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Effects of cangrelor in coronary artery disease patients with and without diabetes mellitus: an in vitro pharmacodynamic investigation

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Abstract Platelets from patients with diabetes mellitus (DM) are hyper-reactive and whether cangrelor, a potent intravenous $P2Y_{12}$ receptor blocker, has differential pharmacodynamic (PD) effects according DM status is unknown. The aim of this investigation was to evaluate the in vitro PD effects of cangrelor in coronary artery disease (CAD) patients with and without DM. This prospective study enrolled 120 clopidogrel-naïve patients with CAD on aspirin therapy. PD assessments using cangrelor (500 nmol/l) in vitro included vasodilator-stimulated phosphoprotein assay to obtain the $P2Y_{12}$ reactivity index (PRI), and multiple electrode aggregometry (MEA). In a 20 patients subgroup, dose-dependent response was assessed following exposure to escalating concentrations (baseline, 5, 50, 500 and 5,000 nmol/l); thrombin generation processes were evaluated by thromboelastography (TEG). PD data were evaluable in 103 patients. No differences in baseline PD parameters were observed in DM ($n = 48$) and non-DM ($n = 45$) subjects. Cangrelor reduced PRI values irrespective of DM status ($p < 0.0001$), yielding no difference in patients with and without DM (16.1 ± 12.3 vs. 16.8 ± 11.3; $p = 0.346$). All MEA values were significantly reduced, although this was of greater magnitude with purinergic compared to non-purinergic agonists. A trend analysis showed a dose-dependent effect on platelet inhibition, with no interaction due to DM status, whereas no significant dose-dependent effect was observed for TEG-derived parameters. Therefore, in vitro cangrelor provides potent and dose-dependent blockade of the platelet $P2Y_{12}$ receptor, with no differential effect in DM and non-DM patients. In addition, in vitro cangrelor exerts moderate inhibitory effects on non-purinergic platelet signaling pathways, without modulating platelet-derived thrombin generation processes.

Keywords Cangrelor · Diabetes mellitus · Platelet inhibition · P2Y12 receptor · Antiplatelet agents

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Diabetes mellitus (DM) has been shown to be associated with impaired response to antiplatelet therapies, particularly to the $P2Y_{12}$ receptor antagonist clopidogrel [1–3]. These pharmacodynamic (PD) findings may contribute to the increased rates of adverse atherothrombotic events observed in DM patients compared with non-DM subjects [4, 5]. Several metabolic and cellular abnormalities contribute to the hyper-reactive platelet phenotype observed in DM patients [6]. In particular, upregulation of $P2Y_{12}$ signaling has been postulated as a mechanism contributing to impaired clopidogrel response in DM patients [7]. Moreover, the functional status of the $P2Y_{12}$ signaling pathway has also been shown to be associated with platelet-derived thrombin generation [8–10], which is also increased in DM patients and thus contribute to their pro-thrombotic status [5, 11]. Overall, these findings underscore the need for more potent $P2Y_{12}$ receptor inhibiting strategies in patients with DM.

Cangrelor is a novel intravenous $P2Y_{12}$ receptor blocker under advanced clinical investigation characterized by a very rapid onset and offset of action (12). Cangrelor

directly, without need for metabolic biotransformation, and reversibly inhibits in a dose-dependent manner the $P2Y_{12}$ receptor, achieving very potent (>90 %) platelet inhibition [12–14]. However, the PD effects of cangrelor in DM and non-DM platelets remain unexplored. Further, if cangrelor can exert additional PD effects other than $P2Y_{12}$ blockade, such as modulating other platelet signaling pathways or thrombin generation processes, is unknown. The present manuscript describes the results of in vitro investigations aimed to provide these insights on the PD effects of cangrelor.

Methods

Subject population and study design

This was a prospective in vitro investigation conducted in patients with stable coronary artery disease. All patients were between 18 and 75 years of age, on maintenance aspirin therapy (81 mg daily), and naïve to treatment with $P2Y_{12}$ receptor inhibitors for at least 30 days prior to inclusion. Patients were classified as having type 2 DM according to criteria from the World Health Organization Report [15]. Patients on any anticoagulant or antiplatelet medication, other than aspirin, within the past 30 days were not eligible for the study. The study had a parallel design in which PD assessments to assess purinergic and non-purinergic mediated signaling were performed at baseline and after in vitro incubation with cangrelor. PD assessments included vasodilator-stimulated phosphoprotein (VASP) and multiple electrode aggregometry (MEA). Cangrelor at a final concentration of 500 nmol/l was chosen for in vitro incubation in line with prior investigations as it approximates that of the mean steady-state plasma concentration of 484 nmol/l at the infusion dose of 4 μg/kg/min, which is also the dose used in large-scale phase III clinical trial investigations [13, 16, 17]. PD assessments were performed in blood samples from 120 patients with and without DM. In a subgroup of patients (n = 20), an escalating concentration range of cangrelor (5, 50, 500 and 5,000 nmol/l) was used with the purpose of investigating the presence of a dose-dependent effect of cangrelor on purinergic and non-purinergic mediated platelet signaling; in addition to VASP and MEA, thrombin-generation processes assessed by thromboelastography (TEG) were also evaluated. This subgroup of patients enrolled to measure the dose-dependent effects of cangrelor represented the last 20 consecutive patients from the overall study cohort with analyzable blood samples.

Patients were screened at the Division of Cardiology of the Shands Jacksonville Hospital-University of Florida College of Medicine. The study complied with the Declaration of Helsinki and was approved by the Institutional Review Board of the University of Florida College of Medicine-Jacksonville. All subjects provided written informed consent.

