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Original article

Assessment of Cilostazol Inhibition Using Whole Blood Samples: Comparison of Three Platelet Function Tests

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Abstract: Introduction: Cilostazol inhibits phosphodiesterase, with resultant increase in intracellular cyclic AMP, leading to platelet inhibition, particularly in the presence of prostaglandin E_1 (PGE_1). This study aimed to establish a cilostazol monitoring assay, using whole blood samples.
Methods: Platelet aggregation in the presence or absence of indicated concentrations of PGE_1 were assessed using VerifyNow®, Multiplate® and Total Thrombus-formation Analysis System (T-TAS®).
Results: In the presence of added PGE_1, cilostazol inhibited *in vitro* platelet aggregation of VerifyNow, with the aspirin test by 19% and the IIb/IIIa test by 44%, respectively. After a single oral uptake of cilostazol in healthy volunteers, cilostazol decreased platelet aggregation, with the IIb/IIIa test by 46% and with the P2Y12 test by 24%, respectively. Multiplate or T-TAS failed to detect cilostazol efficacy both *in vitro* and *ex vivo*. VerifyNow IIb/IIIa tests were used to monitor cilostazol efficacy on cerebral infarction patients. Compared with pre-therapy blood samples, those after cilostazol uptake showed significant inhibition of platelet aggregation in the presence of 3 nM (37%) and 10 nM PGE_1 (69%).
Conclusion: The IIb/IIIa tests of VerifyNow in the presence of 10 nM PGE_1 is the most suitable tool for monitoring assessing cilostazol.

Key Words: cilostazol, whole blood platelet function test, prostaglandin E_1, anti-platelet therapy

INTRODUCTION

Anti-platelet therapies are considered to be the mainstream strategy for the prevention of heart attack and stroke, which are caused by arterial thrombosis. Recently, there is an increasing body of evidence to suggest that high on-treatment platelet reactivity, which is often termed as "resistance to anti-platelet agents", leads to an increased rate of vascular events. On the other hand, intensified anti-platelet therapies may increase the risk of bleeding, and therefore platelet function monitoring during anti-platelet therapy is increasingly applied to patients at high risk of re-thrombosis or bleeding[1)]. With regards to

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aspirin and ADP receptor blockers such as clopidogrel, there have been a large number of reports which described various monitoring methods to detect "resistance". On the other hand, there has been few reports on the monitoring method for cilostazol. Cilostazol is an inhibitor of phosphodiesterase 3 (PDE3) which degrades cyclic AMP (cAMP) with resultant increase in the intracellular cAMP concentration[2]. Since cAMP negatively regulates various signal transduction pathways that lead to platelet activation, cilostazol therapy could be termed as a 'signal sedation therapy'[3]. TASC II international guidelines recommend cilostazol as the frontline drug for peripheral arterial disease treatment, based on the lines of evidence that it improves the various symptoms caused by ischemia[4]. Cilostazol also reduces the risk of stroke[5], and the CSPS2 trial indicates that cilostazol is non-inferior, possibly superior to aspirin for the prevention of secondary strokes with fewer hemorrhagic events[6].

We have previously reported that the addition of low concentrations of prostaglandin E_1 (PGE_1) increases the inhibitory effects of cilostazol on platelet aggregation assessed with the conventional optical density method[3]. We suggest that this method for monitoring cilostazol can be useful for estimating the efficacy of cilostazol on platelet aggregation *ex vivo*; however, the conventional optical density method necessitates high technical skill and complicated procedures for sampling, which precludes its use in clinical setting. More easy-to-use, point of care tests (POCT) for platelet function are desirable for clinical application. In this study, applying this PGE_1 method, we evaluated the efficacy for monitoring cilostazol with three POCT platelet function tests which use whole blood samples.

MATERIALS AND METHODS

This study was conducted in compliance with the Declaration of Helsinki and was approved by the ethics committee of the Faculty of Medicine, University of Yamanashi, Japan. Informed consent was obtained from all patients and healthy volunteers prior to their participation in the experimental procedures.

Materials

Arachidonic acid sodium salt (Sigma Aldrich Corporation, MO, USA) and PGE_1 (Cayman Chemical Company, MI, USA) were purchased from the manufacturers. Cilostazol (OPC-13013) was kindly gifted by Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). The test cassettes, VerifyNow® aspirin, VerifyNow IIb/IIIa and P2Y12, were purchased from Accumetrics (CA, USA). The ADPtest, ASPtest, TRAPtest and ADPtestHS were obtained from the manufacturer (Dynabyte Medical, Munich, Germany). Platelet thrombus formation by T-TAS was evaluated with type 1 collagen-coated platelet (PL)-chips (Nitta Gelatin, Osaka, Japan).

