Studies of the interaction of ticagrelor with the $P2Y_{13}$ receptor and with $P2Y_{13}$-dependent pro-platelet formation by human megakaryocytes

Anna Björquist[1]; Christian A. Di Buduo[2,3]; Eti A. Femia[4]; Robert F. Storey[5]; Richard C. Becker[6]; Alessandra Balduini[2,3,7]; Sven Nylander[1]; Marco Cattaneo[4]

[1]AstraZeneca R&D Mölndal, Sweden; [2]Department of Molecular Medicine, University of Pavia, Pavia, Italy; [3]Biotechnology Research Laboratories, IRCCS San Matteo Foundation, Pavia, Italy; [4]Medicina 3, Ospedale San Paolo – Dipartimento di Scienze della Salute, Università degli Studi di Milano. Milan, Italy; [5]Department of Infection Immunity and Cardiovascular Disease, University of Sheffield, Sheffield, UK; [6]University of Cincinnati College of Medicine, Heart, Lung and Vascular Institute, Tufts University, Medford, Massachusetts, USA; [7]Department of Biomedical Engineering, Tufts University, Medford, Massachusetts, USA

Summary

Ticagrelor is an antagonist of the platelet $P2Y_{12}$ receptor for ADP, approved for the prevention of thromboembolic events in patients with acute coronary syndrome. Previous studies showed that ticagrelor has no significant activity versus P1 receptors for adenosine and other known P2Y receptors, with the exception of $P2Y_{13}$, which was not tested. The $P2Y_{12}$ antagonist cangrelor has been shown to also inhibit $P2Y_{13}$ and to decrease the $P2Y_{13}$-regulated capacity of megakaryocytes to produce pro-platelets. We tested whether or not ticagrelor inhibits $P2Y_{13}$ signalling and function. The *in vitro* effects of ticagrelor, its active (TAM) and inactive (TIM) metabolites, cangrelor and the $P2Y_{13}$ antagonist MRS2211 were tested in two experimental models: 1) a label-free cellular response assay in $P2Y_{13}$-transfected HEK293 T-REx cells; and 2) pro-platelet formation by human megakaryocytes in culture. Ticagrelor, TAM, cangrelor and MRS2211, but not TIM, inhibited the cellular responses in $P2Y_{13}$-transfected cells. In contrast, only MRS2211 and cangrelor, confirming previous results, inhibited pro-platelet formation by megakaryocytes *in vitro*. The platelet count of patients randomised to treatment with ticagrelor in the PLATO trial did not change during treatment and was comparable to those of patients randomised to clopidogrel. In conclusion, ticagrelor and TAM act as $P2Y_{13}$ antagonists in a transfected cell system *in vitro* but this does not translate into any impact on pro-platelet formation *in vitro* or altered platelet count in patients.

Keywords

Ticagrelor, $P2Y_{12}$, $P2Y_{13}$, megakaryocytes, platelets

Correspondence to:
Marco Cattaneo
Medicina 3, Ospedale San Paolo
Dipartimento di Scienze della Salute
Università degli Studi di Milano. Milan, Italy
Tel.: +39 0250323095, Fax: +39 0250323089
E-mail: marco.cattaneo@unimi.it
or
Sven Nylander
AstraZeneca R&D Mölndal
431 83 Mölndal, Sweden
Tel.: +46 31 7762149, Fax: +46 31 7763761
E-mail: sven.nylander@astrazeneca.com

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Introduction

Ticagrelor, a direct acting, reversibly-binding $P2Y_{12}$ receptor antagonist (1), is an oral anti-platelet agent approved for the prevention of thromboembolic events in patients with acute coronary syndromes (ACS). Compared to the thienopyridine pro-drug clopidogrel, whose active metabolite irreversibly inhibits $P2Y_{12}$, ticagrelor decreased the incidence of major adverse cardiovascular events (MACE) and total mortality when given on top of aspirin in patients with ACS (2). An additional clinically relevant mechanism of action of ticagrelor that has been identified is inhibition of adenosine cell uptake via the equilibrative nucleoside transporter-1 (ENT1) (3–5).

P2Y12 is a member of family A of G-protein-coupled receptors (GPCRs) (6). Within this receptor family there are 12 cloned human purinergic receptors: four P_1 receptors (A1, A2A, A2B, A3) and eight P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$). All P_1 receptors are activated by adenosine, while P2Y receptors are activated by nucleotides such as ATP, ADP, UTP, UDP and UDP-glucose (6). From a phylogenetic and structural point of view, two distinct P2Y receptor subgroups have been identified, $P2Y_1$-like receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$), which couple to Gq, and $P2Y_{12}$-like receptors ($P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$), which couple to Gi (6).

Secondary pharmacology screening showed no significant activity of ticagrelor versus the four P1 receptors (3) or the other P2Y

receptors that have been tested (AstraZeneca data on file). In a range of functional and binding assays (human $P2Y_1$, rat $P2Y_6$, human $P2Y_{11}$, human $P2Y_{14}$), ticagrelor demonstrated no significant agonist or antagonist activity at concentrations up to 3 μM or higher (AstraZeneca data on file). In a functional human $P2Y_2$-receptor system, ticagrelor was inactive at 10 μM in two of three experiments. Based on these data, we may conclude that ticagrelor has no clinically relevant effects on these receptors, considering that its mean maximal plasma concentration following four weeks of treatment (90 mg bid) was about 1.5 μM (770 ng/ml) (7), corresponding to a mean maximal free active concentration of 12 nM considering that the protein un-bound fraction is very low ≤0.02 % (8).

The only P2Y receptor that has not been tested with ticagrelor is $P2Y_{13}$, whose functional roles described so far include regulation of ATP release from red blood cells (9), bone remodelling (10, 11), reverse cholesterol transport (12–14), neuropathic pain (15), and pro-platelet formation (16).

The $P2Y_{12}$ antagonist cangrelor has been shown to inhibit $P2Y_{13}$ signalling in a functional assay (17) and to inhibit pro-platelet formation through its interaction with $P2Y_{13}$ (16). Given this prior data with cangrelor and the high homology of $P2Y_{12}$ and $P2Y_{13}$ (63 % using protein-protein BLAST and default settings at NCBI webpage), ticagrelor might also be able to inhibit P2Y13. In order to test this hypothesis, we evaluated the ability of ticagrelor to inhibit ADP-induced $P2Y_{13}$ signalling and function using two different approaches: 1) activation response of human $P2Y_{13}$-transfected cells using a label-free cellular response assay; and, 2) $P2Y_{13}$-induced pro-platelet formation in human primary megakaryocytes.

