INTRODUCTION

The exposure of the subendothelial thrombogenic surface after atherosclerotic plaque rupture and vascular injury during the percutaneous coronary intervention (PCI) facilitates platelet activation. Interestingly, pharmacotherapy with agents that inhibit platelet function has proven to be effective in the treatment of acute coronary syndrome. Proper re-endothelialization after angioplasty prevents adverse cardiovascular events. Therefore, in this in vitro study we examined how antiplatelet P2Y₁₂ receptor blockers can affect endothelial cells’ angiogenic properties. Endothelial cells were exposed to ticagrelor, prasugrel and clopidogrel in their highest concentrations obtained in serum after the treatment with loading and clinical doses. Further, the viability, apoptosis, and necrosis were tested and the following angiogenic properties such as proliferation, migration, invasiveness, tube formation, wound healing and the production of angiogenic mediators (bFGF, PDGF, Mmp-2, Ang-2, Timp-1). The results of this study showed that P2Y₁₂ receptor blockers in the tested concentrations are safe for endothelial cells. They neither induced necrosis or apoptosis nor changed the endothelial cell viability, migration, invasiveness, tube formation, wound healing, the production of VEGF or its receptors. However, they reduced cell proliferation. It was shown that out of these three drugs, ticagrelor in its loading concentration had the most potent angiogenic property. It reduced cell proliferation and changed the production of angiogenic (bFGF, Mmp-2) and angiostatic mediators (Ang-2). In conclusion, P2Y₁₂ receptor blockers in the concentrations obtained in the serum during standard therapy reduced endothelial cell proliferation. Despite this slight antimitogenic effect, they did not change endothelial cell tube formation or wound healing. Out of the three tested drugs, ticagrelor had the most potent angiogenic effect in vitro, but not strong enough to disturb tube formation and wound healing.

Key words: acute coronary syndrome, endothelial cells, P2Y₁₂ receptor blockers, angiogenesis, vascular endothelial growth factor, regeneration, cytokines, cell viability, apoptosis
needs active metabolites of thienopiridines, endothelial cells in vitro respond to either thienopyridine metabolites and thienopyridines themselves, leading to their reduced proliferation, the production of ECM proteins and release of vasoactive NO and PGI₂ (16-18). Extensive clinical studies highlight the improvement of endothelial cell function upon antiplatelet treatment (19-22). Ticagrelor seems to be particularly promising. As reported by Tornegren et al. (21) and Vieceli Dalla Sega et al. (22), ticagrelor improves endothelial vasodilatory function by increasing peripheral arterial function and eNOS level. Additionally Campo et al. showed that patients with stable coronary artery disease undergoing PCI intervention and treated with ticagrelor demonstrated a reduction of endothelial apoptosis, ROS production and increased NO level as compared to clopidogrel (19). Preservation of endothelial cell function is one of the most important goals of cardiac pharmacotherapy, which prevents restenosis, thrombosis, and is responsible for a proper vascular tone (23). Recent studies have revealed the potent antineoplastic effect of P2Y₁₂ receptor blockers (15, 17, 24-26), indicating their potential antiangiogenic effect (15, 17, 25). The impact of P2Y₁₂ receptor inhibitors on endothelium is independent of the antiplatelet effect (27). Gehbremskyn et al. reported that the antiangiogenic effects of ticagrelor are present both in vivo and in vitro. However, the results are not conclusive (24).

Most of the scientific works that examine the impact of P2Y₁₂ receptor blockers are based on clinical trials. In vitro studies have investigated the effect of thienopyridine (16-19, 22) and cyclopentyl-triazolopyrimidine (CPTP) on endothelium (19, 22). The concentrations of P2Y₁₂ receptor blockers tested in in vitro are too high (16-18, 24) and do not correspond to the levels detected in the blood during standard therapy (28-30). There is no comparative in vitro study of thienopyridines and CPTP group, in low concentrations, obtained in the blood after standard medical treatment.

In this study we intended to test how P2Y₁₂ receptor blockers, in the concentrations achievable in patients’ serum following standard medical treatments with loading and clinical doses, affect endothelial cell angiogenic properties that are so crucial for re-endothelization after coronary angioplasty in patients with ACS.

MATERIALS AND METHODS

Cell culture

The tests were performed using human umbilical vein endothelial cells HUVECs line EA.hy926 (kindly provided by Dr. CJ Edgell, University of North Carolina, Chapel Hill, USA) (31). The cells were routinely maintained in the Earl’s-buffered M199 culture medium, supplemented with amphotericin (2.5 µg/ml), gentamycin (50 µg/ml), L-glutamine (2 mmol/l), hydrocortisone (0.4 µg/ml), and 10% v/v fetal calf serum (Invitrogen, USA).