Blood sampling

Blood samples were taken from the antecubital veins with vacutainer tubes containing 3.13% trisodium citrate (9:1, v/v) (Nipro, Osaka, Japan) and hirudin (20 μg/ml final concentration, Verum Diagnostica GmbH, Munich, Germany). The tubes were then kept standing at room temperature for 1 h without agitation.

Measurements of platelet function

a) VerifyNow®

Platelet aggregation in whole blood samples was assessed using VerifyNow (Accumetrics, CA, USA), which uses fibrinogen coated beads for the assessment of platelet aggregate formation[7].

The measurement procedure were performed according to the instructions of the manufacturer, with some modifications. Briefly, citrate-treated blood in the vacutainer tube was incubated with or without 3 μM of cilostazol solution for 2 min, and then the sample was further incubated with or without the specified PGE_1 concentration for 2 min at 37°C. The vacutainer tube was then inserted into either the VerifyNow Aspirin (which uses arachidonic acid), P2Y12 (which uses adenosine diphosphate [ADP] and PGE_1) or IIb/IIIa test cassette (which used TRAP, a thrombin receptor agonist peptides), and platelet aggregation was automatically analysed with the device. Parameters in each of these test cassettes that reflect overall platelet activity are aspirin reaction units (ARU), P2Y12 reaction units (PRU), and platelet aggregation units (PAU), respectively. The relevant parameter was displayed by the device in units, and all measurements were completed within 3 h following blood collection. For the *ex vivo* study, incubation with cilostazol was not performed.

b) Multiplate®

Platelet aggregation induced by agonists in whole blood was assessed using Multiplate (Dynabyte, Munich, Germany), which is based on impedance aggregometry[8]. The measurement procedure was performed following the instructions of the manufacturer, with some modifications. Briefly, 300 μL of hirudin-treated blood was added to 300 μL saline (0.9%) and preheated to 37°C in the test cell for 3 min. Diluted blood was then incubated with or without 3 μM of cilostazol for 2 min, and the blood was further incubated with or without PGE_1 (9.4 nM) for 2 min at 37°C. Finally, platelet aggregation was induced using arachidonic acid (0.5 mM, ASPtest), ADP (6.5 μM, ADPtest) or TRAP (32.2 μM, TRAPtest). Concentrations of reagents were used in accordance with the recommendations of the manufacturer. The parameter that reflects overall platelet activity is the area under the aggregation curve (AUCa), displayed by the device in units. All measurements were completed within 3 h after blood collection, and for the *ex vivo* study, incubation with cilostazol was not performed.

c) Total thrombus-formation analysis system (T-TAS®)

The microchip flow-chamber system T-TAS (Fujimori Kogyo, Kanagawa, Japan) was used to analyze flow-based thrombus formation[9]. Measurements of platelet thrombus formation were performed using type I collagen-coated PL-chips (Nitta Gelatin, Osaka, Japan). Hirudin-treated blood samples were placed in a reservoir connected to a precision pump which pushed the blood through an inlet port into a flow path. Thrombus formation in the flow path generated a back pressure that was monitored by a flow-pressure sensor located between the pump and blood reservoir. Thus, a flow-pressure curve that plots pressure against time creates a reflection of the process of thrombus formation in the flow path in the PL-chip.

Hirudin-treated whole blood was incubated with or without 3 μM cilostazol for 2 min at 37°C. The blood was perfused at flow rates of 12 and 24 μL min^{-1}, corresponding to initial shear rates of 1000 and 2000 s^{-1}, respectively, and platelet thrombus formation on the collagen-coated surface of PL-chips was quantitatively assessed as the area under the flow-pressure curve (AUCp) at a pressure less than 80 kPa. All measurements were completed within 3 h after blood collection, and for the *ex vivo* study, incubation with cilostazol was not performed.

Ex vivo experimental procedure

In the single dose experiment, healthy individuals who took no medication within the past 14 days and patients with cerebral infarctions

who had no anti-platelet therapy within the past 14 days were given a single oral dose of 100 mg of cilostazol. Blood treated with citrate or hirudin was drawn 2 h after the intake of cilostazol.

In the sequential intake experiment, we selected the patients with cerebral infarctions (70.8 ± 9.8 years of age) who were given cilostazol (50–150 mg/day) for at least 2 weeks. No other anti-platelet agents were given to the patients. Blood was withdrawn 1.5 - 3.2 h (median 2.0 h) after the last cilostazol intake.

