Combination Therapy with DETA/NO and Clopidogrel Inhibits Metastasis in Murine Mammary Gland Cancer Models via Improved Vasoprotection

Kseniia Porshneva,† Diana Papiernik,† Mateusz Psurski,† Marcin Nowak,‡ Rafał Matkowski,§∥ Marcin Ekiert,$∥ Magdalena Milczarek,† Joanna Banach,† Joanna Jarosz,† and Joanna Wietrzyk*,†

†Department of Experimental Oncology, Hirsfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw, Poland
‡Department of Pathology, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, 50-375 Wroclaw, Poland
§Division of Surgical Oncology and Clinical Oncology, Department of Oncology, Wroclaw Medical University, 50-367 Wroclaw, Poland
∥Lower Silesian Oncology Center, 53-413 Wroclaw, Poland

Supporting Information

ABSTRACT: Vascular endothelial dysfunction and platelet activation play a key role in tumor metastasis, and therefore, both of these processes are considered important therapeutic targets in cancer. The aim of our studies was to analyze antimetastatic activity of combination therapy using nitric oxide donor DETA/NO and antiplatelet drug clopidogrel. Nitric oxide acts as a vasoprotective mediator, while clopidogrel inhibits ADP-mediated platelet aggregation. 4T1-luc2-tdTomato cell line transplanted intravenously (i.v.) and 4T1 cell line transplanted orthotopically were used as metastatic mammary gland cancer models. Moreover, antiaggregation action of compounds was tested ex vivo on the blood samples taken from breast cancer patients. We have shown that in selected dosage regimes, DETA/NO combined with clopidogrel significantly reduced lung metastatic foci formation in an i.v. model, and such inhibition was transiently observed also in an orthotopic model. The antimetastatic effect was correlated with a significant increase of prostacyclin (PGI2) metabolite and reduction of endothelin-1, sE-selectin, sI-CAM, and TGF-β plasma levels as well as decreased V-CAM expression on the endothelium. Combination therapy decreased fibrinogen binding to the resting platelets at the early stage of tumor progression (day 14). However, at the later stages (days 21 and 28), the markers of platelet activation were detected (increased JON/A antibody bound, P-selectin level, binding of fibrinogen, and vWf). Decreased aggregation as well as a lower release of TGF-β were detected in platelets incubated ex vivo with compounds tested from metastatic breast cancer patients. Although combination therapy increases E-cadherin, the increase of N-cadherin and α-SMA in tumor tissue was also observed. The results showed that at the early stages of tumor progression, combined therapy with DETA/NO and clopidogrel improves vasoprotective and antiplatelet activity. However, in advanced tumors, some adverse effects toward platelet activation can be observed.

KEYWORDS: NO donors, DETA/NO, clopidogrel, breast cancer, metastasis, vasoprotection, platelet activation, endothelial dysfunction

1. INTRODUCTION

Platelet activation and endothelium dysfunction are the two main factors that sustain the metastatic process. During metastasis, circulating cancer cells and disrupted endothelium contribute to platelet activation. Activated platelets interact with...
migrating cancer cells, thus helping them to evade immune response. Activated platelets express P-selectin and activated glycoprotein (GP) IIb/IIIa (integrin αIIbβ3, CD41/61) on their surface. Besides that, von Willebrand factor (vWF) binding to the GPⅡb (CD42) facilitates platelet aggregation and adhesion to vascular endothelium. P-selectin helps cancer cells to adhere both to the platelet surface and blood vessel wall. Accordingly, activated platelets contribute to cancer cell adherence to the vessel wall and subsequently to transmigration to the distant organs. On the other hand, vascular endothelium plays a crucial role in tumor spreading and invasion. Under normal physiological conditions, activated vascular endothelium controls vascular permeability, inflammation, thrombosis, and hemostasis. Endothelium dysfunction develops when endothelial cells remain in an activated stage for a prolonged period of time, which occurs during cancer progression. Endothelium dysfunction is characterized by downregulation of vasoprotective mediators such as nitric oxide (NO) and carbon monoxide (CO). This leads to overexpression of surface adhesion molecules I-CAM 2, 3, CD41/61) on their surface. I-CAM, thromboksyne (TX), and endothelin-1 (ET-1). Moreover, downregulation of NO and CO, which under normal conditions sustains hemostasis, contributes to uncontrolled platelet activation and enhanced blood coagulation.

Nitric oxide is a gaseous signaling molecule with a variety of properties. It is produced from l-arginine by NO synthesize during a process that requires multiple substrates and cofactors. There are three isofoms of NO synthesize: nNOS (inducible), nNOS (neuronal), and eNOS (endothelial). iNOS is activated by TNF-α and IFN-γ, while nNOS and eNOS are Ca2+-dependent. Nitric oxide produced by eNOS is one of the antithrombogenic agents that inhibits platelet aggregation by a cGMP-dependent mechanism. The platelet inhibitory effect of cGMP is mediated by cGMP-dependent protein kinase. Protein kinase phosphorylation results in inactivation of small G-proteins of the Ras and Rho families, Ca2+ release inhibition, and actin cytoskeleton dynamics modulation. It was demonstrated that endogenously produced NO stimulates cancer cell proliferation, whereas iNOS inhibition as well as exogenous delivery of NO suppress the growth of these cells. Diazoniumdiolates (also known as “NONOates”) are one of the most promising NO donors. NONOates decompose spontaneously in a solution at physiological conditions and generate up to two molar equivalents of NO. Presently, NONOates are not used clinically, but they are tested frequently in different experimental models.