Methods

Reagents

Ticagrelor, AR-C124910XX (the main circulating metabolite of ticagrelor with similar $P2Y_{12}$ potency, here referred to as ticagrelor active metabolite, TAM), AR-C133913XX (the main metabolite in the urine, inactive vs $P2Y_{12}$ and here referred to as ticagrelor inactive metabolite, TIM), AZD1283 (an alternative $P2Y_{12}$R antagonist stopped in phase I), elinogrel (an alternative $P2Y_{12}$R antagonist stopped in phase II), cangrelor (an alternative $P2Y_{12}$ antagonist approved for intravenous use), clopidogrel active metabolite (CAM), prasugrel active metabolite (PAM), and AZD6482 (an unrelated compound targeting PI3Kβ used as negative control), were from AstraZeneca R&D (Mölndal, Sweden); MRS2211 ($P2Y_{13}$ antagonist) and MRS2279 and MRS2179 ($P2Y_1$ antagonists) were from Tocris Bioscience (Bristol, UK); 2-methylthio-ADP (2MeSADP) was from Sigma-Aldrich (St. Louis, MO, USA). Thrombopoietin (TPO), interleukin (IL)-6 and IL-11 were from PeproTech (London, UK). Stem Span medium was from Stem-Cell Technologies (Vancouver, BC, Canada). The FITC-conjugated antibody against CD41 was from eBioscience Inc (San Diego, CA, USA); antibodies against AKT, phospho-AKT and ERK1/2 were from Cell Signaling Technology (Danvers, MA, USA); the antibody against phospho-ERK1/2 was from Millipore (Milan, Italy); the antibody against β-actin was from Sigma-Aldrich.

Studies with transfected cells

Cell line development

The T-REx™ system is a tetracycline-regulated mammalian expression system that uses regulatory elements from an *Escherichia coli*-encoded tetracycline resistance operon. Tetracycline regulation in the T-REx™ system is based on the binding of tetracycline to the tetracycline repressor and de-repression of the promoter controlling expression of the gene of interest. HEK293 T-REx cells (HEKTRex) were stably transfected with plasmid containing human P2Y13 cDNA (Astra Zeneca reference id pAM2223) using Lipofectamine plus (Thermo Fisher Scientific, Waltham, MA, USA). Doxycycline (Sigma-Aldrich) was used to induce transcription of P2Y13. Single cells were sorted into 96-well plates by flow cytometry (FACSAriaTM, Becton Dickinson, Sweden) and clones were expanded and screened for 2MeSADP response. The response in doxycycline-induced cells was compared to that in non-doxycycline-induced cells and a clone with maximal response above background (response in non-induced cells) was selected. In the following text, "(+)dox" will be used for "doxycycline-induced" P2Y13 expressing cells and "(–)dox" will be used for "non-doxycycline-induced control (not expressing $P2Y_{13}$) cells". Thus, the system allows the use of (–)dox cells as an optimal negative control cell that does not express $P2Y_{13}$.

Effect on 2MeSADP-induced response in cell clones transfected with human recombinant $P2Y_{13}$ receptor

P2Y13-transfected cells were cultured in DMEM +10 % FBS (tetracycline free) + Blasticidin 5 μg/ml + Zeocin 200 μg/ml in T-75 flasks. On the day before the experiment, cells were detached with accutase, counted, diluted in media without antibiotics and seeded (4000/well) in fibronectin-coated 384-well Epic plates (Corning, Corning, NY, USA) with or without the addition of doxycycline (0.3 μg/ml). Plates were left at room temperature for 30–60 minutes (min) and then incubated overnight in a CO_2 incubator at 37 °C. Each cellplate contained (+)dox and (–)dox cells for parallel analysis of compound effect. Triplicate cellplates were used for each experiment.

A label-free cellular response assay measured on the Epic® System reader (Corning) was used. The technology employs unique microplates that contain a resonant waveguide optical biosensor in each well. The cells are grown in contact with the biosensor as an adherent monolayer. When a broadband light source is applied to the bottom of the optical biosensor, a specific wavelength of light is reflected back. The wavelength of light is dependent on mass changes detected near the surface of the waveguide. The Epic® System measures changes in local index of refraction due to ligand-induced dynamic mass redistribution (DMR) within the bottom region (150 nm) of the cell. A positive DMR indicates an increase in mass at the sensor surface whilst a negative DMR indicates a de-

crease in mass at the sensor surface. Intracellular recruitment, endocytosis, receptor recycling and cellular morphological changes all contribute to DMR. The shift in wavelength can be monitored over time relative to a baseline measurement and is measured in picometers (pm).

Antagonist potency, expressed as the concentration that gave half-maximum inhibition of the response (IC_{50} values), was calculated from DMR raw data fitted to the equation, DMRX=[A+((B-A/(1+(x/IC50)s))], where DMRX = DMR in the (+)dox cells (P2Y13 expressing) stimulated with 10 nM 2MeSADP in the presence of test compound, A = curve min, B = curve max, s = slope of the concentration response curve and x = test compound concentration.

Studies with human megakaryocytes in culture

Megakaryocyte differentiation from human cord blood haematopoietic progenitor cells and analysis of pro-platelet formation

Human cord blood was collected from the local blood bank following normal pregnancies and deliveries with informed consent of the parents, in accordance with the ethical committee of the IRCCS Policlinico San Matteo Foundation and the principles of the Declaration of Helsinki. $CD34^+$ cells from cord blood samples were separated by immunomagnetic bead selection (Miltenyi Biotec, Bologna, Italy) and differentiated, as previously described (16), in Stem Span medium supplemented with 10 ng/ml thrombopoietin, IL-6 and IL-11 at 37 °C in a 5% CO_2 fully humidified atmosphere.

Pro-platelet yields were evaluated at the end of the culture (13 days), as previously described (16). Briefly, cells were seeded in a 24-well plate and incubated at 37 °C in a 5% CO_2 fully humidified atmosphere. After 16 hours (h), pro-platelet-bearing megakaryocytes were counted by phase-contrast microscopy (Nikon TMS-F, Tokyo, Japan). Pro-platelets were identified as cells displaying long filamentous structure, ending with tips of the size of a platelet and their number was expressed as percentage of total cell count. Before being seeded, cells were incubated in Stem Span medium containing 10 μM test compound (ticagrelor, TAM, TIM, MRS2211) or vehicle, dimethyl sulfoxide (DMSO) for 16 h.

Flow cytometric analysis of megakaryocyte differentiation and ploidy

The analysis of DNA content was performed as previously described (16): after 13 days of culture, megakaryocytes were incubated with 10 μM test compound or vehicle, as described above. At the end of treatment, cells were harvested and fixed with cold ethanol 70% and frozen at –20 °C overnight. Frozen cells were centrifuged at 500xg for 10 min and pellets were incubated in phosphate-buffered saline (PBS) supplemented with 50 μg/ml propidium iodide and 100 μg/ml RNAse, at room temperature for 30 min. Finally, megakaryocytes were stained with a FITC-conjugated CD41 antibody. All samples were analysed using a Navios flow cytometer (Beckman Coulter, Indianapolis, IN, USA). A minimum of 10,000 megakaryocytes were acquired and data analysis was performed using Beckman Coulter Navios software package. The percentage of megakaryocytes as defined by $CD41^+$ cells was 91 ± 5%. Ploidy was analysed upon gating $CD41^+$ events.