Sample collection and platelet function assays

Blood samples were collected from an antecubital vein, discarding the first 2–4 ml of blood to avoid spontaneous platelet activation. Tubes were immediately incubated at 37 °C in a waterbath and cangrelor was added to the whole blood to reach the final concentrations desired and incubated for 5 min [14, 18]. The same procedure was followed with tubes used to perform baseline assessments, but without adding cangrelor. After incubation, samples were processed in parallel (all measurements of each assay at the same time) by trained laboratory personnel. Samples were processed within 2 h of blood drawing. PD assessments included flow cytometric analysis of the phosphorilation status of VASP, MEA and TEG.

VASP

The $P2Y_{12}$ reactivity index (PRI) was calculated as a measure of the functional status of the $P2Y_{12}$ signalling pathway. PRI was determined through assessment of phosphorylation status of vasodilator-stimulated phosphoprotein (VASP), a key and specific intraplatelet mediator of $P2Y_{12}$ signaling, according to standard protocols [19, 20]. In brief, VASP phosphorylation (VASP-P) was measured by quantitative flow cytometry using commercially available labelled monoclonal antibodies (Biocytex Inc., Marseille, France). The PRI was calculated after measuring the mean fluorescence intensity (MFI) of VASP-P levels following challenge with prostaglandin E_1 (PGE_1) and PGE_1 + adenosine diphosphate (ADP). PGE_1 increases VASP-P levels through stimulation of adenylate cyclase (AC); ADP binding to purinergic receptors leads to inhibition of AC; thus, the addition of ADP to PGE_1-stimulated platelets reduces levels of PGE_1-induced VASP-P. The PRI was calculated as follows: ([MFI PGE1] − [MFI PGE1 + ADP]/[MFI PGE1]) × 100 %. A reduced PRI is indicative of greater inhibition of the $P2Y_{12}$ signaling pathway. The relative decrease in platelet reactivity was defined as the percentage of inhibition of platelet aggregation and calculated as follows: (PRI value at baseline − PRI value after incubation with cangrelor 500nM) × 100/PRI value at baseline.

MEA

Blood was collected in hirudin-treated tubes. MEA was assessed in whole blood with the Multiplate analyzer (Dynabyte Medical, Munich, Germany) as previously described

[21, 22]. This instrument can perform up to five parallel aggregometry measurements assessing the change in impedance caused by the adhesion of platelets onto sensor units formed by silver-covered electrodes. Curves were recorded for 6 min and platelet aggregation was determined as area under the curve of arbitrary aggregation units (AU * min). The relative change in platelet aggregation was defined as the percentage of inhibition of platelet aggregation and calculated for each agonist as follows: (AU * min at baseline − AU * min after incubation with cangrelor 500 nM) × 100/AU * min at baseline. In the present investigation, the following 5 different agonists were used to assess for purinergic and non-purinergic mediated platelet signaling: (a) purinergic: 6.4 µmol/l ADP and 6.4 µmol/l ADP + 9.4 nmol/l PGE_1; and (b) non-purinergic: 0.5 mM arachidonic acid (AA), 32 µmol/l thrombin receptor activating peptide (TRAP), and 3.2 µg/ml collagen.

TEG

The Thrombelastograph® (TEG®) Hemostasis System (Haemoscope Corporation, Niles, IL, USA) equipped with automated software for the determination of the first derivative was used according to the manufacturer's instructions [8, 11]. Several parameters related to the rate of development of the tensile strength of the developing clot are derived from the first derivative of the waveform generated by the TEG system. In brief, TEG is a viscoelastic monitor that measures platelet–fibrin-mediated clot strength through a rotating sample cup with a stationary pin suspended by a torsion wire. The torque of the rotating cup is transmitted to the pin immersed in the blood sample and the movement of the pin, which depends of the contribution of platelets to the clot strength through platelet–fibrin binding, is transformed into an electrical signal generating a tracing. The reaction time (R), expressed in minutes, is a measure of time to initial thrombin induced platelet–fibrin clot formation and has been correlated with the velocity of thrombin generation [23]. The analytical software of the TEG system also allows use of the first derivative of the waveform generated by the system to determine the time to maximum rate of thrombin generation (TMRTG), also expressed in minutes. About 1 ml of heparinised blood is transferred to a vial containing kaolin and mixed by inversion. Afterwards, 500 µl of the activated blood is transferred to a vial containing heparinase and mixed to neutralize he heparin effect. The neutralised blood (360 µl) is immediately added to a heparinase-coated cup and assayed in the TEG analyser. Two TEG System devices were available, thus, up to four parallel measurements could be performed simultaneously.

Study endpoints and sample size calculation

The primary endpoint was the comparison of VASP-PRI values in DM and non-DM achieved after incubation with 500 nmol/l of cangrelor. Assuming that the standard deviation of the PRI is 10, we will be able to perform an equivalence analysis, being ± 6 % the limit of equivalence, with 80 % power and 2-sided alpha = 0.05 with 48 subjects per group. Considering an approximate dropout of 20 %, recruitment of up to 120 patients was allowed to ensure that complete data from 96 subjects was available for analysis. Other endpoints included the comparison of platelet function in DM versus non-DM patients with MEA using different stimuli, purinergic (ADP and ADP + PGE_1) and non-purinergic agonists (AA, TRAP, collagen). For the subgroup of 20 patients undergoing PD testing with escalating concentrations of cangrelor, the endpoints included: (a) evaluation of the dose-dependent effect achieved with escalating doses of cangrelor using VASP and MEA, investigating if DM status is an interaction factor; and (b) evaluation of the effect of escalating doses of cangrelor in platelet-derived thrombin generation processes measured with TEG.