Unless indicated otherwise, all the reagents were purchased from Sigma-Aldrich (USA). Cell culture plastics came from Nunc (Denmark) and Costar (USA).

Experimental design

Endothelial cells were exposed to the culture medium supplemented with P2Y₁₂ receptor blockers (ticagrelor, prasugrel, clopidogrel) in their highest concentrations obtained in serum after its treatment with loading and clinical doses for 24 h (28-30). The following concentrations of the tested drugs were used in the experiment:

- ticagrelor (T): loading dose – 2 µmol/l, clinical dose – 1 µmol/l;
- prasugrel (P): loading dose – 2 µmol/l, clinical dose – 0.3 µmol/l
- clopidogrel (C): loading dose – 0.4 µmol/l, clinical dose – 0.04 µmol/l.

Cell viability, apoptosis, and necrosis

The effect on endothelial cell viability, apoptosis and necrosis after the exposure of P2Y₁₂ receptor blockers to the tested drugs was assessed by using an Apoptosis/Necrosis/Healthy Cells Detection kit (PromoKine, Heidelberg, Germany) according to the manufacturer’s instructions. First, the cells were harvested using trypsin-EDTA and collected by centrifugation at 150 × g for 10 min. Then, 2 – 3 × 10⁶ cells/ml were resuspended in a binding buffer and incubated for 1 hour with FITC-Annexin V, Ethidium Homodimer III, and Hoechst 33342 in adequate dilutions to detect apoptotic, necrotic, and healthy cells, respectively. Finally, the number of apoptotic, necrotic, and healthy cells in each population was determined by flow cytometry FACSaria III (Becton-Dickinson, Oxford, UK) with appropriate positive and negative controls. The viability of endothelial cells after their exposure to P2Y₁₂ receptor blockers was also determined using the Trypan blue exclusion test. HUVECs were suspended in Hanks’ solution and mixed in a ratio of 1:1(v/v) with 0.4% Trypan blue solution. After 10 min the number of blue-stained nonviable cells was counted in a hemocytometer.

Proliferation assay

Cell proliferation was measured using the MTT assay which measures the metabolic conversion of the MTT salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) by active mitochondrial dehydrogenases (32). The test was performed as described previously (33). Briefly, after exposure to P2Y₁₂ receptor blockers, the cells were incubated in a medium containing 1.25 mg/mL of the MTT salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) for 4 hours at 37°C. The formazan product generated was dissolved with the addition of an acidic solution of 20% w/v sodium dodecyl sulfate and 50% v/v N, N-dimethylformamide. The absorbance of the converted dye was recorded at 595 nm. The data were expressed as a percentage of the control group (cells cultured in a standard medium).

Migration/invasion assay

The endothelial migration was assessed using a 96-well Cell Migration Chamber (Boyden chamber) with 8 µm pore size membranes (Cultrex, R&D Systems USA). The cells at ~70% confluence were incubated in a culture medium supplemented with ± P2Y₁₂ receptor blockers for 24 hours. Next, the cells were harvested, washed, resuspended in a serum-free medium (SFM) and placed in an upper migration chamber previously coated with a coating buffer (for the migration assay) and a basement membranes extract (for the invasion assay), at a density of 20,000 cells per 50 µl SFM. The cells were then stimulated with the standard medium ± P2Y₁₂ receptor blockers for 24 h at 37°C. The cells that migrated were then detached, lysed, and labelled with a calcine AM according to the manufacturer’s instructions. Sample fluorescence was measured with a fluorescence microplate reader (Perkin Elmer, USA) using 480 nm and 520 nm wavelengths for excitation and emission, respectively. The data were expressed as a percentage of the control group (cells cultured in standard medium).
Tube formation

Cells at ~70% confluence were incubated in a culture medium (supplements and FBS). Then, they were harvested, washed, and resuspended in a culture medium ± P2Y₁₂ receptor blockers, VEGF and seeded on Geltrex (reduced growth factor basement membrane matrix, Invitrogen, USA) in 96-well plates. 30 µL/well of Geltrex was distributed on ice-cold 96-well plates. The gel was allowed to solidify by incubating the plate for 30 min at 37°C. The cells were then seeded at a density of 7.5 × 10⁴ cells/well in a culture medium supplemented with P2Y₁₂ receptor blockers and VEGF (20 ng/mL) for 24 hours. Tested drugs were also analysed in the presence of agonist (+ VEGF 100 ng/mL) and antagonist (+ sulforaphane 1 mM) of tube formation. The extent of tube formation was quantified by measuring the total tube length in three random fields in the well, using the software of microscope Carl Zeiss, Axio Observer D1 (Carl Zeiss, Germany). The mean of the total tube length (µm tube/mm²) was calculated using the microscope’s software for each well, with runs in duplicate. The control group consisted of the cells treated with a standard culture medium supplemented with VEGF (20 ng/ml).