Assessment of cell viability

Trypan blue exclusion assay was employed to determine the number of viable cells in cultures, as previously described (16). Megakaryocytes were treated with 10 μM test compound or vehicle, as described above. At the end of the incubation, 100 μl of cell suspension were mixed with 100 μl Trypan blue and visualised by phase-contrast microscopy (Nikon TMS-F, Tokyo, Japan). Viable cells (unstained) and dead cells (blue-stained) were counted and expressed as percentage of total cell count.

Western immunoblotting

In vitro differentiated megakaryocytes after 13 days of culture were pre-incubated with 10 μM test compound or vehicle, as described above. After 16 h, samples were lysed with Hepes-glycerol lysis buffer (Hepes 50 mM, NaCl 150 mM, 10% glycerol, 1% Triton X-100, $MgCl_2$ 1.5 mM, EGTA 1 mM, NaF 10 mM, PMSF 1 mM, Na_3VO_4 1 mM, 1 μg/ml leupeptin, 1 μg/ml aprotinin), as previously described (16). Lysis was performed for 30 min on ice followed by centrifugation at 15,700 x g at 4 °C for 15 min. Protein concentration was measured by the bicinchoninic acid assay (Pierce, Milan, Italy). Samples containing equal amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted. PVDF membranes were finally probed with antibodies against phospho-AKT (Ser473, 1:1000), total AKT (1:1000), phospho-ERK1/2 (Thr185/Tyr187, 1:1000), total ERK1/2 (1:100) or β-actin (1:5000), following the conditions recommended by the manufacturers. Immunoreactive bands were detected by horseradish peroxidase-labeled secondary antibodies (BioRad, Milan, Italy) using enhanced chemiluminescence reagent (Millipore, Milan, Italy).

Measurement of platelet shape change in human washed platelet suspensions

Venous blood samples were collected in acid-citrate-dextrose (ACD) anticoagulant (6:1, v:v) from five healthy volunteers and centrifuged at 200 x g for 10 min to obtain platelet-rich plasma (PRP), which was used to prepare twice-washed platelet suspensions, according to the method described by Mustard et al. (18), with the exception that 500 nM prostaglandin I_2 was added during the first and second wash. Platelet counts in washed platelet suspensions were adjusted to 4×10^{11}/l. Samples of washed platelet suspensions (0.45 ml) were stirred at 1,000 rpm in an aggregometer (Lumi-aggregometer, Chrono-log Corp., Havertown, PA, USA) and incubated with 10 μl ethylenediaminotetracetic acid (EDTA, 5 mM, final concentration), 10 μl of Tyrode solution or different concentrations of MRS2179 or MRS2211 (0.01, 0.1, 1.0 and 10.0

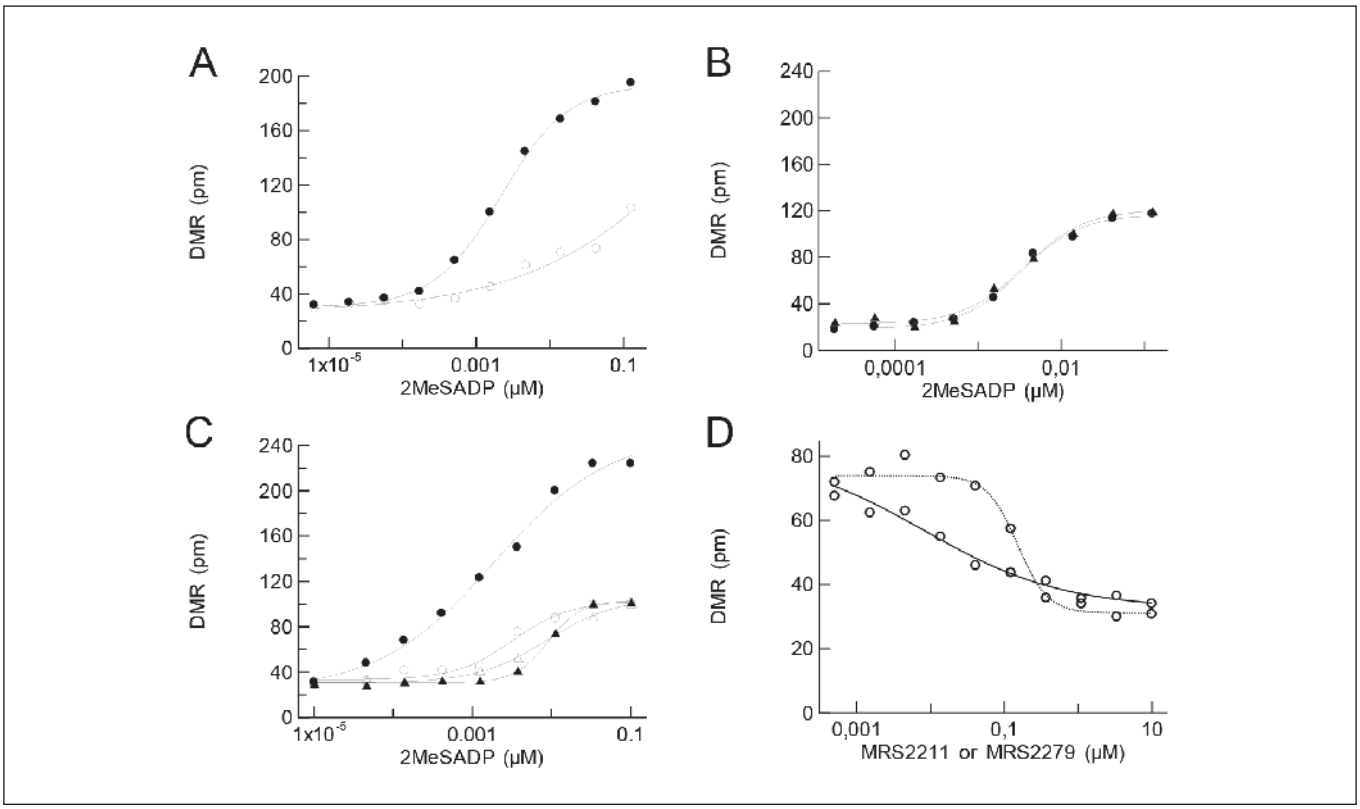

Figure 1: Characterisation of transfected cells. A) 2MeSADP concentration-response in (+)dox cells ($P2Y_{13}$ expressing) (●) and (-)dox cells (control) (▲). B) 2MeSADP concentration-response in untransfected parental cells in the presence of (●) and absence of dox (▲). C) 2MeSADP concentration-response in (+)dox cells ($P2Y_{13}$ expressing) (●), (+)dox cells treated with PTX (○), (-)dox cells (control) (▲) and (-)dox cells treated with PTX (Δ). D) Inhibition of 2MeSADP (10 nM)-induced response in PTX treated (+)dox cells by MRS2279 (solid line) and MRS2211 (dotted line). Data are means of triplicate measurements in four (A) and two (C) experiments. Data in B and D are means of triplicate measurements in one single experiment.