Statistical analysis

For baseline characteristics, continuous variables are expressed as mean ± SD and categorical variables as frequencies and percentages. Normal distribution was evaluated for continuous variables with the Kolmogorov–Smirnov test. Comparisons of quantitative variables were made with non-paired Student's t test or Mann–Whitney's U test as appropriate, while qualitative variables were compared with Chi square test or Fisher's exact test (if expected value in any cell was fewer than 5). An ANCOVA method with a general linear model was used to evaluate the primary endpoint and all other between-groups comparisons, using as covariates the baseline value of the corresponding platelet function test, as well as unbalanced demographic or clinical variables ($p < 0.10$) in the univariate analysis. A repeated measures ANOVA model was used to evaluate intragroup comparisons, such as the comparison of functional assessments before and after cangrelor incubation, as well as the effect of escalating concentrations of cangrelor. In addition, p values for trend analyses to assess platelet reactivity with escalating doses of cangrelor were obtained using a polynomial contrast in the ANOVA method, considering concentration as a categorical variable with an ordinal scale. A two-tailed p value of less than 0.05 was considered to indicate a statistically significant difference for all the analyses performed. Results are reported as least squares mean (LSM) ± standard error of the mean (SEM) for the above detailed analyses. Statistical analysis was performed using SPSS version 16.0 software (SPSS Inc., Chicago, IL).

Results

Study population

A total of 470 patients were screened; of these, 218 refused to participate and 132 did not meet study inclusion criteria as they were not clopidogrel naïve or had been on other antithrombotic medications in the past 30 days. Therefore, a total of 120 patients were finally included in the study. A total of 17 samples were invalidated due to inability to measure platelet function for reasons including hemolysis, insufficient volume obtained or inaccurate processing of blood samples. Therefore, samples from a total of 103 patients (DM = 48; non-DM = 55) were available to assess the in vitro PD effects of a fixed concentration of cangrelor (500 nmol/l); in a subgroup of 20 patients (DM = 10; non-DM = 10) an escalating concentration range of cangrelor (5, 50, 500 and 5,000 nmol/l) was used. Baseline demographics and clinical characteristics of the overall study population are shown in Table 1. Among DM patients, HbA1c levels were 7.8 ± 2.2 and approximately half (n = 26; 54.2 %) were on insulin therapy. Baseline characteristics were overall well balanced between groups, with the exception of body mass index and creatinine concentration, which were higher among DM patients (Table 1) and were accordingly included in the statistical analyses as covariates.

Table 1 Baseline demographic data and clinical characteristics stratified according to diabetes mellitus status

	DM (n = 48)	Non-DM (n = 55)	*p* value
Age (years)	62.8 ± 9.4	62.5 ± 8.8	0.845
Male	28 (58.3 %)	40 (72.7 %)	0.124
BMI (kg/m^2)	33.3 ± 6.6	29.9 ± 6.2	0.012
Race			0.674
Caucasian	30 (62.5 %)	40 (72.7 %)	
Africanamerican	13 (27.1 %)	12 (21.8 %)	
Other	5 (10.4 %)	3 (6.5 %)	
Risk factors			
Current smoking	7 (14.5 %)	14 (27.3 %)	0.268
Hypertension	41 (91.1 %)	45 (81.8 %)	0.286
Dyslipidemia	41 (91.1 %)	45 (81.8 %)	0.286
Family history	28 (58.3 %)	31 (56.4 %)	0.793
Medical history			
Prior MI	24 (50.0 %)	30 (54.5 %)	0.680
Prior stroke	3 (6.25 %)	3 (5.5 %)	0.845
Prior PCI	28 (58.3 %)	28 (50.9 %)	0.293
Prior CABG	6 (12.5 %)	8 (14.5)	0.810
Symptomatic PAD	6 (12.5 %)	5 (9.1 %)	0.720
Multivessel CAD	31 (64.6 %)	31 (56.4 %)	0.462
Medical therapy			
Beta-blockers	39 (81.3 %)	40 (72.7 %)	0.420
ACEI/ARB	34 (70.8 %)	33 (60.0 %)	0.235
Nitrates	19 (39.6 %)	17 (30.9 %)	0.369
Calcium antagonists	18 (37.5 %)	16 (29.1 %)	0.269
Statins			0.699
CYP3A4 metabolism	37 (77.1 %)	39 (70.9 %)	
Non-CYP3A4 metabolism	5 (10.4 %)	8 (14.5 %)	
Proton-pump inhibitors			0.953
Omeprazole	8 (16.7 %)	10 (18.2 %)	
Other	15 (31.2 %)	19 (34.5 %)	
Oral antidiabetic agents	34 (70.8 %)	0 (0 %)	
Insulin	26 (54.2 %)	0 (0 %)	
Laboratory data			
Platelet count (10^3/mm^3)	225.0 ± 58.9	219.9 ± 59.3	0.432
Hematocrit (%)	40.2 ± 5.8	41.5 ± 4.4	0.145
Creatinine (g/dl)	1.3 ± 0.8	1.0 ± 0.3	<0.001

Values are expressed as mean ± SD or n (%)

ACEI/ARB angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, *BMI* body mass index, *CABG* coronary artery bypass graft, *CAD* coronary artery disease, *CYP* cythochrome P450, *DM* diabetes mellitus, *HbA1c* glycated hemoglobin A1c, *PAD* peripheral artery disease

In vitro PD effects of a fixed (500 nmol/l) cangrelor concentration

VASP-PRI

There were no statistical differences at baseline in PRI values between DM patients compared with non-DM subjects (84.3 ± 5.6 vs. 86.0 ± 3.8 %; $p = 0.072$). A significant reduction in VASP-PRI after in vitro incubation with 500 nmol/l of cangrelor was observed in the overall population, in whom there was a 80.6 ± 14.0 % relative reduction in PRI. This reduction was consistent in DM and non-DM patients ($p < 0.0001$ for both comparisons), with no difference in PRI values between groups (16.1 ± 12.3 vs. 16.8 ± 11.3; $p = 0.346$), as shown in Fig. 1a.

MEA

No differences in baseline values were found for all MEA measurements between DM and non-DM patients (Table 2). In the overall population, a marked decrease in platelet aggregation after in vitro incubation with 500 nmol/l of cangrelor was observed independently of the agonist used ($p < 0.0001$ for all comparisons, Table 2). When expressed as percentage of inhibition of platelet aggregation, the reduction of platelet reactivity was higher when using stimuli to assess purinergic mediated signaling (ADP and ADP + PGE_1) (Fig. 1b).

