Pharmacokinetics of clopidogrel after administration of a high loading dose

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Introduction

Besides the gold-standard antiplatelet drug aspirin, the thienopyridine derivative clopidogrel, which inhibits platelet aggregation through irreversible antagonism of the adenosine diphosphate (ADP) receptor subtype P2Y12 (1, 2), has become a second mainstay of antiplatelet therapy during both short-term and long-term treatment (3-5). In patients undergoing percutaneous coronary intervention, pretreatment with a loading dose of clopidogrel (300-600 mg) was shown to improve clinical outcome (6, 7). However, recent findings revealed a marked interindividual variability to clopidogrel inhibition of ADP-induced platelet aggregation, estimating that up to 30% of patients do not achieve an adequate antiplatelet effect from the initial clopidogrel load (8, 9). The reasons for individual heterogeneity in responsiveness to clopidogrel are largely unknown. ADP elicits platelet aggregation through simultaneous activation of P2Y1 receptors (coupling to Gq and mobilizing intracellular calcium) and P2Y12 receptors (coupling to Gi, inhibiting adenylylcyclase and activating the phosphoinositide 3-kinase pathway) (10). Thus, polymorphisms of P2Y1 and P2Y12 genes, differences in receptor expression or posttranscriptional regulation or in the downstream signaling cascades are proposed to contribute to individual variations in ADP-induced response to P2Y12 inhibition with clopidogrel (11-13). Furthermore, interindividual differences in the activity of the cytochrome P450 iso-
zymes 3A4 and 3A5 that metabolize the inactive clopidogrel prodrug to its active thiol form were suggested to underlie the response variability (14, 15). To evaluate these hypotheses we assessed the pharmacokinetics of clopidogrel and its relation to platelet inhibition.

**Materials and methods**

**Materials**

Iscover™ tablets containing clopidogrel bisulfate were from Bristol-Myers Squibb, Munich, Germany. Free clopidogrel base was obtained by extraction of the crushed tablets with methanol, followed by potassium carbonate/cyclohexane treatment and purification over Celite™ 545 (Mallinckrodt Baker, Phillipsburg, NJ, USA). The carboxyl derivative of clopidogrel was synthesized by treating the clopidogrel base with potassium hydroxide/ethanol followed by purification with acetoxyethyl ester/methanol. Identity and purity of the products were determined by LC-MS/MS, 1H-NMR, and 13C-NMR. Purity of clopidogrel base and the carboxyl derivative was >99.9%. Adenosine 5’-diphosphate and diltiazem were purchased from Sigma-Aldrich, Deisenhofen, Germany.

**Study population and interventions**

The study population included ten healthy volunteers (9 men, 1 woman, age 25-47 years, mean ± SD: 34.9 ± 7.5 years). After a run-in of 14 days without medication and a 12-hour overnight fast, participants received a single oral dose of 600 mg clopidogrel (Iscover™).

For pharmacokinetic determinations, EDTA-blood from the antecubital vein was collected immediately predose and at 0.5, 1, 1.5, 2, 3, 6, and 9 hours postdose; plasma was obtained by centrifugation at 1500 g for 10 min at 4°C and stored at -70°C. For aggregometry citrate-blood was collected prior and 6 hours after clopidogrel administration.

The study was approved by the Institutional Ethics Committee of the Technische Universität München, and informed consent was obtained.

**Platelet aggregation**

Platelet-rich plasma (PRP) was prepared from citrate-anticoagulated blood by centrifugation at 150 g for 10 min at room temperature. The platelet count of PRP was adjusted to 300 × 10^9/l with autologous platelet-poor plasma (PPP), which had been centrifugated from PRP (1500 g, 10 min). Platelet aggregation was induced with 5 and 20 μM ADP and recorded by optical aggregometry for 5 min at 37°C and constant stirring (1000 rpm) using a Chronolog Lumi-Aggregometer (Probe&go Labordiagnostica, Endingen, Germany). Aggregation was expressed as maximal percent change in light transmission, with unstimulated PRP (0% light transmission) and PPP (100% light transmission) serving as references. Inhibition of aggregation was described as absolute difference (Δ aggregation, %) between baseline aggregation and 6-hour posttreatment aggregation.