μM) at 37 °C for 60 seconds (sec). After incubation, 10 μl of 2MeSADP (10 nM, final concentration) was added and the extent of increase in optical density (expression of platelet shape change) was recorded for 3 min. Antagonist potency, expressed as IC_{50}, was calculated from the percent inhibition data (mean of the 5 separate experiments) fitted to the same equation as described under the cell line experiments above.

Effect of ticagrelor on platelet count in ACS patients

In the PLATO trial, ticagrelor was compared with clopidogrel when given on top of aspirin in 18,624 ACS patients, with or without ST-segment elevation (2). Platelet count was measured in a central laboratory (Quintiles Laboratory, Durham, NC, USA) by electronic cell counter (Beckman Coulter Gen-s) at seven prespecified visits. Visit 1: Randomisation (n=4393 ticagrelor, n=4375 clopidogrel), Visit 2: 1 month ± 10 days (n=3749 ticagrelor, n=3741 clopidogrel), Visit 3: 3 months ± 10 days (n=3456 ticagrelor, n=3468 clopidogrel), Visit 4: 6 months ± 10 days (n=3132 ticagrelor, n=3110 clopidogrel), Visit 5: 9 months ± 10 days (n=178 ticagrelor, n=172 clopidogrel), Visit 6: 12 months ± 10 days (n=2840 ticagrelor, n=2839 clopidogrel), Follow up: End of treatment + 1 month ± 10 days (n=2804 ticagrelor, n=2773 clopidogrel).

Statistics

All data are expressed as mean ± standard deviation (SD). $P2Y_{13}$ antagonist potency, expressed as IC_{50} calculated from EPIC DMR data, was compared using a two-tailed distribution Student's t-test with two-sample equal variance. For studies with *in vitro* differentiated human megakaryocytes data were obtained in independent experiments performed by using different cord blood samples and analysed using ANOVA with Bonferroni adjustment of the pairwise comparisons of subgroups. As for platelet count data, a one-sample t-test was performed to analyse the mean change from baseline at each visit by treatment. A normal distribution was assumed. A value of $p<0.05$ was considered statistically significant.

Results

Studies with transfected cells

Characterisation of selected $P2Y_{13}$-transfected clone

Both (+)dox cells and (-)dox cells concentration-dependently responded to 2MeSADP, but the response of (+)dox cells was much higher compared to that of (-)dox cells (►Figure 1A). The 2MeSADP-induced response of (-)dox cells was similar to the response

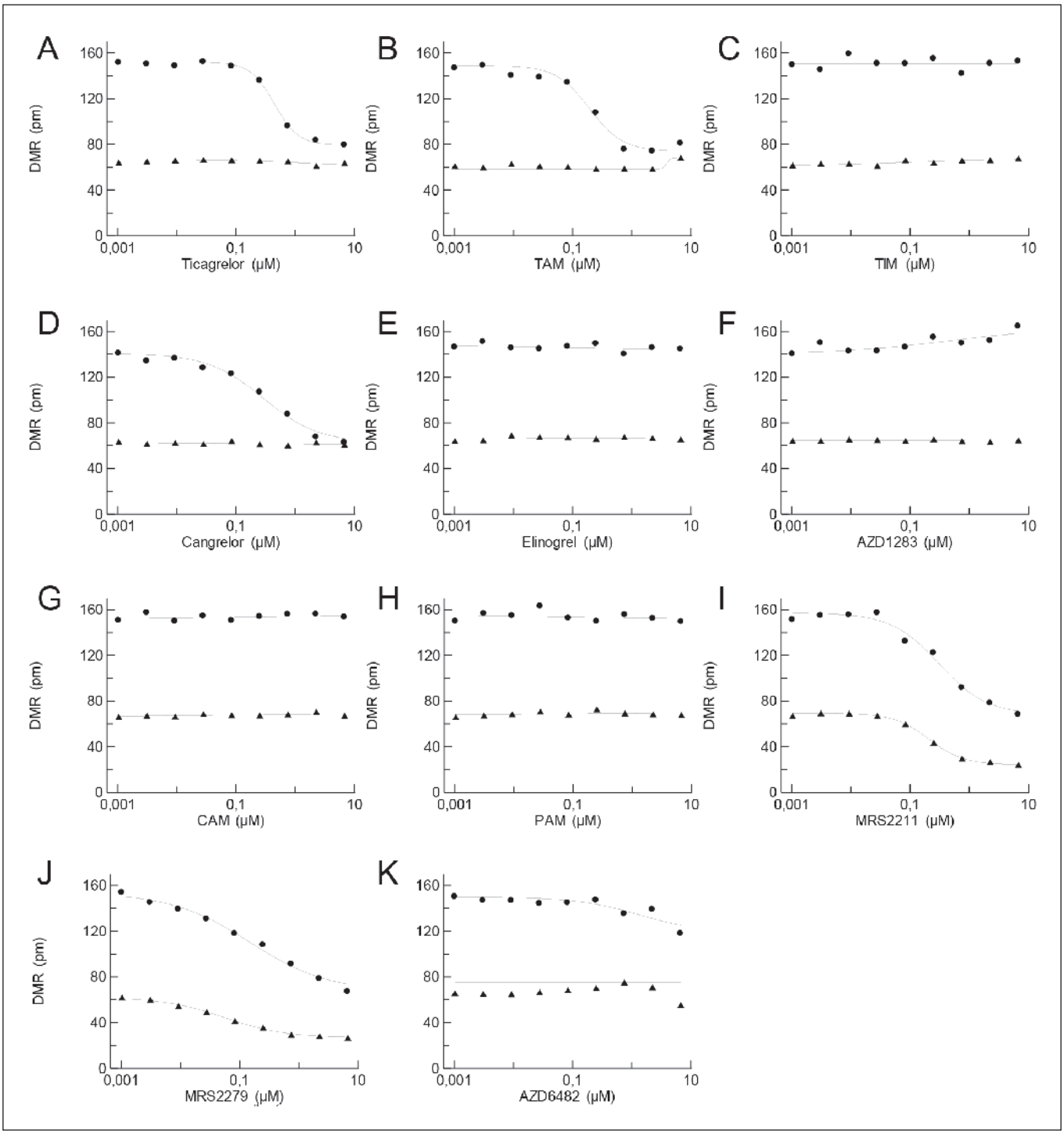

Figure 2: Inhibition of 10 nM 2MeSADP-induced responses in (+)dox cells ($P2Y_{13}$ expressing) (●) and (–)dox cells (control) (▲) by increasing concentrations of ticagrelor (A), TAM (B), TIM (C), cangrelor (D), elinogrel (E), AZD1283 (F), CAM (G), PAM (H), MRS2211 (I), MRS2279 (J) and AZD6482 (K). Data are expressed as means of four separate experiments.

of untransfected HEKTRex parental cells, which was unaffected by dox (▶Figure 1B).

