Clopidogrel variability: role of plasma protein binding alterations

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- The large inter-individual variability associated with clopidogrel pharmacotherapy is a major concern in clinical practice. While pharmacogenetics has advanced our understanding regarding the variability, additional information is needed to optimize clopidogrel dosing.
- Clopidogrel is a pro-drug with both an inactive, carboxyl metabolite and an active thiol metabolite.
- Clopidogrel and both metabolites are extensively protein bound. However, the role of protein binding of the active and inactive metabolites in altering the antiplatelet actions of clopidogrel is still unknown.

WHAT THIS STUDY ADDS

- The inactive metabolite could potentially displace active metabolite from plasma protein binding sites. The plasma protein binding mechanism would offer additional therapeutic strategies to optimize clopidogrel pharmacotherapy.
- The active metabolite, as well as the inactive metabolite, correlates strongly with antiplatelet activity of clopidogrel. Hence, the inactive metabolite could be a robust surrogate marker for the highly unstable active metabolite.

AIM

The large inter-individual variability in clopidogrel response is attributed to pharmacokinetics. Although, it has been used since the late 1990s the pharmacokinetic fate of clopidogrel and its metabolites are poorly explained. The variable response to clopidogrel is believed to be multi-factorial, caused both by genetic and non-genetic factors. In this study, we examined whether the inactive metabolite can alter the plasma protein binding of the active metabolite, thus explaining the large inter-individual variability associated with clopidogrel response.

METHODS

Female subjects ($n = 28$) with stable coronary disease who were not taking clopidogrel were recruited. Serial blood samples were collected following 300 mg oral dose of clopidogrel, plasma was isolated and quantified for total and free concentrations of active and inactive metabolites. Inhibition of platelet aggregation was measured using the phosphorylated vasodilator stimulated phosphoprotein (VASP) assay.

RESULTS

A significant correlation was observed between VASP and both free ($r = 0.49, P < 0.05$) and total ($r = 0.49, P < 0.05$) concentrations of the active metabolite. Surprisingly, we observed a significant correlation with both free ($r = 0.42, P < 0.05$) and total ($r = 0.67, P < 0.001$) concentrations of the inactive metabolite as well. Free fractions of the active metabolite rose with increasing protein binding of the inactive metabolite ($P < 0.05$).

CONCLUSIONS

The above *in vivo* data suggest that the inactive metabolite displaces the active metabolite from binding sites. Thus, the inactive metabolite might increase the free concentration of the active metabolite leading to enhanced inhibition of platelet aggregation. The plasma protein binding mechanism would offer an additional therapeutic strategy to optimize clopidogrel pharmacotherapy.

Introduction

Acute coronary syndrome and related complications are a major cause of morbidity and mortality worldwide [1, 2]. Cardiovascular disease (CVD) is responsible for 42% of deaths in European women under the age of 75 years and 38% of deaths in men under 75 years [2]. About 785 000 Americans had new acute cardiac events while 470 000 have recurrent events in the year 2010 [3]. Coronary heart disease caused one of every six deaths in the United States in 2006. The therapies for acute coronary syndrome are targeted to avoid platelet activation and aggregation. Regrettably, the risk of cardiovascular events in patients taking such therapies still remains high [3].

Clopidogrel, either alone or in combination with aspirin, remains the cornerstone of modern antiplatelet strategies [4]. While two newer thienopyridines have shown some modest improvement in outcomes compared with clopidogrel, clopidogrel remains widely used [5, 6]. Studies show that 4–30% of patients treated with clopidogrel do not display adequate therapeutic response [7], while about 10% of patients face unpleasant nuisance bleeding [8]. Compared with the newer thienopyridines, clopidogrel is associated with a greater inter-patient variability [9, 10]. While this variability is known to be multifactorial, it is primarily pharmacokinetic rather than pharmacodynamic with the active metabolite of clopidogrel showing equal platelet inhibition compared with prasugrel [7, 11, 12]. The predominant factor of intrinsic variability appears to be genetic polymorphisms in cytochrome P450 2C19 (CYP2C19). While 12% of the variance is attributed to

genetic polymorphisms in CYP2C19, recent studies disagree on the importance of CYP2C19 genotype for patient outcomes [13–15]. Additional factors beyond P4502C19 are needed to explain the pharmacokinetic variability of clopidogrel.

