and *CYP2C19* were determined using DNA sequencing (Huada Biotechnology Company, China).

Determination of clopidogrel thiol metabolite H4

Five mL of blood was drawn from patient 0.5, 1, 2, 3, 4, 6, 12 and 24 h after clopidogrel administration into vacuum systems containing EDTA-K (Yangpu Medical Technology Company, China). Immediately after collection, to stabilize the highly labile thiol metabolite, 25 μL aliquot of 500 mM bromo-3′-methoxyacetophenone solution was added to the systems [20]. Centrifuged plasma was stored at -20°C until further analysis. Clopidogrel thiol metabolite H4 (H4) was measured by a validated liquid chromatography - tandem mass spectrometry (LC-MS/MS) [21] with the line arranged from 0.25-50.00 ng/mL.

Statistical analysis

Statistical analysis was performed using the SPSS version 16.0 for Windows (SPSS Inc., USA). Continuous variables, presented as means and standard deviations (mean ± SD), were tested using Student's t test. Categorical variables were compared using the Pearson chi-square test. PAR was tested using the general linear model-repeated measurement. H4 was tested using the Sphericity Assumed. Repeated measure Analysis of Variance (ANOVA) was used to compare platelet function and H4 changes. Correlation between the genotype and allele frequency distribution differences and the CR were analyzed using multivariate regression models. Multivariable logistic regression analysis analyzed the relationship between DCR and risk factors. P<0.05 was considered statistically significant.

Data availability statement

In this study, the researchers showed that all the data of participants whose individuals were dis-identified would be shared, including the general clinical health status of the study subjects, platelet aggregation rate measured by three different methods or data to determine the occurrence of CR. The design scheme of this study (which has been clearly stated in the method) and statistical analysis will be shared. Such data will be available on the Shared platform for a long time; Access to data is usually requested by the type of meta-analysis. The data not provided in this article will be available in a trusted data repository, available from a Shared database.

Results

Patient demographics and comparison

Among the original 160 patients with CI, 30 cases (18.75%) occurred CR were eliminated at 1 weeks after clopidogrel treatment, 130 cases were NCR but only 98 cases completed the 6-month follow-up (51 cases in AC and 47 cases in RC group). There was no significant difference in general demographic information between the AC and RC groups (P>0.05). Among the AC patients, 32 (62.7%) were male, with an average age of 67.69 ± 8.88 years, NIHSS score 4.51 ± 2.28, and 32 (68.1%) of the RC group were male, with an average age of 65.66 ± 11.80 years, NIHSS score 4.98 ± 2.96. The AC group and RC groups were comparable with respect to the incidence of co-existing hypertension (68.6% *vs.* 76.6%, respectively), diabetes mellitus (37.3% *vs.* 27.7%) and hyperlipidemia (37.3% *vs.* 36.2%) (P>0.05).

Comparison of Platelet function between AC and RC in different time periods

PAR was tested at visit 0 (baseline), visit 1 (1 week after clopidogrel alone treatment) and visit 2 to 4 (1, 3 and 6 months after clopidogrel plus statins treatment). PAR was not significant difference between AC and RC groups (P=0.825), suggesting that non-CYP3A4-metabolized and CYP3A4-metabolized statins were not related to DCR. However, there was significant difference for PAR at different time within-groups. At visit 0, the AC-PAR was40.57 ± 8.95% and the RC-PAR was 40.79 ± 8.35%, at visit 1, the AC-PAR was 33.91 ± 14.11% and the RC-PAR was 34.20 ± 11.47%, at visit 2, the AC-PAR was 31.01 ± 14.48% and the RC-PAR was 30.55 ± 12.79%, at visit 3, the AC-PAR was 31.48 ± 13.67% and the RC-PAR was 30.90 ± 12.12% and at visit 4, the AC-PAR was 33.75 ± 16.04% and the RC-PAR was 35.98 ± 16.34% (P=0.001).

Flow cytometry evaluated VASP phosphorylation (platelet function) at visits 0, 2, and 4 in the 2 groups. There was no significant difference for PRI between AC and RC groups P=0.667, but there was significant decreases at different time within-groups. At visit 0, the AC-PRI was 71.02 ± 10.40% and the RC-PRI was 71.40 ± 10.74%, at visit 2, the AC-PRI was 29.23 ± 7.91% and the RC-PRI was 28.21 ± 7.75%, and at visit 4, the AC-PRI was 39.29 ± 18.44% and the RC-PRI was 37.85 ± 18.67% (P=0.000).