Incubation with pertussis toxin (PTX, a selective Gi inhibitor), 100 ng/ml, for 19 h attenuated the 2MeSADP-induced response in (+)dox cells, confirming that part of this response is Gi mediated, compatible with $P2Y_{13}$ involvement. The residual response to 2MeSADP was equal to that in (-)dox cells, which was unaffected by PTX treatment, confirming that it is Gi-independent and thus likely Gq-mediated via endogenous $P2Y_1$ (▶Figure 1C). In line with this observation, the specific $P2Y_1$ antagonist MRS2279 in-

Table 1: IC_{50} of the tested compounds on 2MeSADP-induced responses of (+)dox cells.

	IC_{50} (μM)	SD
Ticagrelor	0.44	0.07
TAM	0.18*	0.09
TIM	>10	NA
Cangrelor	0.26	0.12
AZD1283	>10	NA
Elinogrel	>10	NA
CAM	>10	NA
PAM	>10	NA
AZD6482	>10	NA

Means of four separate experiments. * P<0.05 vs ticagrelor.

hibited the residual, Gi-independent response (►Figure 1D). We observed that also the $P2Y_{13}$ antagonist MRS2211 inhibited the residual Gi-independent response (►Figure 1D), suggesting limited specificity for $P2Y_{13}$.

$P2Y_{13}$ antagonist activity

Ticagrelor, cangrelor, and TAM, displayed $P2Y_{13}$ antagonist activity, as they concentration-dependently inhibited 10 nM 2MeSADP-induced $P2Y_{13}$ responses in (+)dox cells (►Figure 2) with mean calculated IC_{50}-values listed in ►Table 1. TIM, AZD1283, elinogrel, CAM, PAM and AZD6482 did not display any significant $P2Y_{13}$ antagonist activity, as their IC_{50}-values were >10 μM. Given the mixed profile of MRS2211 and MRS2279 inhibiting the MeSADP response in both (+)dox cells and (-)dox cells, no IC_{50}-values were calculated. None of the other test compounds inhibited the MeSADP response in (-)dox cells (►Figure 2). No

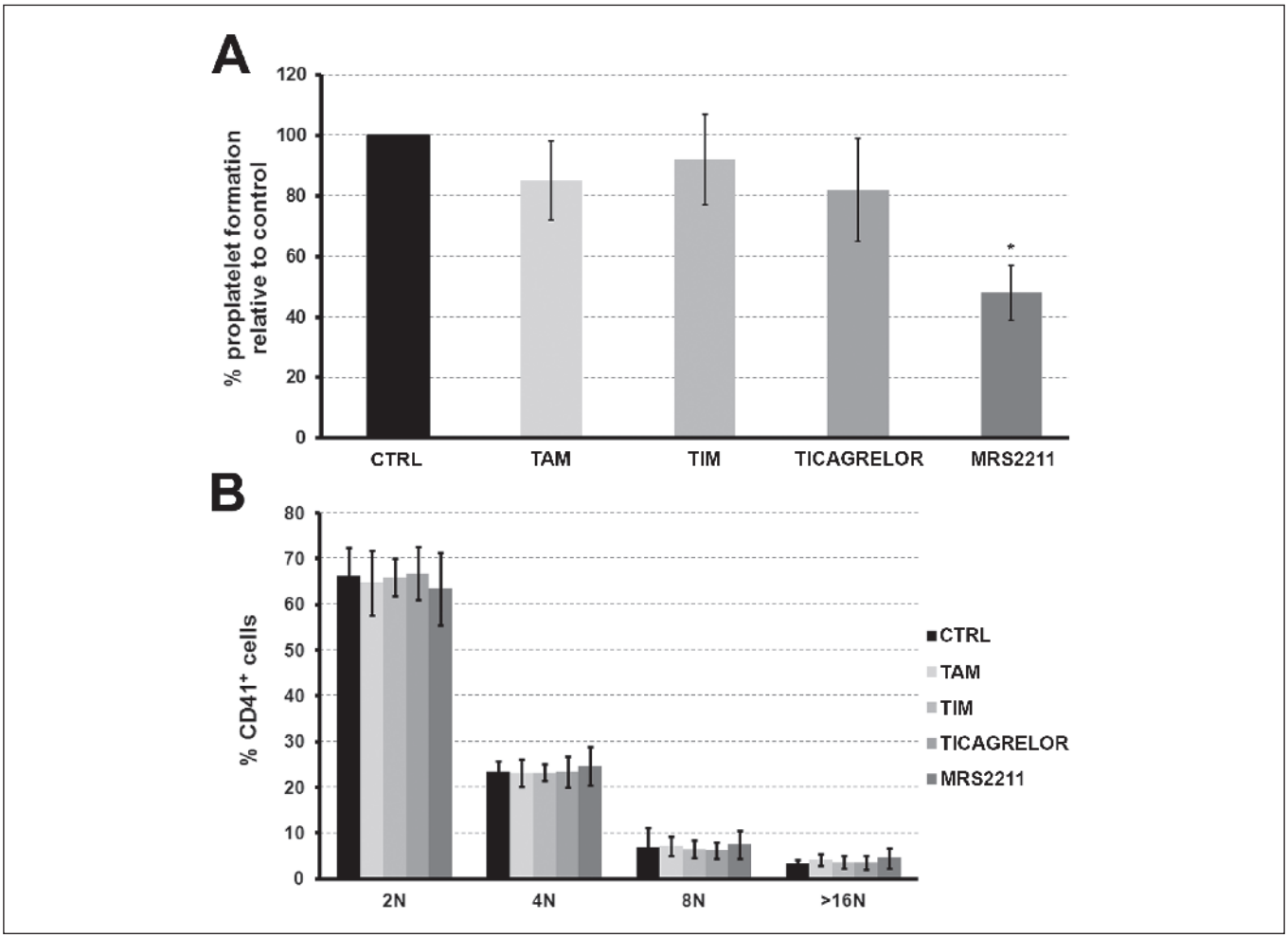

Figure 3: Analysis of the effects of tested compounds on pro-platelet formation and megakaryocyte ploidy. At day 13 of maturation, cord blood derived-megakaryocytes were seeded in the presence or absence of tested compounds for 16 h. A) Pro-platelet formation was quantified as percentage of megakaryocyte count and normalized relative to samples treated with vehicle alone (CTRL) (ANOVA and Bonferroni t-test as post-hoc test). Data are expressed as mean ± SD of eight independent experiments performed using *in vitro* differentiated megakaryocytes obtained from eight different cord blood samples ($p<0.05$). B) Effects of tested compounds on megakaryocyte ploidy. Data are expressed as mean ± SD of three independent experiments performed using *in vitro* differentiated megakaryocytes obtained from three different cord blood samples (p=NS).