Wound healing

Endothelial cells were grown to confluence in 6-well dishes. Under these conditions the cells remained viable in a nonproliferating state. The quiescent monolayer was injured by scraping with a disposable cell scraper (Nunc, Denmark). In order to compare the data from all the tests, it was critical to make a comparable injury in all the monolayers exposed to the medications (34). The monolayer was washed twice with the culture medium and then incubated with M199 after adding ± ticagrelor, prasugrel and clopidogrel. The closure of the denuded area was monitored using a Zeiss Axio Observer D1 inverted microscope (Carl Zeiss, Germany) equipped with a CO₂ module and incubator. During the experiment the cells were maintained in a 5% CO₂ humidified atmosphere at 37°C for the duration of the experiment. A video camera was attached to the microscope, and the images of the wounded area were captured as a digitalized sequence every 30 min, using the Axio-Vision Rel. 4.6.3. image analysis software. The results were calculated as a percentage of the healed area, 12 h after the initial wound. The assay is a well-established method to study wound healing in vitro and is thought to be particularly suited for measuring cell migration (35). In our study migration was additionally verified by using a Boyden chamber, as described in the Migration/Invasion section.

Cytokine measurements

Endothelial cells were cultured with M199 with supplements ± ticagrelor, prasugrel, and clopidogrel for 24 hours. The media were collected and analyzed for the constitutive concentrations of angiogenic/angiostatic mediators: bFGF, PDGF, MMP-2, and TIMP-1. The mediators were measured by using DuoSet Immunoassay Development Kits (R&D Systems) according to the manufacturer’s instructions. The sensitivity of the assay was: 12.7 pg/ml for the bFGF, 28.1 pg/ml for the PDGF, 319.2 pg/ml for the MMP-2, 14,4 pg/ml for the Ang-2, and 16.9 pg/ml for the TIMP-1. The results were normalized per cell protein in the culture wells. The protein concentration was measured using the Bradford method.

Intercellular VEGF and its receptors VEGF R1 and VEGF R2

For the purpose of the experiment, the cells were seeded into six-well plates and allowed to adhere for 24 hours. After the incubation with ± P2Y₁₂ receptor blockers for the next 24 hours, the cells were harvested with trypsin/EDTA and washed twice in PBS. In order to detect the expression of VEGF receptors, the endothelial cells (2.5 – 5 × 10⁵) were suspended in 100 µL PBS with 5 µL VEGF R1 and VEGF R2 (both R&D System, USA) monoclonal antibodies (both PE - conjugated)
and incubated for 30 min in darkness and RT. In order to find the intracellular VEGF expression, the endothelial cells (2.5 – 3 × 10⁵) were fixed in 2% PFA in PBS for 10 min (RT in darkness). Next, the cells were washed twice in PBS, and stained with 5 µL VEGF monoclonal antibodies (R&D System, USA; APC conjugated) in 100 µL 0.1% Triton X-100 for 30 min (RT in darkness). Finally, the surface receptors and the intracellular VEGF stained cells were washed twice, resuspended in 400 µL PBS and measured by a FACSAria III cytometer (Becton Dickinson, USA).

The data were analyzed by FACS Diva Software. The isotype controls such as IgG1 (R&D System, USA) for VEGF R1/R2, and IgG2 for VEGF (R&D System, USA) were used. At least 10,000 events were collected to create each histogram. The results of flow cytometric analysis were expressed as a percentage of the positively labelled cells (on both VEGF receptors). The results for intracellular VEGF were obtained by means of the MdFI ratio. It was calculated as a proportion of MdFI of specific VEGF antibody to MdFI of isotype control.

**Statistical analysis**

Statistical analysis was performed using GraphPad PrismTM 6.00 (GraphPad Software Inc, USA). The data were interpreted with repeated measures analysis of variance using a post hoc test for multiple comparisons (Dunn’s or Tuckey’s tests). A P-value < 0.05 was considered significant. The data were expressed as means ± SD.

**RESULTS**

**The effect of P2Y₁₂ receptor blockers on endothelial cell viability**

The exposure of HUVECs to P2Y₁₂ receptor blockers in their highest concentrations obtained in serum after its treatment with loading and clinical doses for 24 hours proved to be safe for cells (Figs. 1 and 2). The same proportion of
live, apoptotic and necrotic cells was observed after their exposure to ticagrelor, prasugrel, clopidogrel compared to the control cells in the standard medium (Figs. 1 and 2). The average percentage of apoptotic and necrotic cells after their exposure to the control medium was: 0.5% and 1.1% respectively (Fig. 1A). The average percentage of apoptotic and necrotic cells after their exposure to the clinical and loading concentrations of the following receptor blockers was as follows: ticagrelor 0.6 – 0.7% and 1.2 – 1.3% respectively (Fig. 1B), prasugrel 0.5 – 1.5% and 1.4 – 1.5% respectively (Fig. 1C), and clopidogrel 0.6 – 0.7% and 1.1 – 1.6% respectively (Fig. 1D). The average percentage of live cells after their exposure to the following receptor blockers was as follows: control medium 98.4% (Fig. 1A), ticagrelor 96 – 98% (Fig. 1B), prasugrel 97 – 98% and (Fig. 1C) clopidogrel 97 – 98% (Fig. 1D).

