Cangrelor inhibits the binding of the active metabolites of clopidogrel and prasugrel to $P2Y_{12}$ receptors *in vitro*

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Running title: Cangrelor prevents thienopyridine binding to $P2Y_{12}$

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Abstract

Cangrelor is a rapid-acting, direct-binding, and reversible $P2Y_{12}$ antagonist which has been studied for use during percutaneous coronary intervention (PCI) in patients with or without pretreatment with an oral $P2Y_{12}$ antagonist. As cangrelor is administered intravenously, it is necessary to switch to an oral $P2Y_{12}$ antagonist following PCI, such as the thienopyridines clopidogrel and prasugrel or the non-pyridine ticagrelor. Previous studies have suggested a negative pharmacodynamic interaction between cangrelor and thienopyridines.

This *in vitro* study evaluated the receptor-level interaction between cangrelor and the active metabolite (AM) of clopidogrel or prasugrel by assessing functional $P2Y_{12}$ receptor number using a ^{33}P-2MeSADP binding assay.

All $P2Y_{12}$ antagonists studied resulted in strong $P2Y_{12}$ receptor blockade (cangrelor: 93.6%; clopidogrel AM: 93.0%; prasugrel AM: 97.9%). Adding a thienopyridine AM in the presence of cangrelor strongly reduced $P2Y_{12}$ receptor blockade by the AM (clopidogrel AM: 7%, prasugrel AM: 3.2%). The thienopyridine AMs had limited ability to compete with cangrelor for binding to $P2Y_{12}$ (% $P2Y_{12}$ receptor blockade after co-incubation with cangrelor 1000 nmol/L: 11.7% for clopidogrel AM 3μmol/L; 34.1% for prasugrel AM 3μmol/L).

In conclusion, *in vitro* cangrelor strongly inhibits the binding of clopidogrel and prasugrel AMs to the $P2Y_{12}$ receptor, consistent with the previous observation of a negative pharmacodynamic interaction. Care may need to be taken to not overlap exposure to thienopyridine AMs and cangrelor in order to reduce the risk of thrombotic complications following PCI.

Introduction

Cangrelor is a rapidly acting, direct-binding, reversible, intravenous $P2Y_{12}$ antagonist, which makes it attractive for use during percutaneous coronary intervention (PCI) in patients who may not have been sufficiently pretreated with an oral $P2Y_{12}$ antagonist, such as may occur in the management of acute ST-segment elevation myocardial infarction (STEMI) [1,2]. In the recent CHAMPION PHOENIX study, a bolus plus 2 hour infusion of cangrelor resulted in a decrease in ischaemic events during PCI with no increase in severe bleeding [3]. In the BRIDGE study, cangrelor was demonstrated to maintain levels of platelet inhibition following discontinuation of thienopyridine therapy prior to CABG with no increase in bleeding with platelet reactivity rapidly returning to baseline upon cessation [4]. In a recent meta-analysis of randomised PCI trials, cangrelor was shown to reduce the incidence of major adverse cardiovascular events and stent thrombosis compared to clopidogrel [5]. Cangrelor has recently been approved by the European Commission for use during PCI.

Cangrelor is administered intravenously at the time of PCI, however, since the risk of ischemic events persists after PCI, it is necessary to switch to an oral $P2Y_{12}$ antagonist such as clopidogrel, prasugrel or ticagrelor following the PCI. Clopidogrel and prasugrel are thienopyridine prodrugs that each require metabolism to their respective active metabolite (AM) which binds irreversibly to the $P2Y_{12}$ receptor. Clopidogrel active metabolite levels peak after 1 hour (0.45μmol/L following a 600mg dose) with maximal inhibition of platelet function after 2-4 hours [6,7] whereas prasugrel achieves higher levels of active metabolite (1.4μmol/L following a 60mg dose) and maximal inhibition of platelet function within 2 hours of dosing [6].