Comparison of H4 between AC and RC in different time periods

The H4 was unaltered when atorvastatin or rosuvastatin was added to clopidogrel Treatment: AC-H4 36.85 ± 11.94 ng/mlvs RC-H4 34.73 ± 12.06 ng/ml at visit 2 and AC-H4 22.76 ± 10.24 ng/ml *vs.* RC-H4 20.60 ± 7.56 ng/ml at visit 4 (P=0.208). But the H4 was a significant difference in the DCR and CNCR groups: DCR-H4

27.63 ± 10.74 ng/ml *vs.* CNCR-H4 38.21 ± 11.31 ng/mlat visit 2, and DCR-H4 17.98 ± 8.26 ng/mlvs CNCR-H4 22.81 ± 9.06 ng/ml at visit 4 (P=0.000). As expected, H4 was reduced in all patients at visit 2 compared to visit 4.

Genotypic analysis of *CYP3A4*, *CYP2C9* and *CYP2C19*

No cases of DCR were observed at visits 2 and 3, but 22 cases of DCR were observed at visit 4, including 12 cases in the DCR-AC group and 10 cases in the DCR-RC group. In addition, the 12 patients in DCR-AC were switched to RC treatment, and their PAR did not decrease 1 week after the change in treatment, they still showed CR (PAR 60.67 ± 5.13% before conversion to rosuvastatin, and 59.35 ± 3.64% after conversion to rosuvastatin, P=0.224).

The *CYP3A4*, *CYP2C9* and *CYP2C19* genotype analyses. The *CYP2C9*3εC* genotype was found in 4 patients in the AC group and 2 in the RC group (P=0.679; Table 2). The *CYP2C9*3* (*A;A*) occurred with a frequency of 93.9% (92/98), while 6.1% (6/98) were for *CYP2C9*3* (*A;C*), and none *CYP2C9*3* (*C;C*). But the *CYP2C9*3εC* in the DCR group was significantly higher than in the CNCR group (P=0.002). There were no significant difference of the *CYP2C19*2* and *CYP2C19*3* frequencies between AC and RC groups. *CYP2C19*2* in AC was 45.1% and in RC was 46.8% (P=0.865). The *CYP2C19*3* in AC was 9.8% in RC was 4.3% (P=0.439). Totally, there were 53 cases (54.1%) of *CYP2C19*2* (G;G, rs4244285), 44 cases (44.9%) of *CYP2C19*2* (A;G) and 1 case (1%) of CYP2C19*2 (A;A), and 91 cases (92.9%) of *CYP2C19*3* (G;G, rs4986893) wild type, 7 cases (7.1%) of *CYP2C19*3* (A;G) and no case of *CYP2C19*3* (A;A). The *CYP2C19*2εA* in the DCR group (81.8%) was also significantly higher than that in the CNCR group (35.5%, P=0.000). *CYP2C19*3 εA* in the DCR group (22.7%) was significantly higher than that in the DCR group (2.6%, P=0.006). None mutant alleles of *CYP3A4* (*T;T*) and *CYP2C9*2*(*C;C*) was noted.

Risk factor analysis for patients with cerebral infarction

Compared the clinical characteristics of patients with CI between DCR and CNCR. In the DCR group, the patients with calcium antagonists and diuretics and their *CYP2C9*3*, *CYP2C19*2* and *CYP2C19*3* frequencies were significantly higher than the CNCR group (P<0.05). The multiple logistic regression analysis indicated that the *CYP2C19*2* (OR 4.584; 95% CI 1.271-16.526; P=0.020) and *CYP2C19*3* (OR 12.448; 95% CI 1.238-125.173; P=0.0032) were independent risk factors for CR in DCR group. There is no obvious causal relationship between *CYP2C9 *3* and DCR (P>0.05).

Adverse events

Within 6 months, we observed 3 cases of lacunar CI (2 cases in the AC group, 1 case in the RC group), 3 cases of increased liver enzymes (2 cases in the AC group, 1 case in the RC group) and 4 cases of increased muscle enzymes (2 cases in the AC group and 2 cases in the RC group). There was no significant difference adverse events between the AC and RC groups (P>0.05).

Discussion

When statins are combined with clopidogrel, they may interact due to competition at the binding site. This co-administration may inhibit clopidogrel activation and reduce antiplatelet effects, but these results are still controversial Atorvastatin is metabolized by CYP3A4 and significantly inhibited clopidogrel biotransformation *in vitro*, whereas rosuvastatin showed no such inhibitory effect [23-25]. Lau et al. reported that atorvastatin can decrease the activation of clopidogrel by 90% and reduce the antiplatelet effect of clopidogrel *in vitro* experiments, which have drawn wide attention. Brophy et al. [24], Gulec et al. [25] studies found that clopidogrel will increase cardiac adverse events in patients with coronary artery stent implantation when it was co-administered with atorvastatin compared with rosuvastatin. In contrast, other studies [26-28] investigating the influence of CYP3A4-metabolized statins on antiplatelet effects of clopidogrel failed to confirm these findings. Few studies of non-CYP3A4-metabolized and CYP3A4-metabolized statins dynamically affecting clopidogrel resistance have been reported.