Clopidogrel, a pro-drug, is predominantly converted to an inactive carboxylate by carboxylesterases and a minor fraction of 15% is converted to its active thiol metabolite (Figure 1) [16]. Conversion of clopidogrel to its active metabolite is a two step process, which is primarily mediated by CYP enzymes. Multiple CYPs are believed to be involved in the process. Initial studies have suggested that CYP2C19 and CYP3A4 play prominent role while with lesser involvement of CYP2B6, CYP2C9 and CYP1A2. Recent studies have pointed out the important role of paraoxonase (PON-1) in the formation of the active thiol metabolite [17, 18].

Clopidogrel and both metabolites are extensively protein bound [19]. The extensive protein binding (>98%) combined with high concentration of competing drugs or substrates is a perfect combination for clinically important protein binding alterations [20]. The major metabolite of clopidogrel is an inactive carboxyl metabolite which is present in high concentrations and could displace the active metabolite from protein binding sites (see Figure 1 for pictorial depiction of the hypothesis) [21]. Therefore, it is likely that protein binding alterations in the active thiol metabolite could explain a significant amount of inter-individual variability associated with clopidogrel. In this study, we examined whether changes in protein binding of active and inactive metabolites could affect the antiplatelet actions of clopidogrel.

Figure 1

Metabolic pathway of clopidogrel. The pro-drug, clopidogrel, is converted to a minor (10–15%) active thiol metabolite (SR26334) upon sequential metabolism by hepatic cytochrome P450 enzymes. Upon secretion into plasma, the active metabolite is either plasma protein bound (>98%) or as the unbound species which is available for irreversible binding with platelets in order to inhibit their aggregation. On the other hand, hepatic carboxylesterase is responsible for the formation of the major (~85%) inactive carboxyl metabolite (R-130964). Our hypothesis is that the inactive metabolite, which is highly plasma protein bound (>98%) and available in high concentrations in plasma, might compete with the active metabolite for the plasma protein binding sites. This would result in decreased plasma protein binding and increased unbound active metabolite, thus leading to enhanced pharmacological response

Methods

This was a single dose trial examining both the pharmacodynamics and pharmacokinetics of clopidogrel and its metabolites on platelet function in women with established coronary heart disease.

Chemicals

1-methyl-4-phenylpyridinium iodide and acetonitrile were obtained from Sigma Aldrich (MO, USA). 2-bromo-3'-methoxyacetophenone (MPB) was obtained from Fisher Scientific (Fisher Scientific, PA, USA). Clopidogrel carboxy inactive metabolite and the MPB derivatized clopidogrel active thiol metabolite hydrochloride (mixture of diastereoisomers) were purchased from Alsachim (Illkirch, France); S-(+)-clopidogrel hydrogen sulfate was obtained from Toronto Research Chemicals Inc., Canada. $PLT_{VASP/P2Y12}$ kit was obtained from Diagnostica Stago (NJ, USA). The Centrifree and MPS micropartition device was obtained from Millipore (MA, USA).

Patient recruitment

The single dose study was approved by the institutional review board at Oregon Health & Science University (OHSU), Portland, OR, USA. Electronic screening of the patients seen at the OHSU cardiology and family medicine clinics was performed using an electronic medical record database. Patients were identified as having ischaemic heart disease by using ICD-9 codes 410–414. A study coordinator contacted eligible patients and a total of 28 female patients with stable coronary disease who were not taking clopidogrel were recruited. Informed consent was obtained at the beginning of the study visit. Subjects were asked to fast for 2 h prior to the visit.

Blood collection

Blood samples from a venous catheter were collected into a 6 ml vial (containing potassium EDTA as anticoagulant and 50 μ l of 500 mM MPB as stabilizer) at approximately 0, 20, 40, 60, 90, 120 and 240 min after administration of a 300 mg oral dose of clopidogrel. Plasma was isolated within 2 h of blood collection, and stored at -80°C for later analysis of clopidogrel parent drug, active and inactive metabolite concentrations. One additional 10 ml blood sample was collected into a vial containing sodium citrate as anticoagulant at time 0 and 240 min for platelet function testing using the vasodilator-stimulated phosphoprotein (VASP) assay.