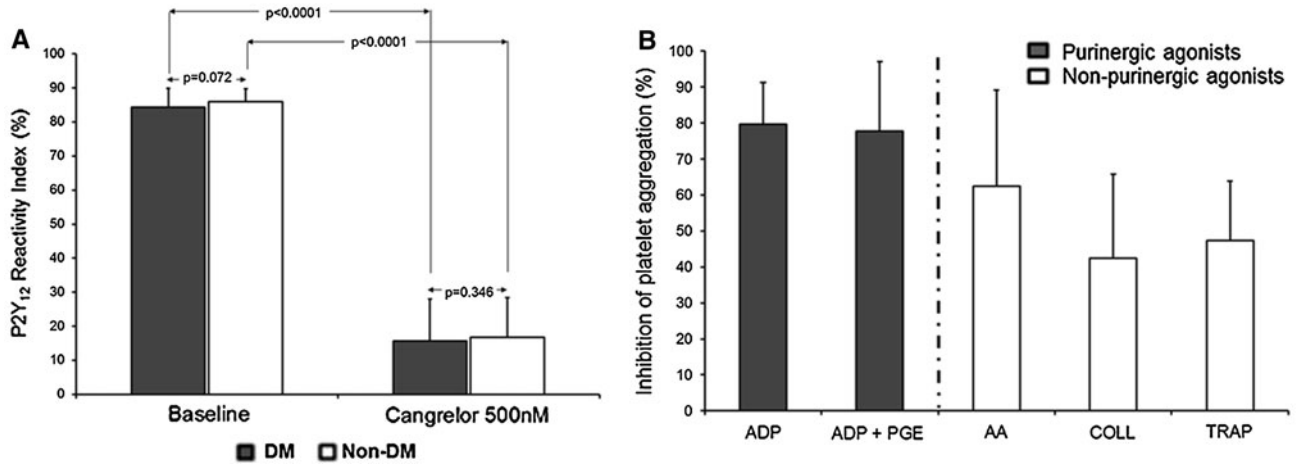

Fig. 1 Platelet function measurements at baseline and after in vitro incubation with cangrelor. **a** Platelet reactivity values according to DM status. **b** Relative reduction of platelet aggregation after in vitro incubation with cangrelor measured with multiple electrode aggregometry and using purinergic and non-purinergic stimuli. The percentage of inhibition of platelet aggregation, calculated as (AU * min at baseline—AU * min after incubation with cangrelor 500 nM) × 100/AU * min at baseline, is higher when using purinergic agonists that assess more specifically the $P2Y_{12}$ signalling pathway. *Values* are expressed as means and *error bars* indicate SD. *AA* arachidonic acid, *ADP* adenosine diphosphate, *COLL* collagen, *PGE* prostaglandin E_1, *TRAP* thrombin receptor activating peptide

Similarly to PRI, there were no significant differences in MEA measurements between DM and non-DM patients for all agonists (purinergic and non-purinergic) used (Table 2).

PD effects of escalating concentrations of cangrelor

VASP-PRI

Trend analysis showed a dose-dependent effect of escalating concentrations of cangrelor on PRI (expressed as LSM ± SEM): baseline: 86.1 ± 1.4 %; 5 nmol/l: 76.4 ± 2.5 %; 50 nmol/l: 48.7 ± 3.8 %; 500 nmol/l: 19.0 ± 3.2 %; 5,000 nmol/l: 9.5 ± 2.0 % (p for trend <0.0001). There was no interaction in this dose-dependent effect according to DM status (Fig. 2a).

MEA

PD results with MEA also showed a dose-dependent effect of cangrelor, irrespective of the agonist used (Fig. 2b). There was no interaction according to DM status for all MEA measurements (Table 3). In addition, no significant differences were observed at any cangrelor concentration between DM and non-DM patients, irrespective of agonists used to stimulate platelet aggregation (Table 3).

TEG

There were no significant differences in the R and TMRTG values at all concentrations of cangrelor ($p > 0.05$ for all between-concentrations comparisons). Accordingly, there was no significant trend for a dose-dependent effect

Table 2 Platelet reactivity values at baseline and after cangrelor incubation according to diabetes mellitus status measured by multiple electrode aggregometry using purinergic and non-purinergic agonists

Assay	Baseline			After cangrelor incubation		
	DM	Non-DM	p value	DM	Non-DM	p value
MEA ADP	633.6 ± 33.7	601.4 ± 31.5	0.976	116.9 ± 7.8	107.5 ± 7.3	0.408
MEA ADP + PGE	449.7 ± 30.7	416.3 ± 28.8	0.497	79.0 ± 8.0	76.5 ± 7.5	0.426
MEA AA	267.5 ± 39.3	269.2 ± 36.6	0.430	96.2 ± 13.7	76.6 ± 12.3	0.127
MEA TRAP	1,082.7 ± 42.9	1,070.0 ± 40.0	0.830	605.4 ± 36.9	544.2 ± 34.4	0.467
MEA COLL	477.2 ± 27.8	450.5 ± 26.0	0.484	251.3 ± 13.1	233.0 ± 12.3	0.365

MEA values are reported as area under the curve of arbitrary aggregation units (AU * min). Values are expressed as LSM ± SEM

AA arachidonic acid, *ADP* adenosine diphosphate, *COLL* collagen, *MEA* multiple electrode aggregometry, *PGE* prostaglandin E_1, *TRAP* thrombin receptor activating peptide, *DM* diabetes mellitus