On using the Trypan blue exclusion test, an alternative method for measuring cell viability, we observed similar results for ticagrelor, clopidogrel and partially prasugrel (Fig. 1, Fig. 3A). Only the cells exposed to prasugrel in the concentrations obtained in serum after its treatment with a loading dose (2 µM) displayed a slightly lower viability (decreased by 17%) when compared with the control cells. The two methods assessing endothelial cell viability differ methodically, using various dyes for labeling viable cells, and the various detection, therefore we observed a slightly different endothelial response to the highest concentration of prasugrel. The trypan blue method showed somewhat lower endothelial cell viability after exposure to the highest level of prasugrel (Fig. 3A).

**The effect of P2Y$_{12}$ receptor blockers on endothelial cell proliferation, migration and invasiveness**

Proliferation represents a crucial step in angiogenesis. The proliferative capacity of endothelial cells treated with increasing concentrations of P2Y$_{12}$ receptor blockers was reduced (Fig. 3B). We have showed that the growth of cells is inhibited after exposing them to the concentration of P2Y$_{12}$ receptor blockers obtained in serum that was treated with clinical and loading doses of: ticagrelor 10% and 18% respectively, prasugrel 11% and 14% respectively, and clopidogrel 7% and 11% respectively. Migration and invasiveness have a pivotal role in the maintenance of microvascular integrity and angiogenesis. The treatment with ticagrelor, prasugrel, and clopidogrel did not significantly alter the migratory and invasive capability of HUVECs (Fig. 3C, 3D).
The effect of P2Y<sub>12</sub> receptor blockers on endothelial cell wound closure and tube formation

The closure of the denuded areas was monitored by time-lapse microscopy. Although the HUVECs displayed a slightly reduced potential to proliferate, they were still able to repopulate the denuded areas. In most cases the regeneration of wounds was completed after about 12 h (Fig. 4A). The surface area of the wounds present at the beginning of the experiment did not differ between the groups (confirmed by statistical analysis, data not shown). The average time needed for regeneration in the control group was 10.5 ± 3.5 h (95% of regeneration). Ticagrelor, prasugrel and clopidogrel in their clinical and loading concentrations regenerate wounds to a comparable extent: 10.2 – 13.0 h (84 – 97% of regeneration), 11.1 – 11.2 h (91 – 92% of regeneration) and 10.3 – 11.8 h (91 – 93% of regeneration) respectively (Fig. 4B).

We also performed an in vitro tube formation to model the reorganization of angiogenesis (Fig. 4C). In vitro tube formation is a multistep process dependent on cell adhesion, migration, differentiation, and growth. When seeded onto Geltrex matrices, endothelial cells cultured in the presence of P2Y<sub>12</sub> receptor blockers and VEGF (20 ng/mL) formed elaborated capillary networks (Fig. 4C, 4D, and Fig. 5). We found that the treatment with ticagrelor, prasugrel and clopidogrel did not change the formation of a capillary-like structure by HUVECs (Figs. 4C and Fig. 5). The tube formation increased in the presence of high concentration of proangiogenic VEGF (100 ng/mL = positive control) and decreased in the presence of angiostatic sulforaphane (negative control). Vascular endothelial growth factor in the high concentration increased almost three times tube formation when compared to the control and tested drugs, while sulforaphane reduced tube formation of nearly 50%. Ticagrelor, prasugrel, and clopidogrel did change tube formation in the presence of a high dose of VEGF and the presence of sulforaphane (Fig. 4D). The summarised data are shown in Fig. 4C, 4D and Fig. 5.