**Detection of clopidogrel and its metabolites in plasma**

LC-MS/MS analyses were performed using a Surveyor HPLC system (Thermo Electron, Dreieich, Germany) coupled to a TSQ Quantum triple-quadrupole mass spectrometer (Thermo Electron) operating in positive electrospray ionization (ESI+) mode with selected reaction monitoring (SRM). 15-μl aliquots of acetonitrile-precipitated plasma samples were injected onto a 5-μm Aquasil C18 column (100 × 3 mm; Thermo Electron), and eluted using a gradient of acetonitrile/0.1% formic acid (10 to 90% v/v) at 0.3 ml/min (run time 13.5 min). Clopidogrel, the inactive carboxyl metabolite and the active thiol metabolite were identified in plasma by the specific collision-induced dissociation (CID) product ions of the respective parent isotopic 35Cl- and 33Cl-ions (M-H+) (16). SRM transitions used for quantification were: m/z 322→212 for unchanged clopidogrel, m/z 308→198 for the carboxy metabolite, and m/z 356→212 for the active metabolite. Detection of the internal standard (IS) diltiazem (1 mg/l) was performed by monitoring the m/z 415→178 transition. The method was validated according to GLP standards. The relative detection responses (i.e. peak area ratios of the analytes over IS) showed linear changes upon dilution with blank plasma; calibration curves of clopidogrel and the carboxyl metabolite obtained from spiked blank plasma were linear (r²>0.993) over the detection range of 0.5-100 ng/ml and 0.5-150 μg/ml, respectively.

Although it was not possible to employ the active metabolite as a calibration standard, absolute concentrations of the active metabolite were approximated from the linear calibration of clopidogrel, due to the following prerequisites: clopidogrel and the active metabolite were eluted from the HPLC column with similar retention times and at constant composition of the mobile phase, the parent ions were fragmented at the same collision energy (25 eV), same fragmentations (transition to m/z 198 for the carboxyl metabolite, and m/z 212 for the active metabolite). Statistical analysis was carried out with SigmaStat™ software (Jandel, San Rafael, CA, USA). Data are presented as means ± SD. Linear association between two variables was tested with Pearson’s test (after confirming normal distribution (K-S test) and constant variance (F test) of the residuals). Deviation from linearity (i.e. whether the association was better described by non-linear regression) was evaluated by the Mandel test. p<0.05 was considered statistically significant. Plasma concentration...
versus time data of each participant were fitted by a one-compartment first order model (Bateman function, $r>0.94$) using WinNonlin™ software (Pharsight, Palo Alto, CA, USA). From the individual regression fits the following pharmacokinetic parameters were calculated: maximal plasma concentration ($c_{max}$), time to reach the maximal plasma concentration after drug intake ($t_{max}$), the area under the plasma concentration time curve from intake ($t=0$) extrapolated to infinity ($t=\infty$) ($AUC_{\infty}$), and the half-life of elimination from the plasma ($t_{1/2}\text{el}$).

**Results**

Subjects showed similar pretreatment platelet aggregation values (88 ± 5%, range 80-94%), whereas 6 hours after the clopidogrel load considerable interindividual differences in aggregation (32 ± 12%, range 15-54%), and thus in inhibition of aggregation (56 ± 13%, range 33-78%) were observed (Fig. 1). All aggregation values were normally distributed ($p>0.2$).

