Utility of 96-well plate aggregometry and measurement of thrombi adhesion to determine aspirin and clopidogrel effectiveness

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Summary

Aspirin and clopidogrel are key anti-thrombotic therapies. Results from platelet reactivity testing during therapy, have been shown to correlate with future events and would allow for the optimisation of therapy. However, there is little agreement among current tests and there remains a clear clinical need for a universal standardised test. It was the objective of this study to explore the potential of 96-well plate aggregometry as a definitive clinical test of platelet reactivity with respect to aspirin and clopidogrel. A small non-blinded trial of 16 healthy male volunteers assigned to seven days of aspirin (75mg/day) or clopidogrel (75mg/day) therapy. Blood was collected before and on day 7 of treatment. Platelet aggregation was measured using a 96-well plate based aggregation method, and thrombi adhesion measured by colourimetric assay. Platelet agonists used were ADP (0.1–30µM), arachidonic acid (0.03–1.3mM), collagen (0.1–30µg/ml), adrenaline (0.001–100µM), ristocetin (0.2–3mg/ml), TRAP6 amide (0.130µM) and U46619 (0.130µM). Concentration response curves were constructed to each agonist under the various conditions and used to extract data such as log EC50, Hill slope, and area under the curve. These demonstrated low intra- and inter-assay variability and strong discrimination of drug effects. This study demonstrates the ability of the 96-well plate based aggregation and adhesion method to detect and differentiate between stable aspirin and clopidogrel treatment in healthy volunteers. Moreover, this assay marries the ability to test subjects or patients using a range of platelet agonists with more rapidity and ease than the current gold standard platelet assay, traditional light transmission aggregometry, making it a serious alternative assay for use in clinical settings.


Introduction

It is frequently remarked how important it is to find easy to perform, robust, reproducible and interrogatable assays of platelet reactivity to assist in optimising anti-thrombotic drug therapy (1–7). Light transmission aggregometry has previously been adapted to 96-well plates (8, 9) and here we report development of such an assay to profile and discriminate ex vivo the anti-thrombotic drugs, aspirin and clopidogrel. Results from the assay can be displayed both as single numerical values, like those produced by point-of-care devices, or interrogated to provide detailed information regarding drug-platelet interactions.

Aspirin and clopidogrel, individually and combined, are proven to prevent thrombotic events in susceptible populations (10–12). Despite the general anti-thrombotic effectiveness of these drugs, as demonstrated in large outcome studies (13–15), there is still much concern regarding ‘resistance’ to both aspirin and clopidogrel (12, 16–21). It has been recently shown, for in-
stance, that testing of post-treatment platelet reactivity is correlated with future thrombotic events (22) and furthermore, that adjusting clopidogrel treatment after testing can reduce the incidence of major cardiovascular events (23).

Unfortunately, the fundamental block that remains in exploring links between anti-thrombotic drug resistance, platelet reactivity and patient outcomes, is the measurement of platelet function. Traditional light transmittance aggregometry (LTA) is seen by many to be the ‘gold-standard’ method for platelet testing (17, 24) but it is laborious and time consuming and requires experienced staff and a specialised laboratory which largely prevents it from being used in a clinical setting. Additionally, despite ongoing efforts, there is no standardisation in LTA methodology which has led to vast differences in laboratory practice (25, 26). For these reasons point-of-care machines such as PFA-100 and VerifyNow® have been developed (19, 27–30). However, there is no consensus on which test or index is predictive for major cardiovascular events, nor indeed much agreement among test results (31). For example, Lordkipanidze et al. (2, 32) recently reported that alternative tests to traditional LTA failed to correlate with either each other or LTA.

Generally, the overriding weakness of many platelet tests, including point-of-care tests, is the use of a single agonist concentration producing only a single numerical result which cannot be probed and provides no opportunity to investigate the dynamic range of platelet responses in any one sample. Clearly an answer to these issues would be to develop LTA so that it becomes accessible to routine use and include the concurrent measurement of platelet responses to multiple agonists across a range of concentrations (33). We have already reported how this approach can be used to measure the in vitro effects of aspirin upon platelet reactivity (34). Here we demonstrate, for the first time, the ability of this technique to detect and discriminate aspirin and clopidogrel therapy in healthy male volunteers.