Clopidogrel active metabolite has a distribution phase plasma half-life of approximately 30 minutes and prasugrel's active metabolite demonstrates a similar profile [8,9,10] with plasma levels of both falling rapidly to less than 0.1 μmol/L in the first four hours during the distribution phase [11]. Due to the irreversible binding of the thienopyridine active metabolites to platelet $P2Y_{12}$ receptors, the inhibitory effects of clopidogrel and prasugrel persist long after the relatively short-lived active metabolites have been cleared from plasma, and normal platelet function is not restored for up to 7 days post dose. As cangrelor is a direct-acting $P2Y_{12}$ antagonist it achieves steady state (401ng/ml, 0.5μmol/L) within 2 minutes of bolus dosing [12] and has a shorter half-life (5 minutes) due to relatively rapid hydrolysis of cangrelor to its inactive metabolite [13]. Normal platelet function is restored 1-2 hours after cessation of cangrelor infusion as a result of the rapid fall in plasma cangrelor levels leading to dissociation of cangrelor from the $P2Y_{12}$ receptor [13].

In a healthy volunteer study, the ability of clopidogrel to inhibit platelet aggregation was reduced if it was administered during a cangrelor infusion [14]. Cangrelor was also found to limit the inhibition of platelet activation (measured by P selectin expression) by prasugrel AM in *in vitro* studies [15]. These previous studies have evaluated the ability of clopidogrel or prasugrel to inhibit platelet activation/aggregation in the presence of cangrelor as a surrogate measure of $P2Y_{12}$ receptor occupancy. In the *in vitro* studies described in this paper, we have directly measured the number of functional (unblocked) $P2Y_{12}$ receptors using a radioligand binding assay following co-incubation with cangrelor and either clopidogrel or prasugrel AM.

Materials and Methods

Materials

Hirudin was Revasc (Rhone-Polenc, Pennsylvania, USA), apyrase, MRS2179, adenosine triphosphate (ATP), di-sodium EDTA and 2MeSADP were from Sigma-Aldrich (Dorset, UK). HEPES-Tyrodes (HT) buffer consisted of 129mmol/L NaCl, 8.9mmol/L $NaHCO_3$, 2.8mmol/L KCl, 0.8mmol/L KH_2PO_4, 5.6mmol/L dextrose and 10mmol/L HEPES. Prostaglandin E_1 (PGE_1) was from Cayman Chemicals (Michigan, USA) and 2-thiomethyl ADP, [β-^{33}P] triethylammonium salt (^{33}P 2-MeSADP, specific activity 2100 Ci/mmol) was from Perkin Elmer (Boston, MA, USA). Prasugrel AM (R-138727) and clopidogrel AM were provided by Daiichi Sankyo Co. Ltd. (Tokyo, Japan). Cangrelor was a gift from The Medicines Company (Parsippany, New Jersey, USA).

Methods

Venous blood was taken from healthy volunteers who were free from drugs that affect platelet function for the previous 7 days. Informed consent was obtained according to a protocol approved by the local research ethics committee. Blood was taken using a 19g needle and syringe and anticoagulated with hirudin (50µg/mL). Platelet-rich plasma (PRP) was then obtained by centrifugation at 180 g for 10 min at room temperature. The PRP was then incubated with cangrelor (0-1000nM) at 37℃ for 5 min.

A subsample for each concentration was then taken and isolated platelets prepared by centrifugation at 400 g for 20 min in the presence of apyrase (0.1U/mL) and PGE_1 (1 mmol/L) before resuspension in HEPES-Tyrodes (HT) buffer. Aliquots were incubated with cangrelor (0-1000 nmol/L) for 5 min before assessing $P2Y_{12}$ receptor blockade using a ^{33}P 2MeSADP radioligand binding assay (see below).

The remaining PRP was incubated with either AM or vehicle (0.1% DMSO v/v) for 30 min at 37℃. Washed platelets were then isolated by centrifugation at 400 g for 20 min in the presence of PGE_1 and apyrase and resuspended in HT buffer.

Cangrelor was then removed by the following steps: a 60 min 37℃ incubation stirring at 1000rpm after the addition of 4mM EDTA, PGE_1, 100µM MRS2179 and 1mM ATP. Apyrase (0.5U/mL) was then added before centrifugation at 400 g for 20 min and resuspension in HT buffer. A second 60 min incubation with gentle agitation (150rpm, orbital shaking incubator) in the presence of EDTA, PGE_1 and apyrase as before was then carried out. Two more washing steps were completed before performing the radioligand binding assay.