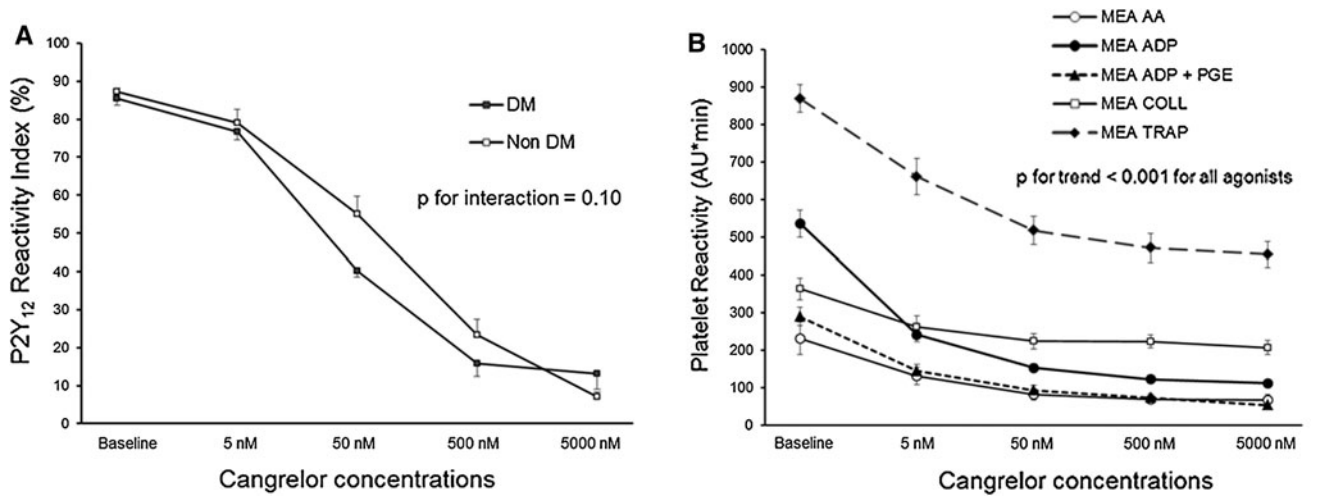

Fig. 2 Effects of escalating doses of cangrelor on: **a** Platelet reactivity index values according to DM status No interaction due to DM status was observed. *Values* are expressed as least standard means and *error bars* indicate SE of the mean. **b** Platelet reactivity measured by multiple electrode aggregometry using multiple agonists. *Values* are expressed as least standard means and *error bars* indicate SE of the mean. *AA* arachidonic acid, *ADP* adenosine diphosphate, *COLL* collagen, *MEA* multiple electrode aggregometry, *PGE* prostaglandin E_1, *TRAP* thrombin receptor activating peptide

Table 3 Platelet reactivity values achieved with increasing concentrations of cangrelor (in vitro incubation) according to diabetes mellitus status measured by multiple electrode aggregometry using purinergic and non-purinergic agonists

Assay	Baseline	Cangrelor 5 nM	Cangrelor 50 nM	Cangrelor 500 nM	Cangrelor 5,000 nM	*p* value for interaction
MEA AA						
DM	263.2 ± 77.8	144.1 ± 39.8	90.8 ± 23.9	74.0 ± 16.8	71.7 ± 24.9	0.509
Non-DM	199.4 ± 29.5	116.7 ± 21.7	71.0 ± 16.2	61.3 ± 13.6	61.3 ± 13.6	
p value	0.473	0.566	0.512	0.572	0.729	
MEA ADP						
DM	500.0 ± 51.1	216.9 ± 22.7	160.3 ± 14.6	135.7 ± 15.3	117.3 ± 13.0	0.645
Non-DM	571.9 ± 51.1	265.4 ± 30.1	144.8 ± 16.1	108.7 ± 17.0	104.7 ± 12.8	
p value	0.333	0.215	0.486	0.253	0.501	
MEA ADP + PGE						
DM	287.0 ± 35.3	134.7 ± 24.4	83.8 ± 18.4	62.8 ± 14.3	52.0 ± 15.6	0.610
Non-DM	292.2 ± 36.0	154.5 ± 22.2	102.1 ± 18.5	82.8 ± 15.9	54.5 ± 14.2	
p value	0.918	0.555	0.490	0.362	0.905	
MEA TRAP						
DM	891.4 ± 42.5	672.6 ± 52.5	530.1 ± 42.0	491.6 ± 48.2	459.8 ± 42.5	0.683
Non-DM	847.0 ± 61.0	648.8 ± 82.0	504.8 ± 61.2	450.7 ± 60.2	448.1 ± 52.7	
p value	0.558	0.809	0.737	0.603	0.865	
MEA COLL						
DM	386.9 ± 43.7	253.5 ± 28.7	219.2 ± 18.9	225.1 ± 19.2	205.4 ± 22.6	0.914
Non-DM	339.7 ± 38.0	270.7 ± 50.3	227.8 ± 37.0	221.5 ± 29.1	207.8 ± 29.0	
p value	0.425	0.770	0.837	0.919	0.948	

MEA values are reported as area under the curve of arbitrary aggregation units (AU * min). Values are expressed as LSM ± SEM

AA arachidonic acid, *ADP* adenosine diphosphate, *COLL* collagen, *MEA* multiple electrode aggregometry, *PGE* prostaglandin E_1, *TRAP* thrombin receptor activating peptide, *DM* diabetes mellitus

observed for any of these TEG-derived thrombin generation parameters. Similar results were obtained when evaluating DM and non-DM subjects separately (Table 4). Additionally, no significant differences were observed between DM and non-DM patients at any cangrelor concentration.

Discussion

Cangrelor is a novel intravenous $P2Y_{12}$ receptor inhibitor. In particular, it is an intravenous ATP analog, which reversibly and directly, thus, not needing any biotransformation, inhibits the $P2Y_{12}$ receptor [12]. It is able to achieve very potent (>90 %) platelet inhibition, with immediate onset of action and because of its ultra-short half-life (3–6 min), it has a very rapid offset of action with return to baseline platelet function within 30–60 min [13, 14]. In the present investigation we performed very comprehensive in vitro assessments to further elucidate the PD effects of cangrelor in patients with CAD, expanding upon prior studies by evaluating the impact of DM status on these findings. Our in vitro PD investigation showed that: (1) cangrelor potency is not affected by DM status; (2) cangrelor provides a potent and dose-dependent inhibition of the $P2Y_{12}$ receptor, as well as a moderate effect on other platelet signaling pathways; and (3) escalating concentrations of cangrelor do not modify platelet-derived thrombin generation processes.