Statistical methods

Continuous variables are presented as mean ± SD. Data was analysed with Microsoft Excel 2007 for Windows 7 (Microsoft, WA, USA). Statistical evaluations used unpaired Student's *t*-tests, with a *P*-value < 0.05 considered statistically significant.

RESULTS

Assessment of in vitro effects of cilostazol on three whole blood platelet function tests in healthy volunteers

Based upon the previous reports, the plasma concentration of cilostazol reaches the level of approximately 3 μM, 2–4 hours following administration of 100 mg cilostazol[10, 11]. Thus, we first evaluated the effects of PGE_1 on platelet aggregate formation assessed by three whole blood platelet function tests in the presence of 3 μM of cilostazol (*in vitro* study).

As platelet agonists were packed into each VerifyNow test cassette, we could not add cilostazol or PGE_1 into the test device reaction chamber. Therefore, citrated-whole blood samples in a vacutainer tube were first preincubated with or without 3 μM cilostazol for 2 min, followed by another 2 min incubation with PGE_1, then and platelet aggregation measurement was started. The aspirin test without PGE_1 show no difference in platelet aggregation in the absence (644 ± 20 ARU) or presence (652 ± 22 ARU) of 3 μM cilostazol (Fig. 1A). In contrast, there was a significant difference between the absence (608 ± 45 ARU) and presence (495 ± 40 ARU) of cilostazol when 30 nM PGE_1 was added (Fig. 1A). These findings confirm our previous hypothesis that an increase of intracellular cAMP in platelets induced by low dose of PGE_1 makes the inhibitory effect of cilostazol on platelet aggregation clearer and easier for the assessment.

With the IIb/IIIa test, preliminary experiments revealed that platelet aggregation is almost completely inhibited in the presence of 30 nM PGE_1 without cilostazol, and 3 nM PGE_1 with 3 μM cilostazol had no inhibitory effect on platelet aggregation (data not shown). We therefore used 10 nM PGE_1 for the assessment of cilostazol inhibition with the IIb/IIIa test. Without PGE_1, there is no difference in the absence (236 ± 12 PAU) or presence (237 ± 17 PAU) of 3 μM cilostazol (Fig. 1B). However, a significant difference is observed between the absence (213 ± 45 ARU) or presence (119 ± 54 ARU) of 3 μM cilostazol with 10 nM PGE_1 (Fig. 1B).

The P2Y12 test is useful for the assessment of anti-platelet drugs that inhibit $P2Y_{12}$ receptors (e.g. clopidogrel and ticagrelor). Platelets have two ADP receptors, $P2Y_1$ and $P2Y_{12}$. Since $P2Y_1$ receptor-dependent signals are more sensitive to cAMP inhibition than $P2Y_{12}$ receptor-dependent signals, 22 nM PGE_1 is added to the reaction chamber of the P2Y12 test to exclude the involvement of $P2Y_1$[12]. We expected that the P2Y12 test was a useful tool for detecting cilostazol inhibition, since 22 nM PGE_1 was prepacked within the test cassette. However, to our surprise, there was no difference in platelet aggregation between the absence (292 ± 9 PRU) and presence (280 ± 21 PRU) of 3 μM

cilostazol (Fig. 1C). We assumed that 22 nM PGE_1 might not have been sufficient for blocking ADP-induced platelet activation even in the presence of cilostazol, and hence 30 nM PGE_1 was additionally mixed with citrated whole blood samples. However, even with additional 30 nM PGE_1, there was no difference between the absence (263 ± 16 PRU) and presence (239 ± 25 PRU) of 3 μM cilostazol (Fig. 1C). We also evaluated the effect of additional 100 nM PGE_1. Platelet aggregation was inhibited by 30~40% in the presence of additional 100 nM PGE_1, and when cilostazol was added in addition to 100 nM PGE_1, error messages were sent out from VerifyNow. Taken together, these findings suggest that the aspirin and IIb/IIIa tests may be more suitable than P2Y12 test for detecting cilostazol inhibition as measured *in vitro* by VerifyNow.