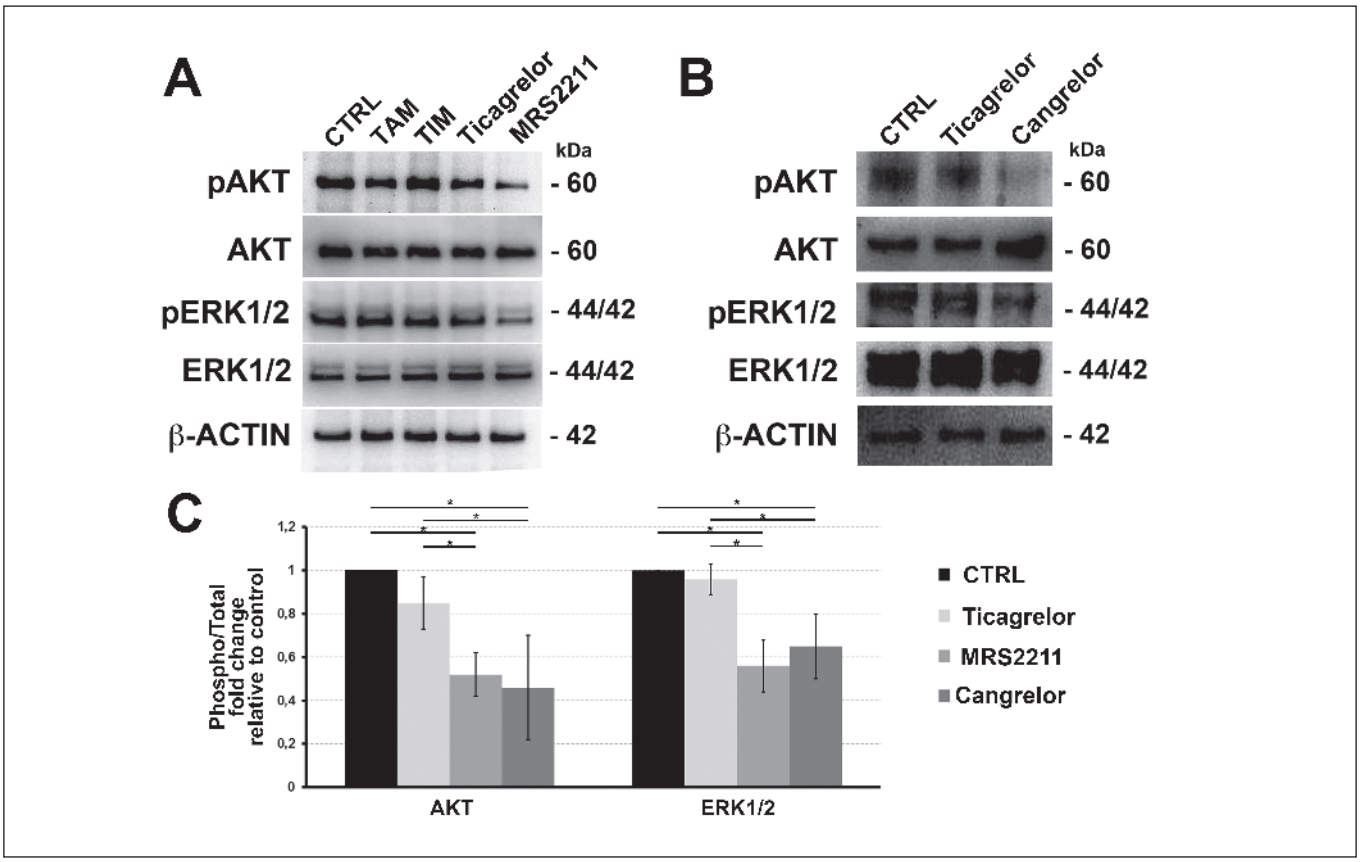

Figure 4: Effects of tested compounds on AKT and ERK1/2 phosphorylation in human megakaryocytes at day 13 of culture. A) Representative western blot analysis of AKT and ERK1/2 phosphorylation in human mature megakaryocytes after 16 h culture in the presence of ticagrelor, ticagrelor-AM (TAM), ticagrelor-IM (TIM) or MRS2211, compared to samples treated with vehicle alone (CTRL). Lysates were also probed with anti-AKT, anti-ERK1/2, and anti-β-actin antibodies in order to ensure equal loading. B) Representative western blot analysis of AKT and ERK1/2 phosphorylation in human mature megakaryocytes after 16 h culture in the presence of ticagrelor or cangrelor, compared to samples treated with vehicle alone (CTRL). Lysates were also probed with anti-AKT, anti-ERK1/2, and anti-β-actin antibodies in order to ensure equal loading. C) Analysis of relative densitometric analysis of phospho/total protein ratio in human megakaryocytes in presence of ticagrelor, MRS2211 or cangrelor relative to CTRL. Data are expressed as mean ± SD of four independent experiments performed using *in vitro* differentiated megakaryocytes obtained from four different cord blood samples (*$p<0.05$).

agonist activity at $P2Y_{13}$ was detected for any of the tested compounds (data not shown).

Studies with human megakaryocytes in culture

Effects of MRS2211, ticagrelor and its metabolites on pro-platelet formation

Pro-platelet formation by *in vitro* differentiated human megakaryocytes was not affected by ticagrelor, TAM or TIM, but, in line with previous observations (16), was significantly inhibited by MRS2211 (►Figure 3A) and cangrelor (not shown) by about 50%. None of the tested compounds significantly affected megakaryocyte ploidy (►Figure 3B) or viability, measured by the trypan blue dye exclusion assay (DMSO, 93 ± 4%; ticagrelor, 89 ± 7%; TIM, 91 ± 4%; TAM, 90 ± 6%; MRS2211, 88 ± 5%).

Effect on AKT and ERK1/2 phosphorylation in human megakaryocytes.

AKT and ERK1/2 phosphorylation is involved in the regulation of pro-platelet formation by human megakaryocytes (16, 19, 20). As shown in ►Figure 4, western blots indicated that the phosphorylation of AKT and ERK1/2 was markedly decreased in the presence of MRS2211 and cangrelor, thus confirming that the activation of these two molecules downstream of $P2Y_{13}$ is important for the control of platelet production by human megakaryocytes (16). In contrast, phosphorylation of AKT and ERK 1/2 was not affected by ticagrelor or its metabolites (►Figure 4). The densitometry analysis of blots revealed that the inhibition of AKT and ERK1/2 phosphorylation by MRS2211 and cangrelor was statistically significant (►Figure 4C).