**Figure 1:** Individual recordings of optical aggregometry in citrated PRP from 10 volunteers. Platelet aggregation was induced by 5 µM ADP. In each panel the upper curve is the baseline recording, the lower curve is the recording 6 hours after ingestion of a 600 mg dose of clopidogrel. Δ represents the absolute difference between maximal baseline aggregation and maximal 6-hour posttreatment aggregation.
Table 1: Plasma cokinetic plasma parameters of clopidogrel and its metabolites. Maximal plasma concentrations (c_max) of clopidogrel and its carboxyl metabolite are given in ng/ml. c_max of the active metabolite is expressed as relative response: i.e. the peak area ratio of the SRM transition m/z 356→212 to the MRM transition m/z 415→178 of the internal standard (1 µg/ml diltiazem). The area under the concentration-time curve (AUC_∞) is determined by multiplication of respective concentrations with the time in hours. t_1/2el is the elimination half-life. t_max is time leading to maximal plasma concentrations after clopidogrel intake. Values are arithmetic means ± SD. Provided that the mean calibration curve for clopidogrel also fits the response to the active metabolite, the mean absolute c_max and AUC_∞ values for the active metabolite can be estimated as 7.5 ng/ml and 17.2 (ng x h)/ml.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>c_max (ng/ml)</th>
<th>t_max (hours)</th>
<th>AUC_∞ (ng x h)/ml</th>
<th>t_1/2el (hours)</th>
</tr>
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<tbody>
<tr>
<td>clopidogrel</td>
<td>38.0 ± 22.9</td>
<td>1.4 ± 0.7</td>
<td>125.5 ± 60.0</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>carboxyl metabolite</td>
<td>43000 ± 16900</td>
<td>1.6 ± 0.9</td>
<td>198600 ± 52400</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>active metabolite</td>
<td>(14.5 ± 6.0)</td>
<td>0.9 ± 0.5</td>
<td>(32.9 ± 13.4)</td>
<td>0.7 ± 0.4</td>
</tr>
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Clopidogrel, its inactive carboxyl metabolite and its active thiol metabolite were reliably identified in plasma of all participants by their specific CID spectra.

The pharmacokinetic plasma parameters (c_max, t_max, AUC_∞, and t_1/2el) of clopidogrel and its metabolites were characterized by considerably interindividual differences (Table 1). The approximate ratio of the mean peak plasma concentrations (c_max, ng/ml) was 0.2 (active metabolite) : 1 (clopidogrel) : 1000 (carboxyl metabolite).

Univariate linear regression revealed strong and statistically significant correlations between reduction in platelet aggregation and the c_max values of unchanged clopidogrel (r=0.76; p=0.01), the inactive carboxyl metabolite (r=0.70; p=0.03), as well as the active metabolite (r=0.73; p=0.02) (Fig. 2). Somewhat closer linear associations (r=0.79-0.89, p<0.01) were observed when platelet aggregation was induced with the higher ADP concentration (20 µM), yielding a maximal baseline aggregation of 94 ± 6% (range 85-105%), a maximal posttreatment aggregation of 43 ± 17% (range 25-70%), and an inhibition of aggregation of 51 ± 19% (range 22-80%) (data not shown).

The c_max values of clopidogrel, the carboxyl metabolite and the active metabolite were also linearly correlated (Fig. 3).

Univariate linear regression represented the best fit to the data. By contrast, AUC_∞ or t_1/2el values were not linearly correlated with the degree of platelet inhibition (p>0.1), and there were no correlations among AUC_∞ or t_1/2el (p>0.1).

Discussion

This is the first report of the pharmacokinetics of clopidogrel in plasma involving the determination of its active metabolite. Correlation of the pharmacokinetic data with platelet inhibition allowed elucidating the possible causes of response variability.

Our results suggest that interindividually variability of ADP-induced inhibition of platelet aggregation to clopidogrel is predominantly explained by a variability in active metabolite concentrations. The association analysis was based on interpretation of the response to 5 µM ADP to be consistent with previous trials evaluating platelet function under clopidogrel treatment (8, 18, 19) as well as the common definition of clopidogrel resistance (8). However, we found that the stronger aggregatory stimulus of 20 µM ADP could also be applied to differentiate individual platelet inhibition to clopidogrel without significant loss in sensitivity.