Patients with DM have been shown to have impaired response to clopidogrel [1–3], the most commonly utilized $P2Y_{12}$ receptor inhibitor, which may contribute to their increased risk of ischemic recurrences, including stent thrombosis, compared with non-DM patients [4, 5]. This may in part be attributed to upregulation of $P2Y_{12}$ mediated signaling in these patients [7], underscoring the need for more potent $P2Y_{12}$ inhibiting strategies. The results of the present study showed that cangrelor achieves a great degree of platelet inhibition irrespective of DM status, which suggests that very potent $P2Y_{12}$ blockade may overcome the hyper-reactive platelet phenotype which characterizes DM patients [5]. This may contribute to the favorable outcomes in DM patients observed with the novel oral $P2Y_{12}$ receptor inhibitors, prasugrel and ticagrelor, which are characterized by more potent PD effects compared to clopidogrel [24–26]. In fact, although studies specifically assessing the PD effects in patients with DM have been conducted only with prasugrel [27], both ticagrelor and prasugrel have been associated with better ischemic outcomes compared with clopidogrel in patients with acute coronary syndromes (ACS) with DM [25, 26]. Indeed, cangrelor represents a potentially promising agent for clinical practice, and this underscores the need for a comprehensive understanding of the PD effects of this drug, particularly in high-risk patients, such as patients with DM. This is the first study evaluating the PD effects of a therapeutic concentration of cangrelor on several platelet signaling pathways other than the $P2Y_{12}$ receptor, the specific target of cangrelor. A marked decrease in platelet inhibition when using non-purinergic agonists to stimulate platelets was observed. Therefore, the findings of our study suggest that strong blockade of $P2Y_{12}$ mediated platelet activation may have an impact on other signaling pathways. This interplay between $P2Y_{12}$ receptor mediated signaling and other platelet activation signaling pathways has been reported previously [9, 10, 28–30]. In fact, our results are in line with those from a previous investigation that observed a reduction in platelet aggregation, in a concentration-dependent manner, after in vitro incubation with two potent $P2Y_{12}$ antagonists, ticagrelor and the active metabolite of prasugrel, using several platelet agonists other than ADP (including arachidonic acid, collagen and TRAP) [31, 32]. However, further studies are warranted to understand the clinical implications of these PD observations.

The functional status of the $P2Y_{12}$ signaling pathway has been associated with platelet-derived thrombin generation profiles. In particular, blockade of the $P2Y_{12}$ receptor with clopidogrel has been associated with a prolongation of the TEG parameters evaluated in this study [8, 11]. However, no effect of cangrelor on TEG parameters related with thrombin generation processes have been revealed in the present investigation. This is in contrast with other investigations, in which cangrelor did show to have an effect on thrombin generation, which however included a different methodological approach and a distinct study population [9]. Indeed, more studies are warranted to better understand the role of cangrelor on modulating procoagulant activities, which to date have been limited and conflicting. Recent observations suggest that cangrelor may exert differential actions from other $P2Y_{12}$ receptors inhibitors on thrombin generation processes due to its effects on intraplatelet signaling which can be mediated through activation of a G protein-coupled pathway separate from Gi, presumably involving Gs [30]. Similarly, the lack of modulating effects on thrombin generation processes has also been shown with other strategies that increase c-AMP

Table 4 Thrombin generation times, assessed by thromboelastography, observed with increasing concentrations of cangrelor (in vitro incubation) in the overall group and according to diabetes mellitus status

Assay	Baseline	Cangrelor 5 nM	Cangrelor 50 nM	Cangrelor 500 nM	*p* value for trend
R					
All	4.4 ± 0.4	4.3 ± 0.4	4.1 ± 0.4	4.2 ± 0.4	0.171
DM	4.3 ± 0.4	4.2 ± 0.3	3.9 ± 0.4	3.9 ± 0.3	0.097
Non-DM	4.4 ± 0.8	4.4 ± 0.8	4.3 ± 0.8	4.5 ± 0.7	0.844
TMRTG					
All	5.4 ± 0.5	5.2 ± 0.5	5.2 ± 0.5	5.2 ± 0.5	0.364
DM	5.4 ± 0.5	5.2 ± 0.4	5.0 ± 0.5	5.0 ± 0.4	0.186
Non-DM	5.5 ± 0.9	5.2 ± 0.9	5.3 ± 0.9	5.5 ± 0.9	0.706

R and TMRTG are expressed in minutes

R reaction time, *TMRTG* time to maximum rate of thrombin generation

levels which in turn are associated with enhanced inhibition of PD markers measuring the activity of the $P2Y_{12}$ pathway [33]. These findings have also been attributed to differential effects on intraplatelet signaling that way occur within the purinergic mediated pathways of platelet activation [34, 35]. These PD observations may explain why the rates of major bleeding and transfusions were not increased with cangrelor in a pooled analysis of the CHAMPION program [36].