Multiplate enables rapid verification of drug-induced and disease-related platelet disorders, and its predictivity has been validated in clinical studies[13, 14]. The manufacturer recommended the addition of 9.4 nM PGE_1 to the samples, and we followed the instruction. The presence of 9.4 nM PGE_1 inhibited arachidonic acid- (Fig. 1D), TRAP- (Fig. 1E), or ADP-induced whole blood platelet aggregation (Fig. 1F), by 28%, 14%, and 36%, respectively. However, the co-presence of 3 μM cilostazol brought significant inhibition in none of the tests, suggesting the Multiplate may not be suitable in assessing the effects of cilostazol. Higher concentrations of PGE_1 were not evaluated, since 9.4 nM PGE_1 alone already had considerable inhibition.

T-TAS is a new analyzer for quantitative estimation of thrombus formation under flow conditions[9]. Because thrombus formation at a shear rate less than 1000 s^{-1} was almost completely inhibited by 30 nM PGE_1 in the absence of cilostazol (192 ± 33 AUC_p vs. 3 ± 3 AUC_p), we assumed that PGE_1 in the range of 3–10 nM is suitable for monitoring the effects of cilostazol (Fig. 1G). However, 3 μM cilostazol brought no further inhibition on thrombus formation in the presence of 3–10 nM PGE_1 (Fig. 1G), using a 1000 s^{-1} shear rate. Even under the higher shear rate of 2000 s^{-1}, the additional effect of 3 μM cilostazol was not observed (Fig. 1H). These data suggest that thrombus formation by T-TAS does not correctly reflect the inhibitory effects of cilostazol *in vitro*.

The results of *in vitro* studies using three whole-blood measurement of platelet aggregation suggest that VerifyNow is suitable for monitoring cilostazol.

Assessment of ex vivo effects of a single oral uptake of 100 mg cilostazol on three whole blood platelet function tests in healthy volunteers

Results of *in vitro* studies were validated by *ex vivo* after a single oral uptake of 100 mg cilostazol in healthy volunteers.

With VerifyNow, as assessed by the IIb/IIIa and P2Y12 tests, platelet aggregation was significantly decreased by 46% (before the uptake of cilostazol 125 ± 53.2 PAU and after the uptake 67 ± 44 PAU) and 24% (before: 233 ± 34 PRU and after: 178 ± 46 PRU), respectively (Fig. 2B, 2C). On the other hand, there was no inhibitory effect of cilostazol with the aspirin test as measured by VerifyNow (Fig. 2A). Thus, there are discrepancies between *in vitro* and *ex vivo* studies; *in vitro* studies, the aspirin test and GPIIb/IIIa test gave positive inhibitory effects, but not the P2Y12 test, and in contrast, in *ex vivo* studies, the IIb/IIIa test and P2Y12 test showed the positive inhibitory effect of cilostazol, but not the aspirin test. We have no reasonable explanation for these discrepancies. However, it is to be noted that the IIb/IIIa test showed the consistent, and clear inhibitory effect of cilostazol.