Methods

Patients
Sixteen healthy male volunteers aged 18–40 years were recruited and participated in the study. The volunteers’ health statuses were determined through their medical histories, physical examination including blood pressure and pulse rate, blood chemistry and urinalysis. Volunteers with normal clinical profiles were included in the study. The study was approved by the St Thomas’s Hospital Research Ethics Committee (07/Q0702/24), conducted according to the Declaration of Helsinki and all volunteers gave written informed consent prior to entering the study.

Study protocol
Prior to starting, all volunteers had abstained from aspirin, non-steroid anti-inflammatory drugs or paracetamol for 14 days. The volunteers received either 75mg aspirin per day (Anettes 75® Bristol-Meyers-Squibb; n=8) or 75mg clopidogrel per day (Plavix® Bristol-Myers Squibb; n=8) for seven days. Compliance was assessed by interview. Blood samples (35ml) were taken on day 0, prior to commencing drug treatment, and on day 7 of treatment.

Platelet aggregation
Platelets were collected and aggregation tested as previously reported (34). Briefly, blood was collected by venepuncture into tri-sodium citrate (3.2% w/v final) and centrifuged at 175g for 15min to obtain platelet rich plasma (PRP). A platelet count was made of all samples to confirm normal platelet numbers; 1.89–3.99x10^10 cells/ml (n=32). Platelet poor plasma (PPP) was obtained by centrifugation of PRP at 15000g for 5min. The PRP (100µl) was then added to the wells of 96-well plates (Nunc, obtained from VWR, Lutterworth, Leicestershire, U.K.) containing the platelet agonists (10µl), adenosine diphosphate (ADP, 0.1–30µM; LabMedics, Salford, Manchester, U.K.), arachidonic acid (AA, 0.03–1.3nM; Cayman Chemical Company, MI, USA), collagren (type I equine tendon 0.1–30µg/ml; Lab-Medics), adrenaline (0.001–100µM; LabMedics), ristocetin (0.2–3mg/ml; Helena, Gateshead, UK), TRAP6 (thrombin receptor activating peptide) amide (SFLRN 0.1–30µM; Bachem, St Helens, Merseyside, U.K.), U46619 (0.1–30µM; Cayman Chemical Company, Ann Arbor, MI, U.S.A.) or vehicle. Plates were then immediately placed in a 96-well plate reader (Tecan Sunrise®) and absorbance determined at 595nm every 15s for 16min between vigorous shaking at 37°C. Changes in absorbance were converted to % aggregation by reference to the absorbances of PRP and PPP.

Human activated platelet adhesion
As we have previously reported (34), the adherence of activated platelets in this assay was determined by the method of Bellavite et al. (35). Briefly, after aggregation, each well was washed twice with saline before buffer (citric acid, 13.7mmol/L; sodium citrate, 43.6mmol/L; Triton X100, 0.1% w/v) containing p-nitrophenolphosphate (0.2mg/ml; Sigma) was added and incubated for 15min at room temperature followed by the addition of 2M NaOH and measurement of absorbance at 405nm. Percentage platelet adhesion was determined by reference to absorbance changes associated with the addition of known quantities of platelets to the assay.

Statistical analysis
All statistical analyses were conducted using GraphPad v4 (GraphPad Software Inc, CA, USA). Agonist concentration response curves were plotted and analysed according to the four parameter logistic equation:

\[ Y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{\left(-\log_{10}EC_{50}-X\right) \times \text{HillSlope}})}{1} \]

Area under curve (AUC) values were calculated using the trapzoid rule from the log-concentration response curve (lower boundary = 0 and upper boundary = maximum % achieved) and were further analysed using receiver-operator curve (ROC) analysis to determine which tests best discriminated drug treatments.