Radioligand Binding Assay

All washed platelet samples were diluted to 400 x 10^9/L. Samples were then incubated in triplicate with 10 nmol/L ^{33}P 2Me-SADP in the presence of the selective $P2Y_1$ antagonist MRS2179 (100 µmol/L) for 10 minutes. Non-specific binding was determined by an excess of unlabelled 2MeSADP. Samples were then washed through with PBS and dried onto glass fibre filtermats (11731, Cox Scientific, Kettering, UK) in a Skatron (Norway) cell harvester. The filters were then placed in Ultima Gold MV scintillant (Perkin Elmer) and counted in a scintillation counter. The number of functional $P2Y_{12}$ receptors was estimated using the following calculation:

((CPM ÷ CPM per fmol) x 6.02 x 10^8) ÷ cell number

where CPM per fmol was calculated using the following formula:

((Ci/mmol x 2.22 x 10^{12}) x counter efficiency) ÷ 10^{12}

Percentage $P2Y_{12}$ receptor blockade was determined using the following equation:

% blockade = [(Total receptor no. - observed receptor no.)/Total receptor no.] x 100

Results are presented as mean ± SEM and were compared using ANOVA. Statistics were performed using GraphPad Prism software version 6. Sample size calculations demonstrated that studying 6 individuals would give a 90% chance of detecting a 15% change in % $P2Y_{12}$ receptor blockade.

Results

Preliminary experiments were carried out to confirm the inhibition of $P2Y_{12}$ receptor binding by cangrelor which demonstrated 93.5 ± 4% inhibition at 1000 nmol/L (figure 1A and table 1). Initial studies showed that simply washing the platelets did not result in removal of cangrelor with a residual 42% inhibition (100 nmol/L cangrelor) still evident after a 24 hour incubation post washing procedure (results not shown). Additional incubation and washing steps were carried out to dissociate cangrelor from the $P2Y_{12}$ receptor which resulted in its complete removal (figure 1B). Further experiments showed that adding prasugrel AM after cangrelor had been washed off achieved the same inhibition as adding prasugrel AM alone at the start of the protocol, suggesting no permanent change to the $P2Y_{12}$ receptor by cangrelor following the washing steps (results not shown).

Thienopyridine AM alone (3μmol/l) led to strong inhibition of ^{33}P 2MeSADP binding with clopidogrel AM achieving 93% and prasugrel AM 97.9% receptor blockade (figure 2 and table 1). However, when either AM was added in the presence of cangrelor, inhibition, following removal of free cangrelor by washing, decreased with

increasing concentrations of cangrelor (figure 2 and table 1), resulting in 7% and 3.2% receptor blockade in the presence of clopidogrel AM or prasugrel AM respectively at 1000nmol/L cangrelor.

Experiments were carried out with increasing concentration of clopidogrel AM or prasugrel AM in the presence of a high concentration of cangrelor (1000nmol/L) to investigate the ability of AM to compete with cangrelor. Both clopidogrel AM and prasugrel AM used alone demonstrated concentration-dependent $P2Y_{12}$ receptor blockade (figure 3A and B). However, they had limited ability to compete with cangrelor 1000 nmol/L, with 3μmol/L clopidogrel AM or prasugrel AM ($p<0.05$) resulting in 11.7 ± 2.2 % and 34.1 ± 8.9 % receptor blockade, respectively, following co-incubation with this concentration of cangrelor and subsequent washing (table 2).

Discussion

An *in vitro* radioligand binding assay was used to provide a direct measure of functional $P2Y_{12}$ receptors and using this methodology we confirmed $P2Y_{12}$ receptor blockade by cangrelor and the AMs of clopidogrel and prasugrel. In agreement with *in vitro* studies assessing platelet function [14,15] when platelets encounter thienopyridine AMs in the presence of cangrelor, the ability of the thienopyridine AM to block the $P2Y_{12}$ receptor is greatly reduced.

Increasing the concentration of clopidogrel or prasugrel AM demonstrated that they have a limited ability to compete with cangrelor (1000 um/L) for binding to the $P2Y_{12}$ receptor. The data suggests that prasugrel AM may be better able to compete with cangrelor than clopidogrel AM despite both thienopyridine AMs having similar IC_{50} values (0.30 μmol/L) [16].