With Multiplate, a single dose cilostazol had

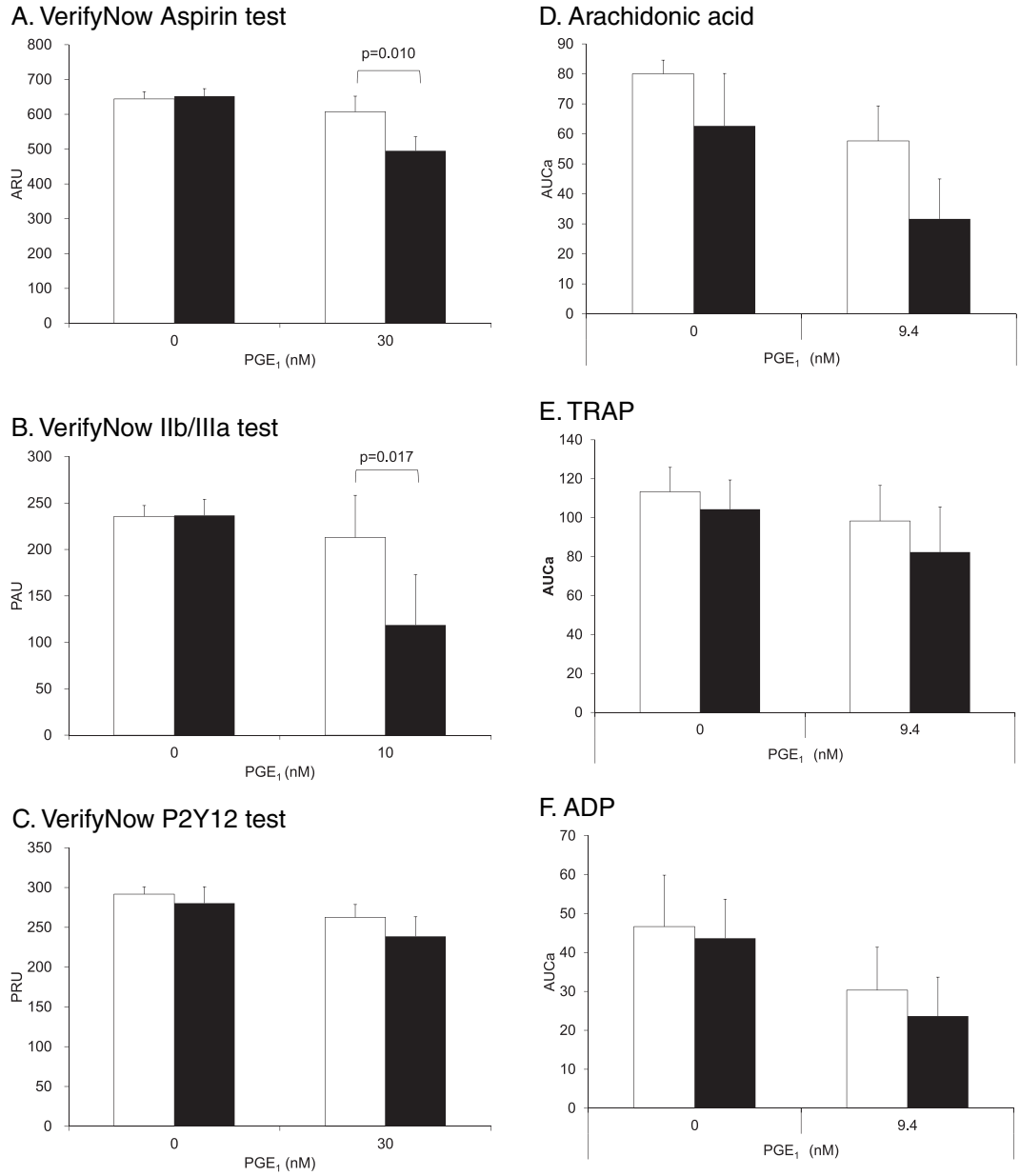

Fig. 1. Assessment of *in vitro* effects of cilostazol using VerifyNow, Multiplate and T-TAS in healthy volunteers.
VerifyNow: Citrate-treated whole blood was incubated without (white column) or with 3 μM (black column) cilostazol for 2 min at 37°C and then further incubated without or with indicated concentrations of PGE_1 for 2 min at 37°C. The vacutainer tube was inserted into the VerifyNow Aspirin (A), IIb/IIIa (B) and P2Y12 tests (C).
Multiplate: Hirudin-treated whole blood was diluted with 300 μL saline preheated to 37°C in the test cell of Multiplate for 3 min. The diluted blood was incubated without (white column) or with 3 μM cilostazol (black column) for 2 min at 37°C and then incubated without or with 9.4 nM PGE_1 (ADPtestHS) for 2 min at 37°C. Platelet aggregation was induced by 0.5 mM arachidonic acid (D), 32.2 μM TRAP (E) and 6.5 μM ADP (F).
The results are expressed as mean ± SD (n = 4–5).