The effect of P2Y<sub>12</sub> receptor blockers on VEGF/VEGFR1/VEGFR2 expression in endothelial cells

VEGF is a specific endothelial cell mitogen with potent angiogenic properties. The most potent stimulus for VEGF...
production in endothelial cells is tissue hypoxia (36). The constitutive production of VEGF in HUVECs in a standard culture condition is meagre and under the detection limit of most commercially available kits (37). Using a specific monoclonal antibody against VEGF and its surface receptors, we documented how P2Y₁₂ receptor blockers can modify their expression. Fig. 6 shows the expression of intracellular VEGF (Fig. 6A) and the percentage of positively stained cells for surface receptors VEGFR1 (Fig. 6B), and VEGFR2 (Fig. 6C). Only a trace number of endothelial cells were positively stained for VEGF and its surface receptors after their treatment with ticagrelor, prasugrel and clopidogrel. (Panel D): cells were exposed for 24 h to standard culture medium supplemented with 20 ng/mL VEGF and angiogenesis inhibitor 1 mM sulforaphane. (Panel E): cells were exposed for 24 h to standard culture medium supplemented with 20 ng/mL VEGF and 2 µM ticagrelor. (Panel F): cells were exposed for 24 h to culture medium supplemented with 20 ng/mL VEGF and 0.4 µM clopidogrel.

**Fig. 5.** The exemplary photomicrographs of tube formation after the exposure of endothelial cells to P2Y₁₂ receptor blockers. Magnification × 100. (Panel A): endothelial cells were exposed for 24 h to standard culture medium supplemented with 20 ng/mL VEGF. (Panel B): endothelial cells were exposed for 24 h to standard culture medium supplemented with 100 ng/mL VEGF. (Panel C): endothelial cells were exposed for 24 h to standard culture medium supplemented with 20 ng/mL VEGF and angiogenesis inhibitor 1 mM sulforaphane. (Panel D): cells were exposed for 24 h to standard culture medium supplemented with 20 ng/mL VEGF and 2 µM ticagrelor. (Panel E): cells were exposed for 24 h to standard culture medium supplemented with 20 ng/mL VEGF and 1 µM prasugrel. (Panel F): cells were exposed for 24 h to culture medium supplemented with 20 ng/mL VEGF and 0.4 µM clopidogrel.

**DISCUSSION**

Human umbilical vein endothelial cells (HUVEC) are widely used in medical experiments. The study of global gene expression in endothelial cells from different vessel areas showed a relatively high degree of similarity between the HUVEC and coronary arteries, which justified their use in experimental studies on coronary circulation (38). In our study, we used immortalized cell line HUVEC EA.hy.926 in which the majority of genes expression at rest and under the stimulation share the same profile as in HUVEC (39). HUVECs line EA.hy926 are broadly used in *in vitro* studies testing the processes crucial for angiogenesis (40, 41).

Prompt regeneration of wounded endothelial cells is a precondition for protecting against vascular restenosis. We have recently demonstrated a model for the regeneration of vascular endothelial cells after injury, which mimics vascular damage after PCI (34). This model consists of a monolayer of endothelial cells that is manually wounded, using a cell scraper. The repopulation of the wounded area can then be continuously monitored by time-lapse microscopy, with the subsequent morphometric analysis. The main mechanism regenerating wounds is the migration process, which only to a small degree is caused by proliferation (34, 42). Using the permanent HUVEC EA.hy926, we also showed that wound healing after PCI could be drug dependent or independent (43, 44). The healing of the endothelial cells after PCI is time-consuming. Complete re-
Fig. 6. The effect of P2Y<sub>12</sub> receptor blockers on intracellular VEGF (panel A), VEGFR1 (panel B) and VEGF R2 (panel C) expression in endothelial cells. Endothelial cells were exposed for 24 h to the control medium, ticagrelor (T), prasugrel (P) and clopidogrel (C) in their highest concentrations obtained in serum after the treatment with loading and clinical doses. The data were analyzed with repeated measures analysis of variance using a post hoc test for multiple comparisons. The results are expressed as mean ± SD and derived from 4 independent experiments. Abbreviations: VEGF, vascular endothelial growth factor; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2.