This study evaluated the difference of DCR between atorvastatin 40 mg/d and rosuvastatin 20 mg/d in patients with CI. We demonstrated that the inhibitory effect of atorvastatin or rosuvastatin on the biotransformation of clopidogrel did not affect the antiplatelet effect of clopidogrel. There was no difference in the incidence of DCR between AC and RC groups. The number of patients with AC-DCR and RC-DCR was 12 and 10, respectively, and there was no significant difference in clinical endpoints between the two groups at 6 months. In addition, the PAR of these 12 AC-DCR patients showed no change after one week of RC treatment and they were still CR. Similar with our results, the multiple studies [29,30] have found no interaction between atorvastatin or rosuvastatin and clopidogrel, and denied that co-administering CYP3A4-metabolized statins with clopidogrel can increase the frequency of clinical adverse events.

In this study, clopidogrel in combination with atorvastatin or rosuvastatin showed a downward trend in PAR from visit 0 to visit 4, however, there was no significant difference between the AC and RC groups. Clopidogrel co-administered with atorvastatin or rosuvastatin simultaneously did not reduce the platelet inhibition of clopidogrel and did not increase platelet activity at different times, suggesting that clopidogrel and atorvastatin or rosuvastatin are safe when used in combination in CI patient.

PL-11 is a new point-of-care platelet function analyzer based on platelet count drop method, which is measured as the single platelet aggregation after the addition of agonists in the citrated whole blood samples, using a standard electrical impedance counter. VASP is an intracellular platelet protein which is non-phosphorylated at basal state. VASP phosphorylation assayed for platelet function [19]. Its phosphorylation is regulated by the cAMP (cyclic Adenosine Monophosphate, cAMP) cascade. PGE1 activates this cascade whereas it is inhibited by ADP through P2Y12 receptors. In the test conditions, VASP phosphorylation correlates with the P2Y12 receptor inhibition, whereas its non-phosphorylation state correlates with the active form of P2Y12 receptor. The effect of clopidogrel can be demonstrated with PLT VASP/P2Y12 by the persistence of VASP phosphorylation induced by PGE1 even with the simultaneous addition of ADP. We found that clopidogrel in combination with atorvastatin or rosuvastatin did not reduce the platelet inhibition of clopidogrel and did not increase platelet activity. The PRI values for DCR-AC and for DCR-RC were similar at the visit 0, 2 and 4, suggesting that CYP3A4-metabolized statins are not associated with DCR.

Clopidogrel [31-33] is a pro-drug requiring hepatic biotransformation for pharmacological activity. The active metabolite of clopidogrel contains a thiol group, which binds irreversibly to a free cysteine in the P2Y12 receptor and blocks activation by ADP. In humans, the majority of drug (85% to 90%) is metabolized to the carboxylic acid metabolite by carboxylesterases, the major metabolite

circulating in the blood. Although this metabolite is inactive, its determination in plasma was used to study the pharmacokinetics of clopidogrel in an indirect manner for many years, because plasma concentrations of the parent drug are very low [34]. Only a small portion of the drug undergoes a two-step activation process, which is mediated by Cytochrome P-450 (CYP). This hydrolysis pathway competes with active metabolite formation catalyzed by hepatic CYP450 enzymes. Clopidogrel's metabolism by CYP450 enzymes forms the inactive intermediate 2-oxo-clopidogrel, which is further metabolized by CYP450 enzymes to three thiol metabolites in humans of which only the H4 metabolite has antiplatelet [35]. Considering some statins similarly to clopidogrel, which are metabolized by CYP3A4, whereas other statins non-CYP3A4-metabolized [23-25], so concentration of H4 in the blood can be used to analyze clopidogrel–statin interactions.

In this study, H4 was measured by LC-MS/MS. The findings of this study were that serum concentrations of clopidogrel active metabolite were unchanged when either atorvastatin or rosuvastatin was added to treatment with clopidogrel. This study showed that neither atorvastatin nor rosuvastatin altered clopidogrel mediated inhibition of platelet aggregation.