Several studies have examined the transition from cangrelor to thienopyridines and have shown a transient recovery of platelet reactivity during the switch from cangrelor to either clopidogrel or prasugrel [14,17]. A recent study by Schneider et al. demonstrated a transient recovery in platelet function in patients with stable coronary artery disease during the transition from cangrelor to prasugrel but this effect was limited if prasugrel was administered 30min before the end of cangrelor infusion [17]. These observations from pharmacodynamic studies are consistent with the known pharmacokinetic profiles of thienopyridine AMs and cangrelor [7-11] and our current findings: it is only when cangrelor plasma levels fall sufficiently for cangrelor to dissociate from the $P2Y_{12}$ receptor that thienopyridine AMs are able to bind to the receptor. Consequently, in order for clopidogrel and prasugrel to achieve irreversible inhibition of the $P2Y_{12}$ receptor, cangrelor plasma levels must fall sufficiently (via hydrolysis to inactive metabolite) before thienopyridine AM levels fall to ineffective levels as a result of distribution to extravascular compartments. We have shown that 0.1 μmol/L of prasugrel AM and clopidogrel AM achieve 28% and 11% receptor blockade, respectively, and higher levels are required for more effective receptor blockade that achieves efficacious levels of platelet inhibition [18]. Previous studies have shown that prasugrel and clopidogrel AM levels fall below 0.1 μmol/L within four hours of dosing, thus explaining the time-sensitive interaction with cangrelor in pharmacodynamic studies.

Experiments described here were performed *in vitro* using blood obtained from healthy volunteers and further studies in patients with acute coronary syndromes undergoing the switch between cangrelor and thienopyridine therapy would be useful to confirm our findings. Of note, in the CHAMPION platelet substudy [19] there was no apparent significant pharmacodynamic interaction when clopidogrel was

administered at the end of the cangrelor infusion in a group of patients undergoing PCI.

The interaction between cangrelor and ticagrelor was not examined in the current study, however a recent study of co-administration of cangrelor and ticagrelor in patients with stable coronary disease did not show an interaction between the two $P2Y_{12}$ antagonists [20]. Since both cangrelor and ticagrelor rely on sustained plasma levels for their inhibitory effects, one might not expect to find a negative pharmacodynamic interaction [19,21].

Conclusions

In conclusion, using a direct measure of functional $P2Y_{12}$ receptors, we have been able to demonstrate an *in vitro* interaction between cangrelor and thienopyridine AMs suggesting a limited ability of the AMs to compete with cangrelor for $P2Y_{12}$ binding. Our observations emphasise the importance of awareness of healthcare teams about the nature of this interaction in order to avoid the risk of potentially life-threatening acute stent thrombosis as a consequence of inadequate $P2Y_{12}$ receptor blockade when co-administering cangrelor and thienopyridines. The findings of our study therefore support the current clinical recommendation of initiating thienopyridine therapy towards the end of a cangrelor infusion [17].

Conflict of interest

HMJ: None to declare.

RJB: None to declare.

JJ: Employee and minor shareholder of Eli Lilly and Company

RFS: institutional research grants, honoraria and/or consultancy fees from Accumetrics, AstraZeneca, Aspen, Correvio, Daiichi Sankyo, Eli Lilly, Medscape, Merck, PlaqueTec, Regeneron, Roche, Sanofi Aventis, ThermoFisher Scientific and The Medicines Company; named as inventor by AstraZeneca on patent application related to the PEGASUS-TIMI 54 study results but no financial interest in this.

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Legend to figures

Figure 1

$P2Y_{12}$ receptor number measured by ^{33}P 2MeSADP radioligand binding in the presence of increasing concentrations of cangrelor (0-1000 nmol/L) (figure 1A) (n=12). In figure 1B, the cangrelor was removed from the $P2Y_{12}$ receptor using a washing and incubation procedure prior to performing the radioligand binding assay (n=6). Mean ± SEM, ***P<0.001 ANOVA

Figure 2

$P2Y_{12}$ receptor number in the presence of both cangrelor and thienopyridine active metabolite (AM). Platelets were incubated with cangrelor (0-1000 nmol/L) for 5 min before adding 3μmol/L clopidogrel or prasugrel AM or saline control for 30min. Cangrelor was then removed from the $P2Y_{12}$ receptor by washing. Data are mean ± SEM (n=6). ***P<0.01, **P<0.001 (ANOVA).

Figure 3

Ability of thienopyridine active metabolite (AM) to compete with cangrelor for $P2Y_{12}$ receptor binding. Platelets were incubated with cangrelor (1000 nmol/L) for 5 min before adding 0 or 3μmol clopidogrel AM (figure 3A) or prasugrel AM (figure 3B) for 30min. Cangrelor was then removed by washing and functional receptor number determined. Data are mean ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 effect of thienopyridine AM, # P<0.05 compared to cangrelor in the absence of thienopyridine AM (ANOVA).