Table 1. The effect of P2Y<sub>12</sub> receptor blockers on the endothelial production of angiogenic and angiostatic mediators released in the culture medium. Endothelial cells were exposed for 24 hours to the control medium, ticagrelor (T), prasugrel (P) and clopidogrel (C) in their highest concentrations obtained in serum after the treatment with loading and clinical doses. The data were interpreted with repeated measures analysis of variance using a post hoc test for multiple comparisons. The results are expressed as mean ± SD and derived from 4 independent experiments. Abbreviations: bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; MMP-2, metalloproteinase 2; Ang-2, angiopoietin 2; TIMP-1, tissue inhibitor of metalloproteinase-1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>T 1 μM</th>
<th>T 2 μM</th>
<th>P 0.3 μM</th>
<th>P 2 μM</th>
<th>C 0.04 μM</th>
<th>C 0.4 μM</th>
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<td>Angiogenic factors, pg/μg cell protein</td>
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<td>bFGF</td>
<td>0.25 ± 0.05</td>
<td>0.57 ± 0.09**</td>
<td>0.55 ± 0.10*</td>
<td>0.30 ± 0.07</td>
<td>0.28 ± 0.06</td>
<td>0.23 ± 0.06</td>
<td>0.26 ± 0.05</td>
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<tr>
<td>PDGF</td>
<td>0.79 ± 0.10</td>
<td>0.69 ± 0.11</td>
<td>0.64 ± 0.15</td>
<td>0.73 ± 0.12</td>
<td>0.71 ± 0.13</td>
<td>0.90 ± 0.17</td>
<td>0.83 ± 0.14</td>
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<tr>
<td>MMP-2</td>
<td>0.11 ± 0.03</td>
<td>0.07 ± 0.01*</td>
<td>0.06 ± 0.01**</td>
<td>0.08 ± 0.02</td>
<td>0.09 ± 0.02</td>
<td>0.10 ± 0.03</td>
<td>0.07 ± 0.01</td>
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<td>Angiostatic factors, pg/μg cell protein</td>
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<td>Ang-2</td>
<td>5.1 ± 0.3</td>
<td>4.7 ± 0.4</td>
<td>4.1 ± 0.2*</td>
<td>5.1 ± 0.4</td>
<td>5.4 ± 0.1</td>
<td>5.4 ± 0.6</td>
<td>5.9 ± 0.8</td>
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<td>TIPM-1</td>
<td>79.4 ± 10.6</td>
<td>73.6 ± 11.3</td>
<td>61.0 ± 12.3</td>
<td>72.4 ± 13.7</td>
<td>61.0 ± 11.1</td>
<td>64.0 ± 14.1</td>
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endothelization was first noted three months after stent implantation (45).

The results of this study showed that P2Y_12 receptor blockers, obligatory used after PCI for 9 – 12 months (3, 12, 46), in the concentrations obtained in patients’ serum, following their standard medical treatments in the loading and clinical doses, are safe for endothelial cells. Ticagrelor, prasugrel, and clopidogrel did neither change endothelial cell viability nor promote apoptosis nor necrosis, which was also observed in patients, especially treated with ticagrelor (19). Crucial angiogenic steps such as migration, invasiveness and the production of proangiogenic VEGF and its receptors were also not disturbed. Moreover, the receptor blockers significantly reduced cell proliferation. Despite this slight antimitogenic effect they did not change the endothelial cell tube formation or wound healing. Tube formation is a holistic process that combines (i) proliferation, (ii) migration, (iii) invasiveness, and (iv) the production of many angiogenic/angiostatic factors in vitro. Although ticagrelor shows the greatest diversity in the production of crucial factors promoting (↑ bFGF, ↓ MMP-2) and inhibiting angiogenesis (↓ Ang-2), it did not affect the in vitro tube formation and wound healing. Its antiangiogenic effect observed in vivo could be more easily explained than effect in vitro. Ticagrelor in vivo: (i) inhibits platelets which release many growth factors (22, 24), (i) modifies endothelial functions (16, 19, 21, 22) and (ii) inhibits the cellular uptake of adenosine, which affects angiogenesis (47, 48). As reported by Gebremeskel et al. (24) its direct in vitro effect on endothelium is weak and depends on platelets inhibition. This observation is consistent with experiments of Viccini Dalla Sega et al. (22) which documented that ticagrelor improved endothelial function by lowering angiogenic EGF leading to the activation of eNOS. In our in vitro study we did not observe any changes in eNOS production by ticagrelor and other tested drugs (our personal observations - data not shown) and the changes in VEGF and its receptors. These observations could partially explain the lack of influence of ticagrelor on tube formation and endothelial healing especially that NO, and VEGF are known to be crucial angiogenic factors (49).

The reduction of cell proliferation after P2Y receptors blockade is mediated by the lack of pro-mitogenic nucleotides. Nucleotides react synergistically with growth factors (PDGF, EGF, TGF-β, IGF-1, insulin, etc.). They are released from endothelial cells, platelets, sympathetic nerves, damaged cells in atherosclerotic plaque, restenosis and ischemia, stimulating proliferation of endothelium and myocytes (neointima formation) (50). The reduction of cell proliferation was previously observed in the endothelial cell culture after its exposure to the high concentration of ticlopidine (6 – 150 µM) (thienopyridine derivate - withdrawn due to side effects) (17) and thienopyridine SR 25989, an enantiomer of clopidogrel lacking antiaggregant activity (75 – 150 µM) (27). In its high

Fig. 7. The exemplary FACS histograms of the intracellular VEGF level after the exposure of endothelial cells to P2Y_12 receptor blockers. (Panel A): endothelial cells were exposed for 24 h to standard culture medium; (panel B): a isotype control; (panel C): cells were exposed for 24 h to standard culture medium supplemented with 2 µM ticagrelor; (panel D): cells were exposed for 24 h to culture medium supplemented with 1 µM prasugrel; (panel E): cells were exposed for 24 h to culture medium supplemented with 0.4 µM clopidogrel.
concentration, SR 25987 (clopidogrel 18 – 150 µM) inhibits endothelial cell migration, regeneration and proliferation \textit{in vitro} by increasing the expression of endogenous thrombospondin-1, which is a natural inhibitor of angiogenesis (27, 51). The inhibition of cell proliferation and migration exerted by clopidogrel was stronger than ticlopidine and was not inhibited by acidic or basic fibroblast growth factor (aFGF, bFGF) or by platelet derived growth factor (PDGF) (27).