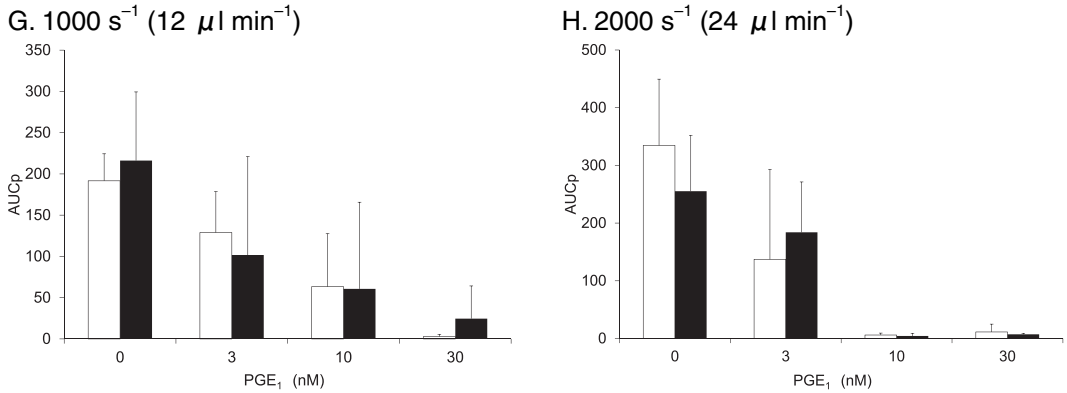

Fig. 1. Assessment of *in vitro* effects of cilostazol using VerifyNow, Multiplate and T-TAS in healthy volunteers. T-TAS: Hirudin-treated whole blood was incubated without (white column) or with 3 μM (black column) cilostazol for 2 min at 37°C, then incubated without or with indicated concentrations of PGE_1 for 2 min at 37°C. The blood was perfused into a PL-chip at flow rates of 12 μL min^{-1} (G) and 24 μL min^{-1} (H). The thrombus formation was quantitatively assessed using flow pressure.
The results are expressed as mean ± SD (n = 4–5).

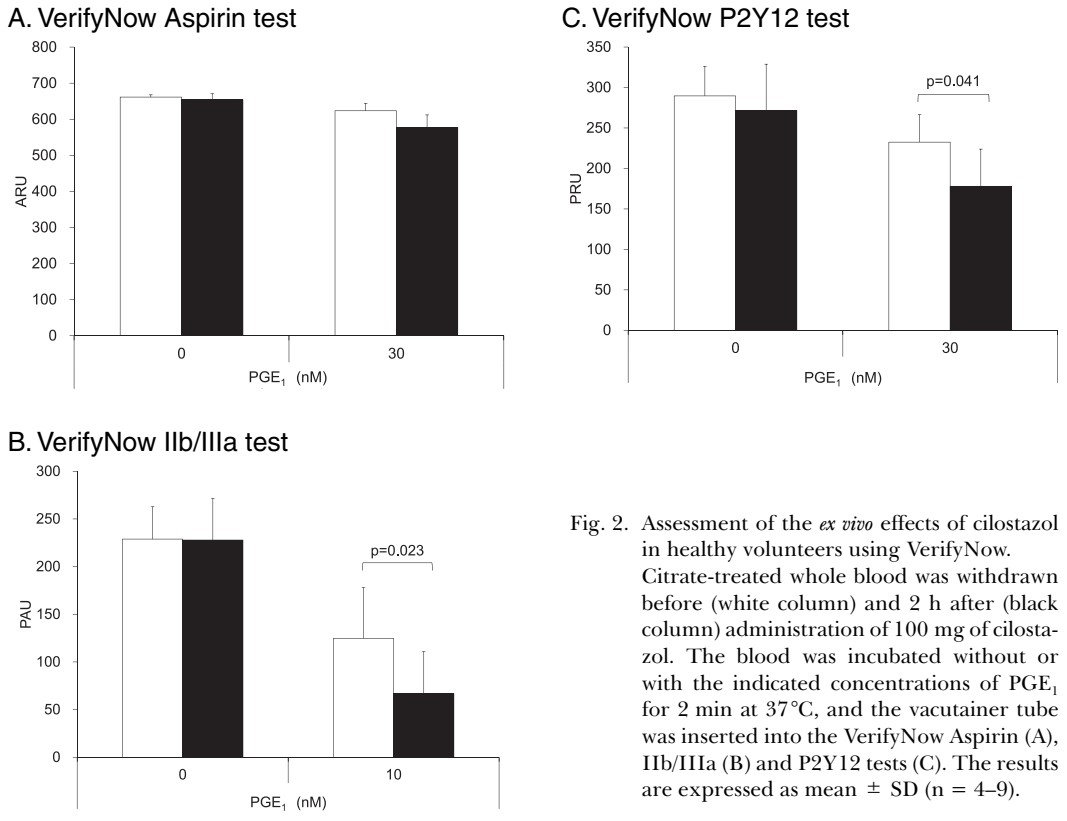

Fig. 2. Assessment of the *ex vivo* effects of cilostazol in healthy volunteers using VerifyNow.
Citrate-treated whole blood was withdrawn before (white column) and 2 h after (black column) administration of 100 mg of cilostazol. The blood was incubated without or with the indicated concentrations of PGE_1 for 2 min at 37°C, and the vacutainer tube was inserted into the VerifyNow Aspirin (A), IIb/IIIa (B) and P2Y12 tests (C). The results are expressed as mean ± SD (n = 4–9).

no inhibitory effect on arachidonic acid-, TRAP-, or ADP-induced platelet aggregation in the presence of 9.4 nM PGE_1 (data not shown). With T-TAS, under 1000 s^{-1} and 2000 s^{-1} shear rates, thrombus formation was not inhibited after cilostazol administration in the presence of 3, 10, or 30 nM PGE_1 (data not shown).