Table 1. Percentage $P2Y_{12}$ receptor blockade with either cangrelor or thienopyridine AMs co-incubated with cangrelor followed by washing

Cangrelor (nmol/L)	Control (no washing)	Prasugrel AM (3 μmol/L) + washing	Clopidogrel AM (3 μmol/L) + washing
0	-	97.9 (0.6)	93.0 (0.6)
10	63.6 (5.4)	95.0 (1.0)	56.1 (12.7)
100	83.1 (2.1)	38.5 (3.4)	8.7 (6.4)
1000	93.6 (0.7)	3.2 (3.9)	7.0 (1.3)

% $P2Y_{12}$ receptor blockade in the presence of cangrelor alone (control) (n=12) and following co-incubation of cangrelor with prasugrel or clopidogrel AM and subsequent washing to remove cangrelor and unbound AM (n=6). Data are mean (SEM).

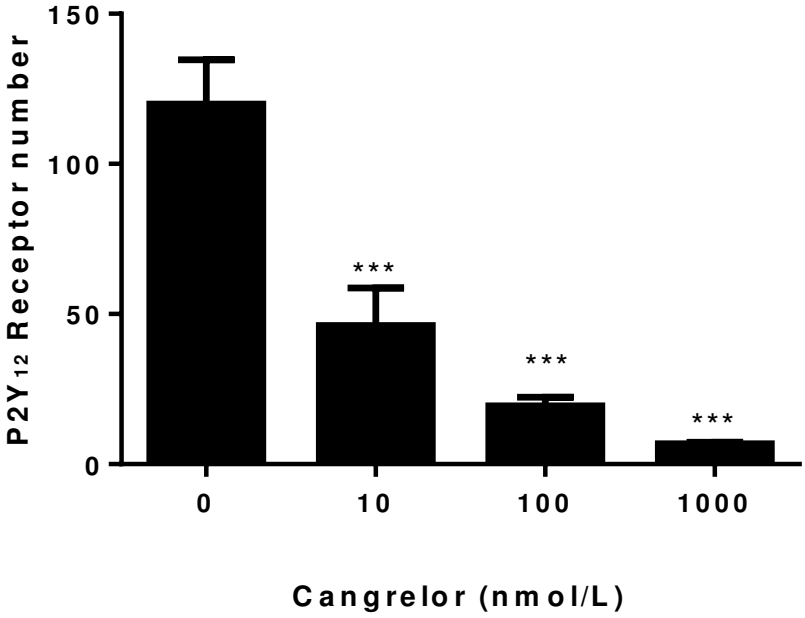
Table 2. Ability of clopidogrel and prasugrel active metabolites to compete with cangrelor for binding to $P2Y_{12}$

Prasugrel AM (μmol/L)	Control	Cangrelor (1000nmol/L)	Clopidogrel AM (μmol/L)	Control	Cangrelor (1000nmol/L)
0	-	4 (2.6)	**0**	-	0 (0)
0.1	28.4 (5.7)	6.2 (3.5)	**0.1**	10.9 (4.1)	0 (0)
0.3	67.9 (4.4)	11.4 (5.6)	**0.3**	39.1 (5.3)	0 (0)
1.0	89.4 (1.4)	19.9 (8.5)	**1.0**	77.0 (5.4)	6.1 (3.1)
3.0	93.7 (1.6)	34.1 (8.9)	**3.0**	89.6 (3)	11.7 (2.2)

% $P2Y_{12}$ receptor blockade in the presence of 1000nmol/L cangrelor and 0-3μmol/L prasugrel or clopidogrel active metabolites (AM). Cangrelor was removed by washing prior to the radioligand binding assay. Data are mean (SEM), n=6.