Results

Platelet aggregation in control conditions: establishing analytical approaches
Conducting platelet aggregometry in 96-well plates permits the concurrent recording of aggregation responses to multiple agonists at multiple concentrations. Figure 1 displays pooled aggregation traces to ADP, arachidonic acid, collagen and adrenaline. The generation of so many concurrent data points permits a par-
particularly detailed analysis of platelet reactivity. To quantify our data we investigated multiple analytical approaches from which we found the following to be the most robust. Firstly, we constructed concentration response curves for each agonist at multiple time points and selected 16min as providing the best end point to allow for response development. From 16min concentration response curves for each agonist we extracted log EC50, Hill slope, area under the curve and maximum aggregation values, in the absence and presence of aspirin or clopidogrel (Table 1).

Effects of aspirin and clopidogrel on platelet aggregation
Consumption of a standard anti-thrombotic dose of aspirin most noticeably inhibited aggregation in response to adrenaline and collagen and abolished the response to arachidonic acid (Figure 1). In contrast, clopidogrel treatment strongly inhibited aggregation to most agonists used here but in particular those to ADP, collagen and adrenaline (Figures 2, 3, 4).

Upon analysing the concentration response curves in more detail, as outlined above, the differential inhibitory profiles of these anti-thrombotic drugs were better defined. For example, following aspirin treatment the aggregation response to 1µM ADP changed from 34 ± 4% to 13 ± 2% but the log EC50 for ADP did not, −5.7 ± 0.1 to −5.5 ± 0.1. Clopidogrel on the other hand, strongly affected the response to the maximum concentration of 30µM ADP reducing it from 79 ± 2% to 39 ± 4% and increasing the log EC50 for ADP from −5.7 ± 0.1 to −4.9 ± 0.4 (Figure 2). To a lesser extent differential inhibition was also seen against the aggregation responses to adrenaline. Aggregation in response to the maximum concentration of adrenaline (100µM) was reduced from 73 ± 3% to 42 ± 8% in the presence of aspirin, and to 57 ± 3% in the presence of clopidogrel. The log EC50 value for adrenaline increased from 6.8 ± 0.1 to −5.2 ± 0.2 and to 6.0 ± 0.1 in the presence of aspirin and clopidogrel, respectively (Figure 3). With regard to collagen, aspirin and clopidogrel had very similar inhibitory effects. For example, the aggregation to 3µg/ml collagen was reduced from 63 ± 5% to 26 ± 5% by aspirin and to 26 ± 4% by clopidogrel, while the log EC50 value for collagen was changed from −5.7 ± 0.1 to −5.3 ± 0.1 by aspirin and to 5.2 ± 0.1 by clopidogrel (Figure 4).

Data for the other agonists used are summarised in Table 1, and show in general terms that aspirin did not affect the aggre-
Table 1: Calculated Platelet Response Values. Log EC50 (log concentration, M, producing half maximum agonist response), maximum response, Hill slope and AUC (area under the curve, log M, maximum) calculated from log-concentration response curves (16min) of platelet responses to each agonist. ADP, adenosine diphosphate; AA, arachidonic acid; TRAP-6, thrombin receptor activating protein-6. Data presented as mean ± s.e.mean, NC = not calculable; n=16 for pre-treatment, n=8 per treatment group. *indicates p<0.05 by one-way ANOVA, comparison of post-treatment to pre-treatment.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Pre</th>
<th>Post Aspirin</th>
<th>Post Clopidogrel</th>
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<tr>
<td></td>
<td>Log EC50</td>
<td>Max</td>
<td>Hill Slope</td>
</tr>
<tr>
<td>ADP</td>
<td>–5.7 ± 0.1</td>
<td>83 ± 5</td>
<td>0.9 ± 0.2</td>
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<tr>
<td>Collagen</td>
<td>–5.7 ± 0.1</td>
<td>91 ± 4</td>
<td>1.3 ± 0.2</td>
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<tr>
<td>Adrenaline</td>
<td>–6.8 ± 0.1</td>
<td>73 ± 3</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>AA</td>
<td>–3.5</td>
<td>55 ± 6</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Ristocetin</td>
<td>–2.9 ± 0.1</td>
<td>90 ± 3</td>
<td>4.6 ± 0.5</td>
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<tr>
<td>TRAP-6</td>
<td>–5.8 ± 0.1</td>
<td>86 ± 1</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>U46619</td>
<td>–6.2 ± 0.1</td>
<td>87 ± 2</td>
<td>3.1 ± 0.5</td>
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Aggregation responses to ristocetin, TRAP-6 or U46619 whilst clopidogrel did, although to a lesser degree than it did to the agonists mentioned above.