Results from *in vitro* and *ex vivo* studies suggest that VerifyNow, particularly using the IIb/IIIa test, is the most useful for monitoring the effects of cilostazol.

Assessment of ex vivo effects of cilostazol in patients under cilostazol therapy with the IIb/IIIa test by VerifyNow

Next, we conducted an experiment in patients with cerebral infarctions. Blood was withdrawn from these patients to assess pre-dosing platelet function before cilostazol administration. Only the patients were selected who had not been on medication with any anti-platelet agents. Patients were treated with 50–150 mg of cilostazol/day over 2 weeks without any other anti-platelet drugs, and blood was withdrawn 1.5–3.2 h after the last cilostazol ingestion for assessment of post-dosing platelet function, using the IIb/IIIa test. Without PGE_1, there was no difference in platelet function assessed with the IIb/IIIa test between the pre- and post-dosing blood samples (221 ± 37 PAU and 238 ± 56 PAU, respectively) (Fig. 3). In contrast, platelet aggregation in the post-dosing blood samples was significantly inhibited in the presence of 3 nM PGE_1 (37% inhibition) and 10 nM PGE_1 (69% inhibition), compared with those in the pre-dosing blood samples, (241 ± 55 PAU) and (192 ± 55 PAU), respectively. These results suggest that platelet aggregation, assessed with the IIb/IIIa test by VerifyNow in the presence of 10 nM PGE_1 is the most suitable tool for cilostazol monitoring.

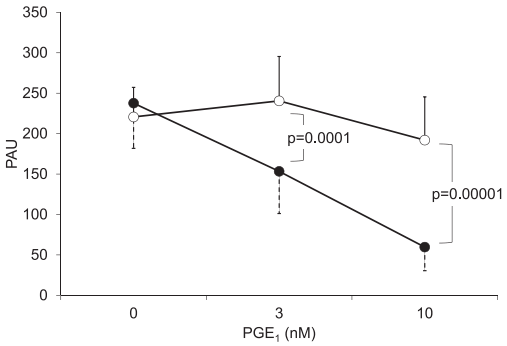

Fig. 3. Assessment of the *ex vivo* effects of cilostazol in patients with cerebral infarction using the IIb/IIIa test of VerifyNow.
Citrate-treated whole blood was withdrawn before (white dot) and 2 h after (black dot) the administration of 50-150 mg/day of cilostazol. Blood was incubated without, with 3 nM, or with 10 nM PGE_1 for 2 min at 37°C, and the vacutainer tube was inserted into the VerifyNow IIb/IIIa test. All results are expressed as mean ± SD (n = 26).

DISCUSSION

We have previously reported that adding low concentrations of PGE_1 increases the inhibitory effects of cilostazol on platelet aggregation, as measured by the conventional optical density method[3]. However, this method requires high technical skill and cumbersome processes of sample preparation. Thus, we sought to investigate whether platelet function tests using whole blood samples could also evaluate the efficacy of cilostazol, when low concentrations of PGE_1 were added to the samples.

Out of three platelet function tests which use whole blood samples, we found VerifyNow was the most suitable for the detection of cilostazol inhibition in the presence of low concentrations of PGE_1. Three types of test devices are available for VerifyNow, namely the aspirin test, the IIb/IIIa test, and the P2Y12 test. These test devices contains as an agonist in the reaction cham-

ber, arachidonic acid, TRAP, or ADP (as well as PGE_1), respectively. While these tests could not detect the inhibitory effects of cilostazol, by themselves, the addition of low concentrations of PGE_1 to the blood samples made it possible to detect the inhibitory effect of cilostazol *in vitro* with the aspirin test and the IIb/IIIa test (Fig. 1A–1C).

On the other hand, in the *ex vivo* studies after a single oral uptake of cilostazol, the IIb/IIIa test and P2Y12 test could detect the positive inhibitory effect of cilostazol, but not the aspirin test. Thus, although the IIb/IIIa test gave consistent results in *ex vivo* studies as well as in *in vitro* studies, there are discrepancies between *in vitro* and *ex vivo* studies with the aspirin test and the P2Y12 test. We have no reasonable explanation for these discrepancies. Since the IIb/IIIa test showed the consistent, and clear inhibitory effect of cilostazol in both *in vitro* and *ex vivo* studies, this test device was used to evaluate the effects of cilostazol in stroke patients. The *ex vivo* inhibitory effect of cilostazol on platelet aggregation was clearly detected with low concentrations of PGE_1 added to the whole blood sample before VerifyNow measurement (Fig. 3).