Figure 1

A.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100
50
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P2Y12 Receptor number
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***
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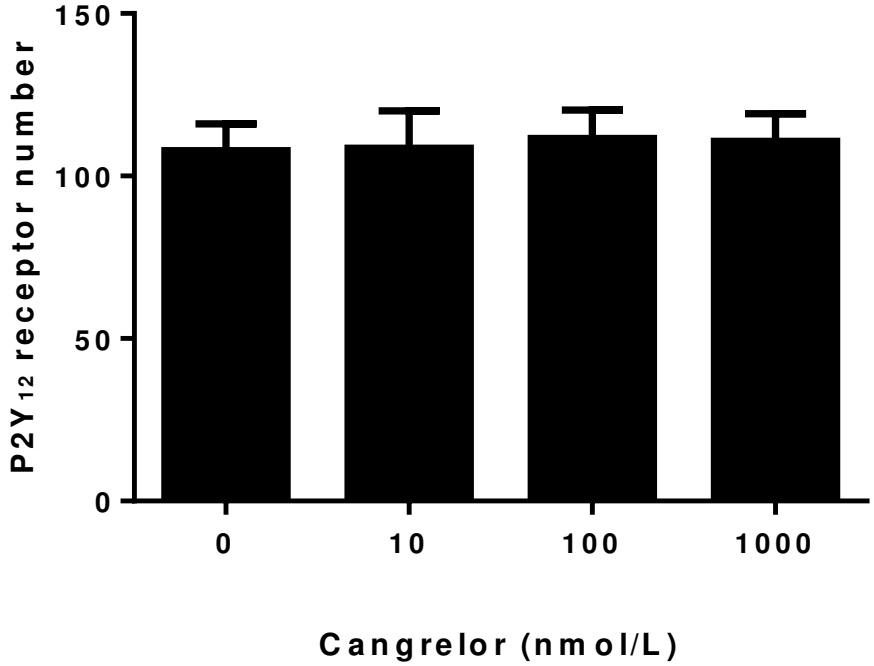
0
10
100
1000
Cangrelor (nmol/L)

B.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100
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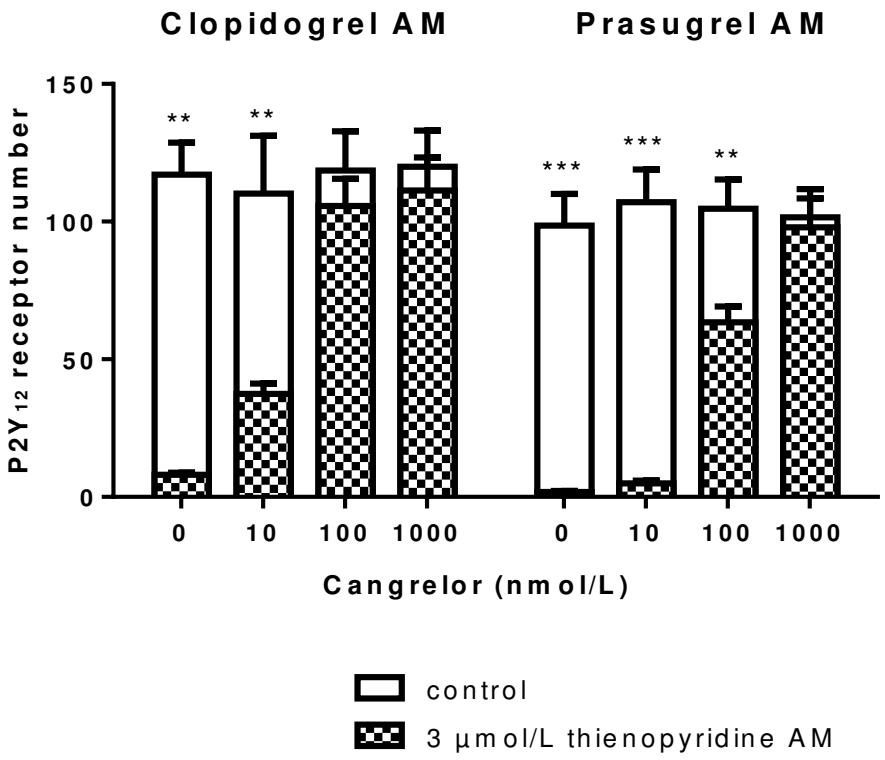
P2Y12 receptor number
0
10
100
1000
Cangrelor (nmol/L)