Sample processing

For quantitation of total concentrations of clopidogrel and both active and inactive metabolites, 50 μ l of plasma was mixed with 100 μ l of acetonitrile containing 5 ng of internal standard (1-methyl-4-phenylpyridinium iodide). The

samples were vortexed for 15 s and centrifuged at 13 400 g for 5 min. The supernatant was analyzed using the LC-MS method described below. For estimation of unbound concentrations of active and inactive metabolites, 1 ml of plasma sample was filtered through a Centrifree and MPS micropartition device with the aid of centrifugation in a fixed angle rotor at 4000 g for 40 min at 10°C . Ultrafiltrate obtained after centrifugation was processed and analyzed similar to the quantitation of total concentrations described above.

LC-MS assay procedure for estimation of clopidogrel and its metabolites

Liquid chromatography-mass spectrometry (LC-MS) analysis was performed with some modification of the previously mentioned method [22]. Briefly, a 30 μ l of processed plasma sample is injected onto a Kromasil C8 column (5 μ , 3.2×100 mm) fitted with a guard column (Fisher Scientific, PA). The mobile phase consisting of 90% (v/v) acetonitrile/0.1% formic acid, and 10% (v/v) deionized water:0.1% formic acid was isocratically run at a flow rate of 0.3 ml min^{-1} (run time 6 min). The following precursor ion to product ion m/z transitions were monitored on a triple-quadrupole tandem mass spectrometer (TSQ Quantum, Thermo Electron, MA, USA) at 25 eV of collision energy and equipped with an auto sampler maintained at 4°C and operating in positive electrospray ionization mode: 322 $\rightarrow$ 212 (clopidogrel), 308 $\rightarrow$ 198 (inactive carboxyl metabolite), 356 $\rightarrow$ 212 (active thiol metabolite) and 170 $\rightarrow$ 127 (internal standard MPB).

The method was validated according to good laboratory practice standards. The linear range of quantitation was 0.1–8.0 ng ml^{-1} (active metabolite), 1.0–80.0 $\mu\text{g ml}^{-1}$ (inactive metabolite) and 1.0–80.0 ng ml^{-1} (clopidogrel). The intra-assay and inter-assay coefficients of variation were less than 10% for clopidogrel and both metabolites.

Pharmacokinetic data analysis

Pharmacokinetic parameters were obtained by non-compartmental methods using WinNonLin (v 5.2; Pharsight, Mountain View, CA, USA). Maximum plasma concentrations (C_{max}) and time to maximum concentration (t_{max}) were observed values. Area under the curve (AUC(0,240 min)) was calculated from time 0 to 240 min using the linear trapezoidal rule. Drug elimination half-life ($t_{1/2}$) was computed as $t_{1/2} = 0.693/\lambda_z$, where λ_z is the terminal elimination rate constant.

Vasodilator stimulated phosphoprotein (VASP) assay

The antiplatelet effect of clopidogrel was monitored using the $PLT_{VASP/P2Y12}$ kit and as described in the kit manual. Briefly, 10 μ l of whole blood samples collected at time 0

and 240 min were incubated with PGE₁ alone or PGE₁ + ADP. After permeabilization for 5 min, VASP is labelled with a specific monoclonal antibody. The immunofluorescence was measured using dual colour flow cytometry in order to compare the capacity of ADP to inhibit VASP phosphorylation. Platelet reactivity index (PRI) was computed using corrected mean fluorescence intensities in the presence of PGE₁ alone or PGE₁ + ADP. The change in PRI between 0 and 240 min ($PRI_{0min} - PRI_{240min}$) was reported as the antiplatelet effect. The intra- and inter-day assay variability was less than 5%.