Although we have not determined formation of covalent adducts of the active metabolite with the platelet P2Y12 receptor, constituting the molecular mechanism of clopidogrel action, the observed linear correlation of response with c_max values, but lack of correlation with AUC_∞ or t_1/2el, is consistent with a rapid and irreversible binding of the thiol metabolite to the P2Y12 receptor. Furthermore, a Pearson’s coefficient of r>0.7 for the correlation between active metabolite concentrations and platelet inhibition implies that differences in gene sequence, expression or regulation of the P2Y12 or P2Y1 receptors or in the signaling cascade downstream of the receptors in all can account for less than 50% of the response variability. Moreover, the closer correlation (r>0.8) found after stimulation with 20 µM ADP (associated with a reduced measurement uncertainty) suggests that less than 30% of the variability depends on individual platelet-related characteristics.

Linear correlation of c_max values between clopidogrel and its active metabolite implies that interindividual differences in the activity of metabolizing enzymes (CYP3A4 or 3A5) are not the rate limiting step for generation of the active thiol. Thus, variability in bioactivation also does not constitute the central mechanism for response variability to clopidogrel. However, this does not contradict recent findings that an efficient inhibition of CYP3A4 (e.g. by co-administration of statins) reduces the ability of clopidogrel to inhibit platelet aggregation (14, 15). Hence, considering the strong association of peak plasma concentrations of unchanged clopidogrel with the degree of platelet...
Figure 2: Linear regression of platelet inhibition against maximum plasma concentrations ($c_{\text{max}}$) of clopidogrel, carboxylmetabolite, and active metabolite. $c_{\text{max}}$ of the active metabolite is expressed as relative response. $\Delta$ Aggregation indicates 5 µM ADP-induced platelet aggregation at baseline minus aggregation at 6 hours after administration of a single 600 mg dose of clopidogrel. Solid lines represent the linear-regression fit to the data; dashed lines show 95% confidence intervals. $r$ and $p$ were obtained with Pearson’s test; $p<0.05$ was considered statistically significant.

Figure 3: Linear correlations of maximum plasma concentrations ($c_{\text{max}}$) of clopidogrel, carboxylmetabolite and active metabolite. $c_{\text{max}}$ of the active metabolite is expressed as relative response. Solid lines represent the linear-regression fit to the data; dashed lines show 95% confidence intervals. $r$ and $p$ were obtained with Pearson’s test; $p<0.05$ was considered statistically significant.
inhibition, interindividual variability of intestinal clopidogrel absorption may be the main determinant of response variability to clopidogrel.

Although the present study is explorative, the potential implications of our findings on clinical practice should be discussed. We measured platelet function by optical aggregometry, since this method is widely available in clinical laboratories, it meets the criteria of an accurate and valid measure, and has been used to assess the antiplatelet effects of clopidogrel in previous studies (which also expressed the inhibitory response to clopidogrel as difference between maximal pretreatment and posttreatment aggregation values) (8, 19, 20). Recently introduced techniques that allow rapid bedside assessment of platelet function, such as the PFA-100, would principally provide advantages over conventional aggregometry as a screening tool, but these assays were found to lack sensitivity and specificity, and have limited reproducibility (21, 22). However, due to the strong correlation between plasma drug concentrations and platelet inhibition, interindividual variability of intestinal clopidogrel absorption may be the main determinant of response variability to clopidogrel. Preliminary data of our group suggest that even a low clopidogrel dose of 75 mg (1 tablet) may be sufficient to discriminate poor clopidogrel responders. Thus, screening for clopidogrel resistance may be routinely performed by measurement of plasma concentrations after administration of a test dose of clopidogrel.

The most straightforward and clinical implication of the present findings is that an adequate level of platelet inhibition or active metabolite concentration may be achieved in poor responders by increasing the clopidogrel dose. However, it appears premature to advise treatment of clopidogrel resistance by elevation of the clopidogrel dose. Unlike in the case of aspirin resistance (23), prospective data providing definite evidence that an inadequate response to clopidogrel correlates with an excess risk of cardiovascular events are still lacking. Moreover, the safety of higher clopidogrel doses than 600 mg, in particular with respect to the hazard of potentially fatal thrombotic thrombocytopenic purpura, has not yet been established. Subsequent studies on a sufficient number of patients are needed to answer these questions.

References