We tested many of the crucial angiogenic and angiostatic factors such as VEGF and its receptors, bFGF, PDGF, MMP-2, angiopeotin-2 and TIMP-1 and their production in small concentrations of thienopyridines (0.04 – 2 µM) and ticagrelor (1 – 2 µM). We observed that only ticagrelor is potent enough to modify some of these factors (bFGF, MMP-2, Ang-2). Smadja et al. documented no changes in angiogenic factors (VEGF and its receptors, SDF-1, PI(3)K) and endothelial cell activation (vWF, sE-selectin) after short (1 day) and long (7 days) time of clopidogrel administration in healthy male volunteers (20). No changes of EGF level after one-month treatment of clopidogrel was also observed by Vieceli Dalla Sega et al. whereas the ticagrelor reduced the concentration of this proangiogenic factor which contributed to increase eNOS expression (22).

The effect of thienopyridines exerted on endothelial cells \textit{in vitro} is easier to detect than on platelets. Platelets respond only to thienopyridine derivates which are created \textit{in vivo} using liver microsomal enzymes (1, 52). As reported by Jakubowski et al. the effect of thienopyridines exerted on endothelium is independent of their antiplatelets action and mediated by unchanged drug and their derivates. Therefore, the authors stated that thienopyridines and their derivatives (thienopyrimidiones) might be regarded as endothelial drugs (16). The tests which examined the impact of thienopyridines \textit{in vitro} revealed a wide variety of action exerted on endothelium such as: (i) reduced cell proliferation and fibronectin production, which diminished platelet adhesion to subendothelium (17), (ii) release of vasoactive NO and PGI$_2$ (16, 18), (iii) reduced chemokine production and monocyte adhesion (53), and (iv) promotion of migration (54) and (v) apoptosis (55). The recent work by Torngeen et al. noted that ticagrelor improves peripheral arterial function more potently than thienopyridines (clopidogrel and prasugrel) do (21). This vasodilatory effect of ticagrelor is achieved by inhibiting the cellular uptake of adenosine (47, 56) and the release of ATP from cells (57). ATP is released from endothelium due to shear stress. The ectoenzymes secreted from cells degrade it to adenosine, which inhibits vascular smooth muscle cell proliferation via P1 receptor (A2) (47) and stimulates endothelial cell proliferation, migration and tube formation (48).

P2 receptors mediate the action of extracellular nucleotides ATP, ADP, UTP, and UDP and regulate several physiologic responses including cardiac function, vascular tone, muscle contraction, cell proliferation, neurotransmission, platelet aggregation, glomerular permeability to albumin, and the release of endothelial mediators (1, 14, 58). P2 receptors can be divided