Figure 2


Clopidogrel AM
Prasugrel AM
P2Y12 receptor number
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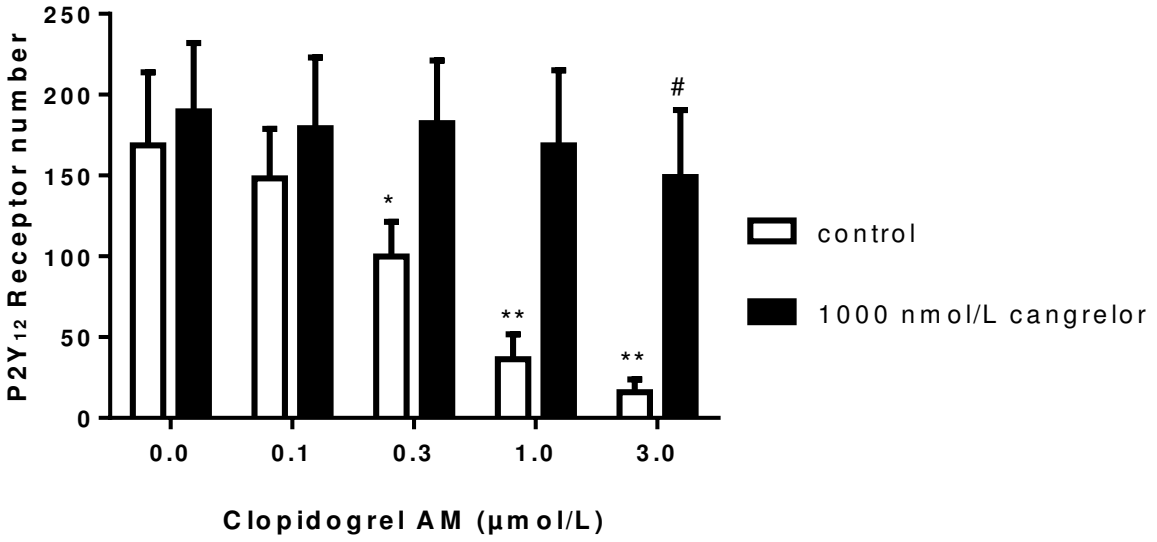
Cangrelor (nmol/L)
control
3 µmol/L thienopyridine AM

Figure 3

A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P2Y12 Receptor number
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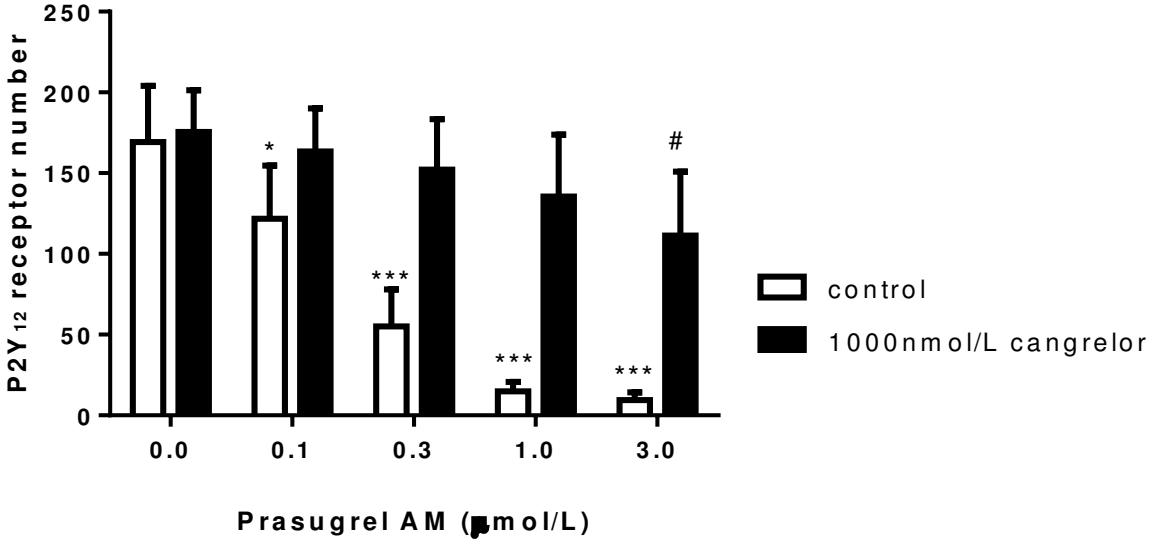
Clopidogrel AM (µmol/L)
control
1000 nmol/L cangrelor

B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P2Y12 receptor number
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0.0
0.1
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1.0
3.0
Prasugrel AM (µmol/L)
control
1000nmol/L cangrelor