CYP2C19 SNP genotyping

Genomic DNA was isolated from whole blood using the DNA isolation kit (QAIGEN, CA, USA) and quantitated with Nanodrop 1000 (Thermo-Scientific, Wilmington, DE, USA). Genomic DNA (250 ng) was added to a reaction mixture containing iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA) and forward and reverse primers (CYP2C19*2: 5'-AATTACAACCAGAGCTTGGC-3', 5'-TATCACTTTCCATAAAAGCAAG-3'; CYP2C19*3: 5'-AAATTGTTTCCAATCATTTAGCT-3', 5'-ACTTCAGGGCTTGGTCAATA-3') at a final concentration of 0.2 µM to a final volume of 25 µL. PCR amplification was performed in the Bio-Rad MyiQ Thermocycler as follows: 2 min at 95°C, followed by 40 cycles of 95°C for 20 s and 50°C for 30 s. PCR product (50 ng) was cleaned with ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA), and incubated at 37°C for 15 min followed by 80°C for another 15 min. Forward and reverse primers were added to a final concentration of 0.27 µM each, and sequenced at Oregon Clinical Translational Research Institute Core DNA Sequencing Laboratory at OHSU. The resulting electropherograms were compared with the reference sequence found on NCBI using Mutation Surveyor® (SoftGenetics LLC, State College, PA, USA).

Data analysis

Four of the total (28) recruited subjects had very low/non-detectable concentrations of active metabolite. This was traced to failure to add MPB stabilizer immediately after blood collection and hence the concentrations were three standard deviations away from the population mean. The data analysis throughout the study was limited to 24 subjects. Demographic and pharmacokinetic data were analyzed using descriptive statistics. Pearson product-moment correlation was employed for regression analysis. All statistical tests were conducted at $\alpha = 0.05$ using SigmaPlot software (v 11.0; Systat Software, Inc., San Jose, CA, USA).

Results

The mean age and BMI of the 24 subjects were 60.2 ± 5.4 years (mean $\pm$ SD) and 31.9 ± 5.8 kg m⁻², respectively. Two

of the 24 subjects self identified as African American with the remainder being Caucasian. Twenty of the 24 subjects were using aspirin at the time of the study visit and one patient was on Aggrenox (aspirin and dipyridamole). Eight patients had diabetes, four patients were active smokers and seven patients were former smokers (quit > 1 year prior).

The pharmacokinetic profile and data after a single dose of 300 mg clopidogrel are shown in Figure 2 and Table 1. A large inter-individual variability was observed in pharmacokinetic parameters, especially AUC. DNA sequencing identified two of the subjects as CYP2C19 *1/*2 expressors. However their exclusion did not affect the %CV of the pharmacokinetic parameters.

Total concentrations of inactive metabolite were strongly correlated with total ($r = 0.83, P < 0.001$) and free concentrations ($r = 0.84; P < 0.001$) of active metabolite (Figure 3). The free concentrations of active metabolite also correlated with free concentrations of inactive metabolite ($r = 0.58, P < 0.01$; Figure 3C).

The plasma protein binding of active and inactive metabolites is presented in Figure 4. Both metabolites were extensively protein bound (<1.0% free fractions). The protein binding (%) was found to be independent of plasma concentrations of either free or total concentrations of metabolites (data not shown). However, the free fraction of the active metabolite increased with increasing protein binding of the inactive metabolite ($r = 0.46, P < 0.05$).

As expected, change in PRI was significantly correlated with both free ($r = 0.49, P < 0.05$) and total ($r = 0.49, P < 0.05$) concentrations of active metabolite (Figure 5). Surprisingly, we observed a significant correlation with both free ($r = 0.42, P < 0.05$) and total ($r = 0.67, P < 0.001$) concentrations of inactive metabolite too.

Discussion

The large inter-individual variability associated with clopidogrel response results in unpredictable clinical outcomes, and remains a concern in clinical practice. The mechanisms underlying the variability are poorly understood. In this study, we have examined the role of inactive carboxyl metabolite in the variability of clopidogrel response. The results of this study suggest that protein displacement of the active metabolite by the inactive metabolite could be a potential mechanism contributing to inter-individual variability.