Numerous studies [35-39] show that several genetic and non-genetic aspects might be influencing this phenomenon of resistance to clopidogrel. CYP3A4 and CYP2C19 are the most important isozymes of cytochrome P450 (CYP450), which activates clopidogrel. Most notably, the *CYP2C19* loss-of-function alleles such as *CYP2C19*2* and *CYP2C19*3* are connected with poor response to clopidogrel, lower H4 metabolite concentrations, and higher clinical adverse events [36]. Among the non-genetic factors, concomitant use of statins is associated with the occurrence of adverse cardiovascular events [35]. Also, lower exposure to the active metabolite is observed when CYP3A4-metabolized statins, most notably atorvastatin and rosuvastatin, are co-administered with clopidogrel. Other genetic polymorphisms occurring in the gene sequences are also considered as potential factors of variable response to clopidogrel. As shown in the literature, *CYP2C9* genotype might be associated with lower exposition to the parent drug [37]. In this study, our data showed that *CYP2C19*2εA* and *CYP2C19*3εA* are independent risk factors for DCR. Statistical correlation suggests that *CYP2C19 *2εA* and *CYP2C19 *3εA* increased the risk of DCR by 4.6-fold and 12.4-fold, respectively, indicating that *CYP2C19εA* can reduce the inhibitory effect of clopidogrel on platelets, increase the incidence of DCR and related clinical adverse events. As with these results, in our previous study [8], we found that *CYP2Y19εA* is a risk factor for CR or DCR. There were no significant differences between *CYP2C9* and *CYP2C19* between the AC and RC groups. No *CYP3A4* was a mutant allelic variant.

Based on our data, clopidogrel in combination with atorvastatin or rosuvastatin did not impact platelet inhibition of clopidogrel and did not increase incidence of DCR. Statins that are not metabolized by CYP3A4 and metabolized by CYP3A4 are not associated with the development of DCR. One explanation may be that drug interactions are influenced by many factors. Clopidogrel is metabolized through CYP450, a variety of isozymes are involved in this process, such as CYP1A2, CYP2C9, CYP2D6, and CYP3A5, except the 2 most important CYP3A4 and CYP2C19 [38,39]. The combination of CYP3A4 and CYP2C19 inhibitors may be not sufficient to affect the metabolism of clopidogrel. Furthermore, clinical doses of statins may not reach the saturation concentration of CYP3A4 and CYP2C19, and thus do not produce competitive inhibition, which may partly explain our results.

CHANCE's [40] reported that the benefits of dual antiplatelet therapy come mainly from patients in CR with poor metabolizer of *CYP2C19* gene. In addition to the use of aspirin, some patients treated with clopidogrel did not show an adequate antiplatelet response in dual antiplatelet therapy. CYP2C19 are the dominant metabolic enzymes in eastern population's CYP450 metabolic enzymes, while CYP2D6 is the dominant metabolic enzymes in western population [41-43]. Whether the differences of genetic metabolic enzymes can lead to significant higher static CR and DCR in eastern populations? PRoFESS and CAPRIE's [44,45] studies found that clopidogrel have an advantage over aspirin in preventing recurrent CI (8.7% advantage), but these results were all western demographics. The current data lacks a positive study of aspirin and clopidogrel in the eastern population. So in the future, we should attach importance to study the clinical risk of DCR, and monitor the occurrence of DCR, and analyze genetic related risk factors of stroke recurrence throughout the treatment. In other aspects, we should further observe whether CR or DCR can reduce platelet inhibition of clopidogrel, and whether the use of aspirin in the eastern population will take precedence over clopidogrel?

Conclusion

Combined administration of clopidogrel with CYP3A4-metabolized statins does not decrease clopidogrel mediated platelet aggregation inhibition. Administration of statins that are metabolized by CYP3A4 in patients in CI treated with clopidogrel does not reduce platelet inhibition of clopidogrel and does not increase the incidence of DCR. In patients with CI, simultaneously using clopidogrel and CYP3A4-metabolized statins is safe and secondary prevention after ischemic stroke. As well, DCR occurred after CI was related to *CYP2C19εA*. The H4 in the CNCR group was higher than the DCR group, suggesting clopidogrel is ineffective in secondary prevention after ischemic stroke and has a high risk of recurrent stroke.

Acknowledgments

This work was supported by the Astra Zeneca international horizontal single center research project (ESR-14-10092) of Guangdong Province, China.

Our project was registered on clinical trials website, which the ID was NCT02411903. The data was uploaded on July 31, 2018.

Ethical Standards

This study is a human clinical study. On January 1, 2015, we applied to the ethics center of the third hospital affiliated to Guang Zhou medical university for ethics review. The protocol was approved by The Ethics Committee of The Third Hospital affiliated to Guang Zhou Medical University at Jan 16, 2015. The Ethics approval report number is 2014-093. Written informed consent has been prepared in this study, and the informed consent has been obtained from all study participants (or their guardians) (study consent).

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