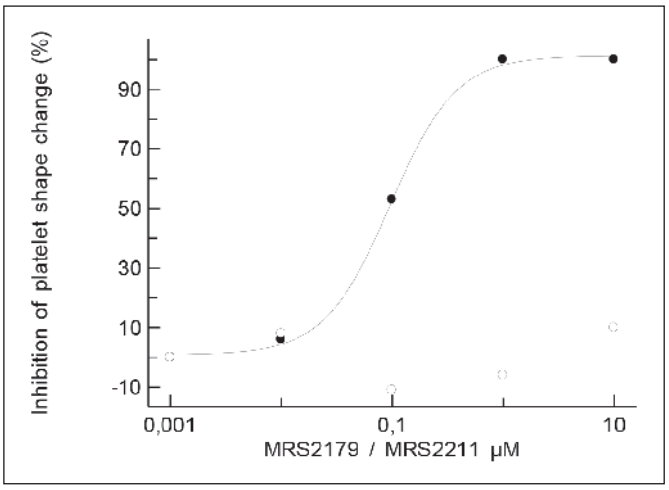

Figure 5: Effects of tested compounds on platelet shape change induced by 10 nM 2MeSADP. Samples of washed platelet suspensions were incubated with increasing concentrations of MRS2179, a $P2Y_1$ antagonist (●), or MRS2211, a $P2Y_{13}$ antagonist (○). Data are expressed as means of five separate experiments.

Effects of MRS2179 and MRS2211 on platelet shape change induced by 2MeSADP

The addition of 10 nM 2MeSADP to washed platelet suspensions caused platelets to change shape (detected by the aggregometer as an increase in optical density), which was not followed by platelet aggregation, due to the presence of EDTA, which prevents platelet aggregation. MRS2179, a $P2Y_1$ antagonist, concentration-dependently inhibited platelet shape change with an IC_{50} of 0.09 µM (►Figure 5). In contrast, the $P2Y_{13}$R antagonist MRS2211 did not inhibit platelet shape change (IC_{50} >10 µM) (►Figure 5).

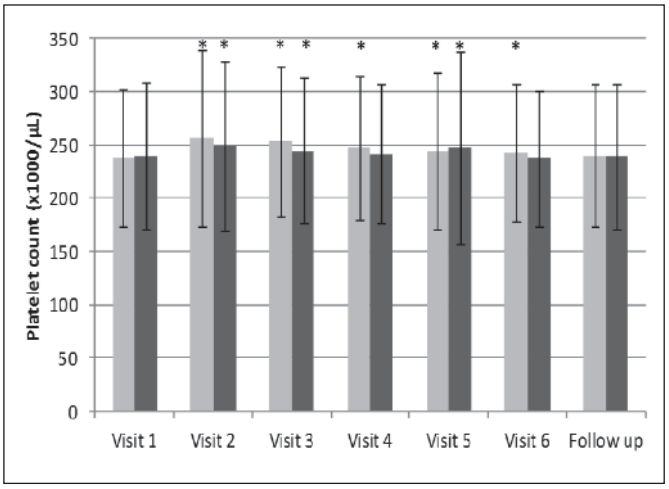

Figure 6: Platelet count data from the PLATO trial. Platelet counts (mean ± SD) in ticagrelor-treated patients (gray) and clopidogrel-treated patients (black). Visit 1: Randomisation (n=4393 ticagrelor, n=4375 clopidogrel), Visit 2: 1 month ± 10 days (n=3749 ticagrelor, n=3741 clopidogrel), Visit 3: 3 months ± 10 days (n=3456 ticagrelor, n=3468 clopidogrel), Visit 4: 6 months ± 10 days (n=3132 ticagrelor, n=3110 clopidogrel), Visit 5: 9 months ± 10 days (n=178 ticagrelor, n=172 clopidogrel), Visit 6: 12 months ± 10 days (n=2840 ticagrelor, n=2839 clopidogrel), Follow up: End of treatment + 1 month ± 10 days (n=2804 ticagrelor, n=2773 clopidogrel). *Significant change from randomisation, visit 1 (p>0.05), see Table 2.

Table 2: Statistical analysis of mean change from baseline in platelet count in PLATO.

Visit	n	Mean	95 % CI of mean		P-value
			Lower	Upper	
Ticagrelor					
2	3749	18.20	16.11	20.28	<0.0001
3	3456	14.62	12.92	16.31	<0.0001
4	3132	10.03	8.29	11.77	<0.0001
5	178	12.42	3.24	21.60	0.008
6	2840	4.87	3.11	6.63	<0.0001
Follow up	2804	1.63	-0.34	3.61	0.106
Clopidogrel					
2	3741	9.69	7.64	11.74	<0.0001
3	3468	5.64	3.91	7.37	<0.0001
4	3110	1.49	-0.24	3.23	0.091
5	172	8.56	0.45	16.67	0.039
6	2839	-1.68	-3.43	0.06	0.059
Follow up	2773	-0.71	-2.68	1.26	0.481

Platelet count (x1000/µl). Visit 2: 1 month ± 10 days, Visit 3: 3 months ± 10 days, Visit 4: 6 months ± 10 days, Visit 5: 9 months ± 10 days, Visit 6: 12 months ± 10, Follow up: End of treatment + 1 month ± 10 days.

Effect of treatment with ticagrelor or clopidogrel on the platelet count in ACS patients

Mean values for platelet counts were similar in ticagrelor- and clopidogrel-treated ACS patients enrolled in the PLATO trial at all the seven programmed visits (►Figure 6). Small but significant changes, mainly increases, in mean platelet count from baseline were noted in both treatment groups at a number of visits (►Table 1). At follow up, mean platelet count had returned to levels not significantly different from randomisation (►Table 2). The percentage of patients who displayed a decrease in platelet count by ≥100×10^9 platelets/l during the follow-up was 1.6 % in the ticagrelor group and 2.5 % in clopidogrel group.

Discussion

The aim of our study was to test whether or not ticagrelor, a direct-acting, reversibly-binding $P2Y_{12}$ antagonist approved for the prevention of thromboembolic events in patients with ACS, inhibits the functional activity of $P2Y_{13}$. Previous studies failed to show any significant activity of ticagrelor versus the four P_1 receptors for adenosine and other P2Y receptors (3) (AstraZeneca data on file).

However, the interaction of ticagrelor with $P2Y_{13}$ has never been tested previously. Given the high homology between $P2Y_{12}$ and $P2Y_{13}$, as well as the fact that cangrelor has been shown to inhibit $P2Y_{13}$ (17) and that this inhibition may have biological consequences (16), we considered that it was important to test whether ticagrelor also inhibits $P2Y_{13}$.