Our findings suggest that the IIb/IIIa test device which uses TRAP as platelet agonist is more suitable for the assessment of cilostazol inhibition, compared with other test devices which use arachidonic acid or ADP for inducing platelet aggregation. In the previous report[2], we found that the addition of low concentrations of PGE_1 results in an increase in the basal level of cAMP, which serves to amplify the inhibitory effect of cilostazol on PDE3, with resultant inhibition of various aspects of platelet activation. These reactions affected by increased cAMP levels include early activatory signals, such as the release of Ca^{2+} from the intracellular stores, granule release, adhesion and aggregation, and a number of intracellular signalling molecules, such as Rap1b, RhoA and the inositol 1, 4, 5-triphosphate receptor[15]. However, these features are common to TRAP-, ADP- and arachidonic acid-induced platelet activation, which does not provide a clue to explain why TRAP-induced platelet activation is superior to ADP and arachidonic acid-induced one for evaluating the efficacy of cilostazol.

One feature of thrombin receptors on platelets needs attention. Human platelets have two thrombin receptors, PAR1 and PAR4, and PAR1 plays a major role in activating platelets. An increased level of cAMP inhibits thrombin from binding to its receptor PARs most probably because of a reduction in the number of PAR expressions on the platelet membrane[16]. Since TRAP binds to PAR1 on the platelet membrane, TRAP-induced platelet aggregation would be most sensitively modified by a decrease in the number of platelet PAR1, which is induced by cAMP levels enhanced by cilostazol. That the effect of cilostazol was more remarkable (69%) with repetitive uptake of cilostazol than with single oral uptake of cilostazol (46%) (Fig. 3 and Fig. 2B) could be well explained with longer exposure of platelets to increased cAMP levels induced by repetitive cilostazol uptake. The measurement of PAR1 on the platelet membrane after cilostazol uptake could be our next challenge. It is also conceivable that the intracytoplasmic accumulation of cilostazol after repetitive administration increases the inhibitory effect of cilostazol *ex vivo*. We have no good method to measure the intracellular concentration of cilostazol, and this also awaits future investigation.

Multiplate is based on the impedance method and has been used to monitor aspirin and $P2Y_{12}$ receptor inhibitors in whole blood samples[17]. However, up to date, there has been no report on the monitoring of cilostazol using Multiplate

ex vivo. There is a report on the anti-platelet effects of cilostazol on collagen-induced aggregation using Multiplate. A graded dose-dependent inhibition curve with an IC_{50} value of 75.4 ± 2.4 μM was obtained in an *in vitro* assay using rat blood [18]. As the plasma concentrations of cilostazol reached approximately 3 μM, 2~4 hours following administration of 100 mg of cilostazol with human [10, 11], it is evident that the conventional measurement methods of Multiplate cannot monitor the efficacy of cilostazol in clinical settings. Therefore, we attempted to modify the measurement method by adding low concentrations of PGE_1. However, since 9.4 nM PGE_1 in accordance with the instructions of the manufacturer already had considerable inhibition, and the addition of cilostazol had no further effect, we concluded that Multiplate is not suitable for cilostazol monitoring.

There is a report that shear-induced platelet aggregation (SIPA), measured by a cone plate type device, was inhibited in the presence of PGE_1 and cilostazol, while it was not inhibited by 10 nM PGE_1 alone [19]. Since a cone plate SIPA measurement requires the preparation of platelet-rich plasma, we sought to use T-TAS, which measures shear stress- and collagen-dependent platelet activation in whole blood. However, thrombus formation was not affected by 3 μM cilostazol, and 3 nM PGE_1 itself inhibited thrombus formation in the absence of cilostazol (Fig. 1G, 1H). Therefore, we could not monitor the effect of cilostazol in the presence of low concentrations of PGE_1, a method we propose to be suitable for monitoring cilostazol.

In conclusion, we found VerifyNow, especially the IIb/IIIa test device, was the most suitable for the detection of cilostazol inhibition in the presence of low concentrations of PGE_1, and this method can be applied in clinical settings.

Conflict of interest statement

K. Hosokawa and T. Ohnishi: Employees of the Fujimori Kogyo Co.

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Author contributions

MO and KS designed the study, analyzed the data, performed the statistical analyses and wrote the manuscript. IF recruited patients and collected samples. KH and TO contributed essential reagents and analysis tools. JN analyzed the data. YO reviewed and edited the manuscript. All authors approved the final version of the manuscript.

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