Effect of aspirin and clopidogrel on platelet adhesion

The adhesion of the activated aggregates was also measured at the end point of the assay and concentration curves constructed and analysed as for aggregation (Figures 2, 3, 4 and Table 1). The trends for adhesion mimicked those seen for the aggregation. For example, the log EC50 for adhesion in response to ADP was increased from –5.7 ± 0.2 to –5.3 ± 0.1 by aspirin, but increased to –4.9 ± 0.3 by clopidogrel. The log EC50 value for adhesion in response to collagen shifted similarly from –5.9 ± 0.1 to –5.5 ± 0.1 for both aspirin and clopidogrel, as did that for adrenaline, from –6.7 ± 0.1 to –5.7 ± 0.2 for both aspirin and clopidogrel. Notably, adhesion in response to U46619 was decreased by clopidogrel, with the log EC50 increasing from –6.2 ± 0.1 to –5.6 ± 0.1, but was unaffected by aspirin.

Area under curve analyses and receiver operating curves

Having carried out many analyses of the platelet reactivity data, made possible by conducting multiple parallel assays, we found that comparison of area under the curve values for both aggregation and adhesion provided a simple way to express the anti-thrombotic effects of the drug interventions. In making this judgement we used receiver-operator curve (ROC) analysis to determine whether area under the curve values obtained in our tests accurately discriminated drug treatments, as this could indicate the ability of our assay to provide a single number read out as an expression of clinical anti-platelet drug effectiveness. When examining the effects of aspirin in our subjects our test was definitive, i.e. a ROC area of 1 (95% confidence interval [C.I.], 1.0–1.0; p=0.0007). The collagen and adrenaline induced aggregation responses were also highly discriminatory for the effects of aspirin with ROC areas of 0.94 (95% C.I., 0.8–1.0; p=0.0008) for both. The effects on aggregation were matched by changes in platelet adhesion, with a ROC area for arachidonic acid of 0.98 (95% C.I., 0.94–1.0; p=0.0012) and for collagen of 0.92 (95% C.I., 0.77–1.0; p=0.0046). Adhesion to adrenaline also scored highly with a ROC area of 0.88 (95% C.I., 0.67–1.0; p=0.0118) but with a more broad confidence interval.

When testing for clopidogrel in this study, the definitive tests were the markedly reduced adhesions of platelets in response to ADP and U46619, with ROC areas of 1 (1.0–1.0 95% C.I.; p=0.0008). In addition, ADP induced aggregation was strongly inhibited by clopidogrel (ROC area,0.97; 95% C.I., 0.89–1.0; p=0.0017), as were the adhesion responses to ristocetin (ROC area, 0.97; 95% C.I., 0.89–1.0; p=0.0016) and TRAP6 (ROC area, 0.94; 95% C.I., 0.82–1.0; p=0.0033). The aggregation re-
sponse to adrenaline also had a high ROC area of 0.89 but with a broader confidence interval (95% C.I., 0.68–1.0; p=0.0088).

Discussion

This study shows the ability of our modified 96-well plate based aggregometry method to discriminate the anti-platelet effects of aspirin and clopidogrel in healthy volunteers. Information generated by the assay can be displayed as single numerical values that can provide immediate simple comparisons of effectiveness such as produced by point-of-care devices; here we have tested the idea of delivering this as area under the curve values. For more in depth analyses the data can also be interrogated to provide detailed information, such as EC$_{50}$ and Hill slope values, that could be of more use to researchers investigating drug platelet interactions. The power of our assay to detect, characterise and discriminate treatment lies in its ability to expose single blood samples to a broad range of agonist concentrations, allowing us to display the full dynamic range of responses to each platelet activator. Such relationships cannot be seen with single point assays such as VerifyNow® or PFA-100, which provide a single number result, e.g. units or time (36, 37).