The inactive carboxyl metabolite is the major metabolite of clopidogrel and accounts for ~85% of the parent drug [23]. *In vitro* and *in vivo* studies have conclusively demonstrated the lack of antiplatelet effect of the carboxyl metabolite when administered alone [16]. However, in the current study and in our previous study [24], the plasma

Figure 2

Pharmacokinetic profile of active thiol metabolite (A), inactive carboxyl metabolite (B) and parent compound clopidogrel (C). Pharmacokinetic profile of 24 women following 300 mg oral dose of clopidogrel is shown here. The error bars depict one SD. spread of data

concentrations of the inactive metabolite generated upon oral administration of clopidogrel strongly correlated with the antiplatelet effect. This interesting correlation, even though counterintuitive, was observed with both total and free concentrations of the inactive metabolite. One could argue that an increase in the bioavailability of clopidogrel would lead to parallel increases in concentrations of both inactive and active metabolites; with the increase in the active metabolite subsequently explaining the greater antiplatelet effect. In this way, the observed correlation between the inactive metabolite and the antiplatelet effect could simply be a surrogate for the true relationship that exists between the active metabolite and the antiplatelet effect. As expected, in the current study, we found a strong correlation between the active metabolite and the antiplatelet effect.

Enhanced bioavailability leading to an increase in antiplatelet effect was reported in earlier studies [25]. However, the possibility of drug–protein displacement resulting in an increase in the free concentrations of the active metabolite and subsequent enhancement in antiplatelet effect has not yet been examined. In theory, displacement of bound drug from plasma proteins increases the pharmacologically active ‘free’ or unbound form of the drug (Figure 1). The effect of drug displacement is dramatic even for extensively bound drugs, such as the active metabolite of clopidogrel which is >99% bound [20]. A slight alteration in drug–protein binding of extensively bound drugs translates into large increases in free concentrations. Furthermore, if the perpetrator molecule, such as the inactive carboxyl metabolite, is also extensively protein bound and present in excess concentration (the inactive metabolite concentration is 100 fold higher than that of the active metabolite), the probability of drug–protein displacement is higher. This study demonstrates that the increase in percent bound of the inactive metabolite correlates strongly with percent unbound of the active metabolite. This observation suggests that as more of the inactive metabolite occupies plasma protein binding sites, less of the active metabolite would have access to binding sites and hence leading to an increase in percent unbound of active metabolite. The exponential relationship further confirms the theory.

The high concentration of inactive metabolite resulting in greater percent binding is likely due to enhanced bioavailability. This could translate into higher concentrations of active metabolite. Simultaneous increases result in two counteracting effects: increased and decreased binding of inactive and active metabolites, respectively. The opposing effects could be due to the differences in binding affinities of these two metabolites. We speculate that the inactive metabolite has relatively higher binding affinity than active metabolite for binding sites on plasma proteins. The higher binding affinity coupled with high plasma concentrations suggests the inactive metabolite as a perpetrator of active metabolite displacement from binding sites.

Table 1

Pharmacokinetic parameters of clopidogrel and metabolites (active thiol and inactive carboxyl)

PK parameter	Active metabolite	Inactive metabolite	Clopidogrel
$C_{\max}$ (ng mL ⁻¹)	17.4 ± 17.9†	6.2 ± 5.2‡	14.5 ± 9.6
$t_{\max}$ (min)	54.6 ± 28.3	72.9 ± 43.2	70.8 ± 33.5
$t_{1/2}$ (min)	45.4 ± 31.5	83.6 ± 32.5	60.0 ± 28.8
AUC(0,240 min) (ng mL ⁻¹ min)	1406 ± 1465	655 ± 515‡	1498 ± 1122

$C_{\max}$, Maximum plasma concentration; $t_{\max}$, time to maximum concentration; $t_{1/2}$, elimination half-life; AUC(0,240 min), area under the curve. †Mean ± SD. ‡Units of $C_{\max}$ and AUC₀₋₂₄₀ are µg mL⁻¹ and µg mL⁻¹ min, respectively.