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**Fig. 8.** The exemplary FACS histograms of the VEGF R1 expression after the exposure of endothelial cells to P2Y$_{12}$ receptor blockers. (Panel A): endothelial cells were exposed for 24 h to standard culture medium; (panel B): a isotype control; (panel C): cells were exposed for 24 h to standard culture medium supplemented with 2 µM ticagrelor; (panel D): cells were exposed for 24 h to culture medium supplemented with 1 µM prasugrel; (panel E): cells were exposed for 24 hours to culture medium supplemented with 0.4 µM clopidogrel.
into two classes depending on their signal transmission mechanisms. P2X (P2X_1 – P2X_7) are the receptors mediated by ion influx into the cells, and P2Y (P2Y_1, P2Y_2, P2Y_4, P2Y_6, P2Y_11, P2Y_12, P2Y_13, P2Y_14) are the receptors mediated by G-proteins. In arteries, extracellular nucleotides regulate the vascular tone by activating both the P2X and P2Y receptors on smooth muscle cells and endothelial cells (1, 14, 59, 60). Various sources investigate the role of the following P2 receptors on platelets: P2X_1, P2Y_1, P2Y_6 (61, 52), human endothelial cells: P2X_4, P2Y_1, P2Y_2, P2Y_6, P2Y_11, P2Y_12 (54, 59, 63, 64) and myocytes: P2X, P2Y_2, P2Y_6, P2Y_12 (59, 65). The data concerning the presence of P2Y_12 receptors on platelets is consistent, but its expression on the endothelial cell and vascular smooth muscle cells is still contradictory (54, 59, 66). Wang et al. documented that P2X_7, P2Y_2, and P2Y_6 are the most expressed P2 receptors in SMC, which probably mediate the contractile and mitogenic action of extracellular nucleotides (59). Expression of P2 receptors on the cell surface vary depending on endothelial cells (HUVEC, HUVEC line926, Bovine Aortic EC, transformed endothelial cells ECV340), which have a high expression on their surface, and do not have a P2Y_12 receptor. Therefore we hypothesized that effect exerted by P2Y_12 receptor blockers on endothelium could be mediated (i) not only by P2Y_12 receptor but (ii) all nucleotide receptors presented on the endothelial surface or is caused by the (iii) direct effect of P2Y_12 receptor blockers on nucleotide concentration which reacts with P1 and P2 receptors. Similarly, the vasoconstriction of vascular smooth muscle cells is not only mediated by the activation of P2Y_12 and also P2Y_2, P2Y_6 and P2X_1 receptors (59, 65) and vasodilatation and antimitotic effect of P2Y_12 receptor blockers might be mediated by all nucleotide receptors on vascular smooth muscle cells (15). Other hypothesis suggested by Jakubowski’s personal observations is that some of the effects exerted by thienopyridines and its derivatives (NO release) might not be a result of the stimulation of the P2 and P1 receptors. Neither non-specific antagonists of P2 and P1 receptor changed the observed results (16). The explanation of how P2Y_12 receptor blockers react with endothelial cells which do not have the P2Y_12 receptor is complicated and requires further insightful analysis.

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**Fig. 9.** The exemplary FACS histograms of the VEGFR1 expression after the exposure of endothelial cells to P2Y_12 receptor blockers. (Panel A): endothelial cells were exposed for 24 h to standard culture medium; (panel B): a isotype control; (panel C): cells were exposed for 24 h to standard culture medium supplemented with 2 µM ticagrelor; (panel D): cells were exposed for 24 h to culture medium supplemented with 1 µM prasugrel; (panel D): cells were exposed for 24 h to culture medium supplemented with 0.4 µM clopidogrel.
Antiplatelets P2Y12 receptors blockers, besides their cardioprotective function, are also useful for treating neoplastic disorders (24, 67, 68). Platelets also play a crucial role in vascular homeostasis through the release of inflammatory mediators, growth and coagulation factors, and by interacting with other cell types, such as endothelial cells, leukocytes, and smooth muscle cells. The involvement of platelets in tumour progression is well recognized. The activation of platelets is a contributing factor to cancer progression by (i) increasing tumour cells adhesion to endothelium, (ii) promoting angiogenesis, and (iii) stimulating tumour cells growth by the release of ATP and ADP nucleotides (69). ATP released from activated platelets interacts with P2Y12 receptors on endothelial surface, accelerating their migration and the transendothelial migration of tumour cells.

The lack of P2Y12 receptor reduces the risk of metastasis (26). The depletion of circulating platelets or pharmacologic inhibition of platelets activation (via P2Y12 or GPIIbIIIa) decreases the metastatic potential of circulating tumour cells in the metastasis mouse model (24). The treatment with either ticagrelor or clopidogrel leads to a significant reduction in lung metastases (24, 68). Gebremskel et al. showed that co-incubation with platelets from ticagrelor-treated mice exhibited reduced adhesion to endothelial monolayers compared to those animals where platelets were co-incubated with PBS. Pre-treatment of endothelial monolayers with ticagrelor alone did not result in a reduced tumour cell adhesion and did not change the expression of another purinergic receptor P2Y2. These results exhibit the role of platelets in tumour progression and show no effect of P2Y12 on endothelium itself (24).

The effect and clinical benefit of P2Y12 receptor antagonist may not be limited to platelet inhibition and the prevention of arterial thrombus formation. Thienopyridines and CCPT derivative differ in the model of action and might provide different potential. On balance, our results indicate that ticagrelor, prasugrel, and clopidogrel at clinically relevant concentrations obtained in the serum retain the endothelial cells’ viability and thus are safe to use. Although all the tested drugs reduce cell proliferation, they do not limit cell regeneration after the injury mimicking those occurring after PCI and tube formation. Out of all the tested drugs ticagrelor seems to have the most potent modifying effect on the production of angiogenic and angiostatic factors in vitro but its potential in vivo, in tested concentrations, is not strong enough to reduce wound healing and tube formation.

Acknowledgments: This work was supported by the Polish Cardiac Society scientific grant funded by ADAMED (2016-2018).

Conflict of interests: None declared.

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Received: June 12, 2018
Accepted: August 30, 2018

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