Some of the advantages of our assay are demonstrated, for instance, by examining the effects of aspirin and clopidogrel on aggregation induced by ADP. At higher concentrations of ADP there is little or no inhibition by aspirin whereas clopidogrel halves the response; conversely at an ADP concentration of 1µM, aspirin reduces the aggregatory response by half (34% to 13%) and clopidogrel reduces the response by more than three quarters (34% to 7%). In this way we can see that aspirin may slightly reduce the potency of ADP but not its maximum response. Clopidogrel reduces both the potency of ADP and its overall ability to
cause platelet aggregation, including maximal response. This differential effect is well reflected in the pharmacologically derived values for EC$_{50}$ and in the values for area under the curve (104 ± 8 to 78 ± 5 for aspirin; 112 ± 15 to 35 ± 6 for clopidogrel).

Further to this we used ROC analysis to test if reducing our data to area under the curve values provided drug discrimination, since this could provide a simple accessible reading in a clinical setting. Aspirin acts by inhibiting the enzyme cyclooxygenase-1 and so blocking the production of thromboxane A$_2$ (TXA$_2$) in platelets (38). Exogenously added arachidonic acid induces aggregation through the production of TXA$_2$ and is used as a test for aspirin in traditional LTA. ROC analysis, to test sensitivity of agonists to drug treatment, identified the definitive test for the effects of aspirin in our study to be the abolishment of arachidonic acid induced aggregation (ROC area, 1; 95% C.I., 1.01.0). We also identified the aggregation induced by collagen and adrenaline to be markedly affected with similar ROC areas of 0.94, but with wider confidence intervals for both. Clopidogrel, on the other hand, is a P2Y$_{12}$ antagonist that blocks the interaction of ADP with platelet receptors (39). Reassuringly, our test reported ADP-induced aggregation to be strongly inhibited by clopidogrel with a ROC area of 0.97 (95% C.I., 0.89–1.0).

Another facet of the 96-well plate method is the combination with a colourimetric assay, allowing for the measurement of adhered activated aggregates after aggregation. In general, the trends seen in the adhesion were similar to those seen in aggregation. However, the adhesion is not simply settling platelets but an active process, as demonstrated by aspirin and clopidogrel significantly inhibiting adhesion induced by ristocetin and not “aggregation” to ristocetin which is predominantly an agglutination in which platelets are essentially inactive (40). Interestingly, following exposure to clopidogrel, adhesion in response to the TP receptor ligand U46619, demonstrated a ROC area of 1.0, whereas aggregation induced by U46619 had a ROC area of 0.75 with broad confidence intervals. This identifies U46619-induced adhesion as another discriminatory test for clopidogrel, and also raises the potential of an involvement of ADP in driving adhesion following activation of TP receptors. Drawing these results together we can define for each inhibitor a characteristic profile that discriminates each from the other.

We therefore present tests using 96-well plate based aggregation and adhesion to identify and discriminate between aspirin and clopidogrel therapy in healthy volunteers. These tests generate large amounts of data that can be interrogated. In the experiments reported here we used a Tecan Sunrise spectrophotometer that allows measurements to be made every 15 seconds between shaking. However, in order to overcome the limitations of using a single model of plate reader, we have recently repeated our studies using a simple incubated plate shaker (data not shown). Using such a device, plates can be readily and cheaply incubated.

### What is known about this topic?
- Platelet reactivity testing, during therapy, has been shown to be associated with future events and could allow for the optimisation of therapy.
- Light transmission aggregometry is the current gold standard for platelet testing but cannot be used for routine clinical testing.
- There is little agreement among other platelet tests and there remains a clear clinical need for another standardised test.

### What does this paper add?
- This study demonstrates the ability of the 96-well plate based aggregation and adhesion method to detect and differentiate between stable aspirin and clopidogrel treatment.
- The assay can be used to test subjects or patients against a range of platelet agonists with rapidity and ease and should be considered for use in future clinical settings.
References