Figure 3

Correlation analysis between active thiol and inactive carboxyl metabolites of clopidogrel. Pearson product moment analysis was performed on total maximum concentration ($C_{\max,t}$) of inactive metabolite vs. $C_{\max,t}$ of active metabolite (A), $C_{\max,t}$ of inactive metabolite vs. free maximum concentration ($C_{\max,f}$) of active metabolite (B) and $C_{\max,f}$ of inactive metabolite vs. $C_{\max,f}$ of active metabolite (C). The solid line represents the line of regression and the broken lines represent the 95% confidence interval

The studies on binding affinities of both metabolites are lacking to attempt a quantitative comparison. Our data that plasma protein binding of either metabolite is independent of their concentrations suggest that the bioavail-

ability is probably a minor factor, and thus highlights the role of binding affinities in drug displacement from plasma proteins. The drug displacement by the inactive metabolite would increase the concentration of the unbound active

Figure 4

Plasma protein binding of active thiol and inactive carboxyl metabolites. A second order polynomial regression analysis was performed on bound (%) inactive metabolite vs. unbound (%) active metabolite. The solid line represents the line of regression and the broken lines represent the 95% confidence interval. ($n = 24$)

metabolite, but not the total concentration, leading to strong correlation between antiplatelet activity and the inactive metabolite.

In this study, the percent unbound of both metabolites was mostly estimated to be <1.0 which appears to be lower than previously reported values of $\sim 2.0\%$ [26]. Greater bio-analytical sensitivity coupled with better sample collection using stabilizer might have led to more accurate estimations in this study. While the current study employed a single dose, maximum anti-platelet effects is observed only after multiple dosing over several days [27]. The delay in achieving maximum effect could be speculated as a result of saturation of plasma protein binding sites. The concentrations of plasma albumin, a major drug binding protein, decreases with age [28], and therefore could result in higher concentrations of free active metabolite leading to undesirable bleeding episodes. Multiple studies link old age with higher incidence of bleeding episodes [29–31]. Using *in vitro* experiments, Weber *et al.* have demonstrated that albumin abolished the antiplatelet effect of clopidogrel [32]. However, it is to be noted that the albumin concentration in the above study (35 g dl^{-1}) was about 7–10 times higher than physiological concentrations found in human plasma. It would be relevant to determine the effect of physiological albumin concentrations on the antiplatelet effect of clopidogrel. Fibrinogen, a soluble plasma glycoprotein, could also potentially render the active metabolite ineffective. Ang *et al.* showed that fibrinogen concentrations inversely correlated with the antiplatelet effect of clopidogrel [33]. The aforementioned studies underscore the importance of plasma protein binding in clopidogrel response. In the current study, the strong correlation between free concentrations of active

metabolite and antiplatelet effect corroborates the importance of plasma protein binding.

This study included a predominantly homogenous group of study subjects: females, narrow age range and similar genotype. While homogeneity can be desirable in a pharmacokinetic drug trial, it can also be a limitation in terms of limiting external validity of the trial data. Similarly, only two of the subjects were not Caucasian. All but two were wild-type genotype for CYP2C19. Two of the subjects were heterozygous CYP2C19 $*1/*2$. While the homogeneity of the study population provided a less-confounding environment to test the protein binding theory, the authors believe that the present study was explorative but with potential clinical implications. Our data suggest that the bioavailability coupled with plasma protein binding characteristics of both metabolites determine the overall antiplatelet effect of clopidogrel therapy. While this is an exciting finding, detailed *in vitro* experiments are needed to establish conclusively this phenomenon. This new knowledge will provide valuable insights into the mechanism that could be tapped into optimizing clopidogrel pharmacotherapy. One potential strategy would be to co-administer clopidogrel with an agent that competes with the active metabolite for plasma protein binding sites. Interestingly, salicylic acid, the main metabolite of aspirin, has strong affinity for albumin. Furthermore, our data indicate a strong correlation between antiplatelet activity and the concentrations of the inactive carboxyl metabolite which is chemically stable, and therefore appeals to its potential use as a surrogate measure of clopidogrel pharmacotherapy.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare (i) no support from any organization for the submitted work, (ii) no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years and (iii) no other relationships or activities that could appear to have influenced the submitted work.

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Figure 5

Correlation analysis between metabolites of clopidogrel and change in platelet reactivity index PRI. Vasodilator-stimulated phosphoprotein (VASP) is quantified to measure change in PRI which is correlated with maximum concentration, both total ($C_{\max,t}$) and free ($C_{\max,f}$), of active thiol (A and B) and inactive carboxyl (C and D) metabolites of clopidogrel. The solid line represents the line of regression and the broken lines represent the 95% confidence interval

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