We used two different experimental approaches, which gave apparently diverging results. The experiments performed using human $P2Y_{13}$ receptor-transfected cells and a label-free cellular response assay showed that ticagrelor inhibits $P2Y_{13}$-mediated cellular responses. Similar results were obtained with its active metabolite (TAM), while its inactive metabolite (TIM) failed to show any activity against $P2Y_{13}$. None of four alternative P2Y12 antagonists (AZD1283, elinogrel, CAM and PAM) or AZD6482, an unrelated compound targeting PI3Kβ, displayed any activity versus $P2Y_{13}$. The potency of ticagrelor and TAM was similar to that of cangrelor. To support interpretation of the data generated, we performed detailed characterisation of the used cells system. ADP is known to activate $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$. In our transfected HEK293 cells, the stable ADP analogue 2MeSADP concentration-dependently activated both (+)dox cells ($P2Y_{13}$ expressing) and, albeit to a lesser extent, (-)dox cells (control). Selective inhibition of Gi signalling by PTX did not affect 2MeSADP-induced signalling in (-)dox cells, whereas it reduced 2MeSADP-induced signalling in (+)dox cells to the same level as (-)dox cells. . Although we did not assess transfection efficiency, our functional data clearly support that the PTX-sensitive response in (+)dox cells is $P2Y_{13}$ specific, because HEK293 do not to contain any $P2Y_{12}$ mRNA (21), which besides $P2Y_{13}$ is the only Gi coupled receptor responding to ADP and its analogue 2MeSADP. Ticagrelor, TAM and cangrelor all inhibited this $P2Y_{13}$ response, but had no activity vs the Gi-independent 2MeSADP response in (-)dox or (+)dox cells.

Previous studies indicated that HEK293 cells contain mRNA coding for $P2Y_1$ and $P2Y_{13}$ (21). However, the HEK293 cells that we used in our experiments clearly did not express any functional $P2Y_{13}$-related activities, as selective Gi inhibition by PTX did not affect the 2MeSADP response in (-)dox cells. It must be emphasised that, to the best of our knowledge, no protein expression or functional role has been attributed to the described $P2Y_{13}$ mRNA expression in HEK293 cells. In contrast, a functional response that is mediated by $P2Y_1$ has been demonstrated (21), which supports our hypothesis that the Gi-independent 2MeSADP response in our HEK293 cells is mediated by $P2Y_1$. Both MRS2211 (claimed to be $P2Y_{13}$ selective) and MRS2279 (claimed to be $P2Y_1$ selective) inhibited the response to 2MeSADP in (+)dox cells. As both compounds inhibited the response mediated by endogenous $P2Y_1$, which is part of the total 2MeSADP response of (+)dox cells, it was not possible to distinguish the $P2Y_{13}$-dependent from the $P2Y_1$-dependent component of their inhibition. Importantly, and in sharp contrast, cangrelor, ticagrelor and TAM inhibited the $P2Y_{13}$-mediated signal only, which is consistent with the previously reported $P2Y_{13}$ activity of cangrelor and selectivity of ticagrelor versus $P2Y_1$.

Our studies of pro-platelet formation by human primary megakaryocytes in culture revealed that ticagrelor, TAM and TIM did not inhibit the ability of megakaryocytes to produce pro-platelets, which, in contrast, was inhibited by antagonists of $P2Y_{13}$, such as MRS2211 and cangrelor (6, 15), confirming the results of our previously published studies (16). In our present study, we also showed that both MRS2211 and cangrelor, but not ticagrelor and its metabolites, inhibit AKT and ERK1/2 phosphorylation, which is involved in the regulation of pro-platelet formation by human megakaryocytes (16, 19, 20).

It appears, therefore, that ticagrelor and TAM inhibit $P2Y_{13}$-mediated signals in HEK293 transfected cells, but that this inhibition, unlike for cangrelor and MRS2211, does not translate into an effect on pro-platelet formation in megakaryocytes that naturally express $P2Y_{13}$.

Additional discrepancies between findings obtained with MRS2211 in HEK293 transfected cells and cells of the megakaryocyte/platelet lineage were observed in our experiments. When we tested MRS2211 in experiments of 2MeSADP-induced platelet shape change, which is mediated by platelet $P2Y_1$, we found that it did not interfere with this platelet function. This finding contrasts with our observation that MRS2211 inhibited the Gi-independent response in HEK293 cells, which is likely P2Y1-dependent. Since $P2Y_1$ is endogenously expressed by both HEK293 cells and platelets, the discrepant results are unlikely explained by artefacts induced by the process of transfection.

The lack of inhibitory effects of ticagrelor and its metabolites on pro-platelet formation by human megakaryocytes is in agreement with the observation that long-term treatment with ticagrelor (90 mg bid) did not significantly reduce the number of circulating platelets in aspirin-treated patients with ACS who were enrolled in the PLATO trial. On the contrary, there was a small, statistically significant but not clinically relevant increase in mean platelet

What is known about this topic?

- Some antagonists of the platelet $P2Y_{12}$ receptor for ADP (e. g. cangrelor) also antagonise the $P2Y_{13}$ receptor.
- $P2Y_{13}$ has been shown to play a role in the formation of proplatelets by human megakaryocytes in culture.
- Ticagrelor, an antithrombotic drug that antagonises $P2Y_{12}$, does not interact with other known P2Y receptors, but its interaction with $P2Y_{13}$ has never been tested.

What does this paper add?

- Our *in vitro* studies showed that ticagrelor inhibited the cellular responses in $P2Y_{13}$-transfected cells, but did not inhibit pro-platelet formation by megakaryocytes *in vitro*, at variance with the $P2Y_{13}$ antagonists MRS2211 and cangrelor.
- The platelet count of patients randomised to treatment with ticagrelor in the PLATO trial did not change during treatment and was comparable to those of patients randomised to clopidogrel.
- Ticagrelor acts as $P2Y_{13}$ antagonists in a transfected cell system *in vitro*, but this does not translate into any impact on pro-platelet formation *in vitro* or altered platelet count in patients.

count that returned to randomisation levels during follow up, both in ticagrelor- and clopidogrel-treated patients. These important data are reassuring in terms of safety of this important antithrombotic drug and provide strong support for the conclusion that the antagonist potency versus $P2Y_{13}$ documented in transfected HEK293 cells is not clinically relevant.

In conclusion, these studies show that ticagrelor and TAM can act as $P2Y_{13}$ antagonists using a transfected cell system *in vitro* but this does not translate into any impact on pro-platelet formation by human megakaryocytes *in vitro* or altered platelet count in patients.

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Conflicts of interest

SN is and AB was (at time of work) employees of AstraZeneca. MC has received research grants from AstraZeneca. RFS has received consultancy fees, research grants and honoraria from AstraZeneca; in addition, he is listed as an inventor on patent applications by AstraZeneca related to the PEGASUS-TIMI 54 trial results but has no financial interest in these.

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