The PD properties of cangrelor make this a potentially desirable antiplatelet agent for clinical practice. Cangrelor may have a role as a bridging strategy in the setting of patients requiring surgery but who may require treatment with a $P2Y_{12}$ inhibitor to prevent thrombotic complications, such as in ACS patients or those treated with coronary stents [37]. However, despite these promising findings, 2 large scale phase III clinical trials conducted in the setting of percutaneous coronary intervention (PCI) were both terminated before completion because of an interim analysis showing insufficient evidence of clinical effectiveness of cangrelor [16, 17]. A PD interaction between cangrelor and clopidogrel was deemed unlikely as a cause of these findings, and pitfalls in trial design, particularly with regards to the definition of myocardial infarction, may have been a potential explanation [38]. Notably, in a pooled analysis of the two CHAMPION trials ($n = 13{,}049$ patients), with the use of the universal myocardial infarction (MI) definition instead of the original definition used, cangrelor was associated with a significant 18 % relative risk reduction in the primary end point (death, myocardial infarction, or ischemia-driven revascularization at 48 h), which included a 66 % relative risk reduction in stent thrombosis [36]. Therefore, these observations have provided the rationale for the design of the ongoing large-scale phase III clinical trial CHAMPION-PHOENIX (NCT01156571), which evaluates the efficacy and safety of cangrelor compared to standard of care in patients undergoing PCI [39].

In conclusion, in vitro cangrelor provides a potent and dose-dependent blockade of the platelet $P2Y_{12}$ receptor, with no differential effect in patients with and without DM. In addition, in vitro cangrelor exerts moderate inhibitory effects on other non-purinergic platelet signaling pathways, without modulating platelet-derived thrombin generation processes. Ex vivo studies are warranted to confirm these in vitro findings.

Study limitations

The main limitation of the present investigation is derived from its very design, since in vitro conditions convert the results of this study in exploratory and ex vivo PD studies are warranted to confirm these findings. No significant differences in baseline platelet reactivity were found between DM and non-DM patients, although an upregulation of $P2Y_{12}$ signaling pathway has been reported in prior investigations [7]. This may be due to the fact that studies with a similar sample size to ours that have shown differences in platelet function profiles between patients with and without DM have usually included patients on dual antiplatelet therapy with aspirin and clopidogrel [29], while a larger sample size may be needed to find baseline differences in patients not taking a $P2Y_{12}$ inhibitor [3]. In addition, the effect of escalating concentrations of cangrelor was evaluated in a relatively small sample size, which may have played a role in the absence of interaction due to DM condition observed and in the lack of effects on TEG thrombin generation parameters found. Further, thrombin generation comprise a number of complex mechanisms that include cell interactions, thus, a cell-based model could have been potentially more fitting for the present investigation [40].

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Conflict of Interest None of the other authors have conflict of interest to report.

References

1. Geisler T, Anders N, Paterok M et al (2007) Platelet response to clopidogrel is attenuated in diabetic patients undergoing coronary stent implantation. Diabetes Care 30:372–374
2. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E et al (2005) Platelet function profiles in patients with type 2 diabetes and coronary artery disease on combined aspirin and clopidogrel treatment. Diabetes 54:2430–2435
3. Serebruany V, Pokov I, Kuliczkowski W, Chesebro J, Badimon J (2008) Baseline platelet activity and response after clopidogrel in 257 diabetics among 822 patients with coronary artery disease. Thromb Haemost 100:76–82
4. Lüscher TF, Creager MA, Beckman JA, Cosentino F (2003) Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part II. Circulation 108:1655–1661
5. Ferreiro JL, Angiolillo DJ (2011) Diabetes and antiplatelet therapy in acute coronary syndrome. Circulation 123:798–813

6. Ferreiro JL, Cequier ÁR, Angiolillo DJ (2010) Antithrombotic therapy in patients with diabetes mellitus and coronary artery disease. Diab Vasc Dis Res 7:274–288
7. Ueno M, Ferreiro JL, Tomasello SD et al (2011) Functional profile of the platelet P2Y12 receptor signalling pathway in patients with type 2 diabetes mellitus and coronary artery disease. Thromb Haemost 105:730–732
8. Gurbel PA, Bliden KP, Guyer K, Aggarwal N, Tantry US (2007) Delayed thrombin-induced platelet-fibrin clot generation by clopidogrel: a new dose-related effect demonstrated by thrombelastography in patients undergoing coronary artery stenting. Thromb Res 119:563–570
9. Leon C, Ravanat C, Freund M, Cazenave JP, Gachet C (2003) Differential involvement of the P2Y1 and P2Y12 receptors in platelet procoagulant activity. Arterioscler Thromb Vasc Biol 23:1941–1947
10. van der Meijden PE, Feijge MA, Giesen PL, Huijberts M, van Raak LP, Heemskerk JW (2005) Platelet P2Y12 receptors enhance signalling towards procoagulant activity and thrombin generation. A study with healthy subjects and patients at thrombotic risk. Thromb Haemost 93:1128–1136
11. Angiolillo DJ, Capranzano P, Desai B et al (2009) Impact of P2Y(12) inhibitory effects induced by clopidogrel on platelet procoagulant activity in type 2 diabetes mellitus patients. Thromb Res 124:318–322
12. Ferreiro JL, Ueno M, Angiolillo DJ (2009) Cangrelor: a review on its mechanism of action and clinical development. Expert Rev Cardiovasc Ther 7:1195–1201
13. Storey RF, Oldroyd KG, Wilcox RG (2001) Open multicentre study of the P2T receptor antagonist AR-C69931MX assessing safety, tolerability and activity in patients with acute coronary syndromes. Thromb Haemost 85:401–407
14. Storey RF, Wilcox RG, Heptinstall S (2002) Comparison of the pharmacodynamic effects of the platelet ADP receptor antagonists clopidogrel and AR-C69931MX in patients with ischaemic heart disease. Platelets 13:407–413
15. Alberti KG, Zimmet PZ (1998) Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 15:539–553
16. Harrington RA, Stone GW, McNulty S et al (2009) Platelet inhibition with cangrelor in patients undergoing PCI. N Engl J Med 361:2318–2329
17. Bhatt DL, Lincoff AM, Gibson CM, CHAMPION PLATFORM Investigators et al (2009) Intravenous platelet blockade with cangrelor during PCI. N Engl J Med 361:2330–2341
18. Bouman HJ, van Werkum JW, Hackeng CM, Clappers N, Ten Berg JM (2009) Cangrelor increases the magnitude of platelet inhibition and reduces interindividual variability in clopidogrel-pretreated subjects. Neth Heart J 17:195–198
19. Aleil B, Ravanat C, Cazenave JP, Rochoux G, Heitz A, Gachet C (2005) Flow cytometric analysis of intraplatelet VASP phosphorylation for the detection of clopidogrel resistance in patients with ischemic cardiovascular diseases. J Thromb Haemost 3:85–92
20. Angiolillo DJ, Shoemaker SB, Desai B et al (2007) Randomized comparison of a high clopidogrel maintenance dose in patients with diabetes mellitus and coronary artery disease: results of the optimizing antiplatelet therapy in diabetes mellitus (OPTIMUS) study. Circulation 115:708–716
21. Sibbing D, Braun S, Jawansky S et al (2008) Assessment of ADP-induced platelet aggregation with light transmission aggregometry and multiple electrode platelet aggregometry before and after clopidogrel treatment. Thromb Haemost 99:121–126
22. Ueno M, Ferreiro JL, Tomasello SD et al (2011) Impact of pentoxifylline on platelet function profiles in patients with type 2 diabetes mellitus and coronary artery disease on dual antiplatelet therapy with aspirin and clopidogrel. JACC Cardiovasc Interv 4:905–912
23. Rivard GE, Brummel-Ziedins KE, Mann KG, Fan L, Hofer A, Cohen E (2005) Evaluation of the profile of thrombin generation during the process of whole blood clotting as assessed by thrombelastography. J Thromb Haemost 3:2039–2043
24. Ferreiro JL, Angiolillo DJ (2012) New directions in antiplatelet therapy. Circ Cardiovasc Interv 5:433–445
25. James S, Angiolillo DJ, Cornel JH, PLATO Study Group et al (2010) Ticagrelor vs. clopidogrel in patients with acute coronary syndromes and diabetes: a substudy from the PLATelet inhibition and patient outcomes (PLATO) trial. Eur Heart J 31:3006–3016
26. Wiviott SD, Braunwald E, Angiolillo DJ, TRITON-TIMI 38 Investigators et al (2008) Greater clinical benefit of more intensive oral antiplatelet therapy with prasugrel in patients with diabetes mellitus in the trial to assess improvement in therapeutic outcomes by optimizing platelet inhibition with prasugrel-Thrombolysis in Myocardial Infarction 38. Circulation 118:1626–1636
27. Angiolillo DJ, Badimon JJ, Saucedo JF et al (2011) A pharmacodynamic comparison of prasugrel vs. high-dose clopidogrel in patients with type 2 diabetes mellitus and coronary artery disease: results of the optimizing anti-platelet therapy in diabetes mellitus (OPTIMUS)-3 trial. Eur Heart J 32:838–846
28. Storey RF, Sanderson HM, White AE, May JA, Cameron KE, Heptinstall S (2000) The central role of the P(2T) receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. Br J Haematol 110:925–934
29. Holinstat M, Voss B, Bilodeau ML, McLaughlin JN, Cleator J, Hamm HE (2006) PAR4, but not PAR1, signals human platelet aggregation via Ca2 + mobilization and synergistic P2Y12 receptor activation. J Biol Chem 281:26665–26674
30. Srinivasan S, Mir F, Huang JS, Khasawneh FT, Lam SC, Le Breton GC (2009) The P2Y12 antagonists, 2-methylthioadenosine 5′-monophosphate triethylammonium salt and cangrelor (ARC69931MX), can inhibit human platelet aggregation through a Gi-independent increase in cAMP levels. J Biol Chem 284:16108–16117
31. Kirkby NS, Leadbeater PD, Chan MV, Nylander S, Mitchell JA, Warner TD (2011) Antiplatelet effects of aspirin vary with level of P2Y12 receptor blockade supplied by either ticagrelor or prasugrel. J Thromb Haemost 9:2103–2105
32. Armstrong PC, Leadbeater PD, Chan MV et al (2011) In the presence of strong P2Y12 receptor blockade, aspirin provides little additional inhibition of platelet aggregation. J Thromb Haemost 9:552–561
33. Angiolillo DJ, Capranzano P, Ferreiro JL et al (2011) Impact of adjunctive cilostazol therapy on platelet function profiles in patients with and without diabetes mellitus on aspirin and clopidogrel therapy. Thromb Haemost 106:253–262
34. Hayashi H, Sudo T (2009) Effects of the cAMP-elevating agents cilostamide, cilostazol and forskolin on the phosphorylation of Akt and GSK-3beta in platelets. Thromb Haemost 102:327–335
35. Gachet C, Léon C, Hechler B (2006) The platelet P2 receptors in arterial thrombosis. Blood Cells Mol Dis 36:223–227
36. White H, Chew DP, Dauerman HL et al (2012) Reduced immediate ischemic events with cangrelor in PCI: a pooled analysis of the CHAMPION trials using the universal definition of myocardial infarction. Am Heart J 163:182–190
37. Angiolillo DJ, Firstenberg MS, Price MJ et al (2012) Bridging antiplatelet therapy with cangrelor in patients undergoing cardiac surgery: a randomized controlled trial. JAMA 307:265–274
38. Angiolillo DJ, Schneider DJ, Bhatt DL et al (2012) Pharmacodynamic effects of cangrelor and clopidogrel: the platelet function substudy from the cangrelor versus standard therapy to

achieve optimal management of platelet inhibition (CHAMPION) trials. J Thromb Thrombolysis 34:44–55
39. Leonardi S, Mahaffey KW, White HD et al (2012) Rationale and design of the Cangrelor versus standard therapy to acHieve optimal Management of Platelet InhibitiON PHOENIX trial. Am Heart J 163(768–776):e2
40. Roberts HR, Hoffman M, Monroe DM (2006) A cell-based model of thrombin generation. Semin Thromb Hemost 32(Suppl 1):32–38