Clopidogrel is an antplatelet agent whose use in antimetastatic therapy is of great interest to researchers. It acts on the P2Y12 platelet ADP receptor, successfully inhibiting ADP-mediated platelet aggregation. A recent study has shown that treatment with clopidogrel combined with a nanoformulation of cissapin results in a strong inhibitory effect of the tumor growth in 4-week-old BALB/c female mice injected with 4T1 breast cancer subcutaneously. Moreover, such combination therapy was associated with a higher survival rate compared with cisplatín nanoparticles alone. These effects were achieved by enhanced permeability of the tumor vasculature induced by clopidogrel, which in turn increases tumor drug delivery. Furthermore, clopidogrel is described as an agent that improves the bioavailability of endothelial NO in patients suffering from coronary artery disease. During the past few years, the interest to the concomitant administration of NO donors and clopidogrel in different therapeutic approaches has been remarkably increased. Lee et al. used sustained released nitrates (SRN) in combination with clopidogrel to enhance the inhibition of platelet aggregation. Furthermore, Kirkby et al. have demonstrated that blockade of platelet P2Y12 receptors strongly elevated the ability of NO to inhibit platelet activation. Nevertheless, there are no studies of the concomitant application of clopidogrel with nitric oxide donors for the inhibition of tumor cell survival in the circulation as well as for the inhibition of cancer metastasis through the normalizing of endothelial metabolism.

Thus, our studies aimed to test the hypothesis that the combined application of clopidogrel with a nitric oxide donor could have a promising effect in antimetastatic therapy. For this reason, we evaluated the antimetastatic effects of clopidogrel combined with DETA/NO in intravenous and orthotopic models of 4T1 and 4T1-luc2-tdTumor mouse mammary gland cancer. We also analyzed the effect of such combination therapy on ex vivo collagen-induced platelet aggregation using platelet-rich plasma (PRP) from breast cancer patients.

2. EXPERIMENTAL SECTION

2.1. Compounds. Clopidogrel was extracted from an antiplatelet drug PLAVIX (Sanofi Pharma, California, USA) in the Laboratory of Biomedical Chemistry, Institute of Immunology and Experimental Therapy in Wroclaw. Its purity was as high as 99% as shown by HPLC. Nitric oxide (NO) donor DETA/NO (Cayman Chemical, Michigan, USA), a diazeniumdiolate with a half-life of 21 h was chosen as an exogenous source of NO.

2.2. Mice. BALB/c female mice (6-8 weeks old) obtained from the Center of Experimental Medicine, Medical University of Bialystok, Poland were used. All experiments were performed according to the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education issued by the New York Academy of Sciences’ Ad Hoc Committee on Animal Research and in line with the European Union rules. Experimental procedures were approved by the Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland (permissions numbers: 46/2013 (with annexes: 38/2015 and 55/2015), 78/2015, 63/2016, 23/2017).

2.3. Cells. Mouse mammary adenocarcinoma 4T1 cells were obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in RPMI 1640 (IIET, Poland) with Opti-MEM (Life Technologies, USA) (1:1 v/v) medium with 5% fetal bovine serum (HyClone, Thermo Fisher Scientific Inc., UK), supplemented with 4.5 g/L glucose and 1.0 mM sodium pyruvate (all from Sigma-Aldrich, Germany). The mouse mammary adenocarcinoma 4T1-luc2-tdTumor cell line expressing stably the firefly luciferase gene and tdTomato fluorescent protein were obtained from Caliper Life Sciences Inc. (USA). These cells were cultured in RPMI 1640 + Gluta-MAX medium (Life Technologies, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Germany). Both culture media were supplemented with 2 mM glutamine (Sigma-Aldrich, Germany) as well as penicillin (100 U/mL) and streptomycin (100 µg/mL) (both from Polfa Tarchomin S.A. Warsaw, Poland). Cell cultures were maintained in a humidified atmosphere at 37°C with 5% CO2.

2.4. Design of Experiments. 2.4.1. Cell Transplantation. Prior to the transplantations, all cells were trypsinized (IIET, Poland), centrifuged (200g, 4°C, 5 min), and counted. For the model of experimental metastasis, 4T1-luc2-tdTumor cells were suspended in Hank’s Balanced Salt Solution (HBSS; IIET, Poland), and then, the suspension of 7.5 × 10⁴ 4T1-luc2-tdTumor cells in 0.1 mL of HBSS was injected into the lateral tail vein (i.v.). For the model of spontaneous metastasis...
(orthotopic), 4T1 cells were suspended in HBSS, and then, the suspension of $1 \times 10^4$ 4T1 cells in 0.050 mL of HBSS was inoculated into the mammary fat pad of mice.

2.4.2. Drug Administration. DETA/NO was administered intraperitoneally (i.p.) every 24 h (QD). We used two different dosages of the compound: 1.6 mg/kg/24 h (~0.01 mmol/kg) or 2.4 mg/kg/24 h (~0.015 mmol/kg). To induce inflammation, mice were injected with 1 mg/kg of LPS i.v. 5 h before i.v. cancer cell inoculation (Table 1).

<table>
<thead>
<tr>
<th>route of cell inoculation</th>
<th>LPS (1 mg/kg)</th>
<th>single dose of DETA/NO (mg/kg/24 h)</th>
<th>CLO and DETA/NO pretreatment</th>
<th>time of experiment termination</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. intravenous</td>
<td>+</td>
<td>2.4</td>
<td>7 days</td>
<td>24 h</td>
</tr>
<tr>
<td>1. model of extravasation</td>
<td>+</td>
<td>1.6</td>
<td>2.5 h</td>
<td>14 days</td>
</tr>
<tr>
<td>2. model of settlement</td>
<td>+</td>
<td>2.4</td>
<td>2.5 h</td>
<td>14 days</td>
</tr>
<tr>
<td>B. orthotopic</td>
<td>-</td>
<td>1.6</td>
<td>treatment started 14, 21, and 28 days after cells inoculation</td>
<td></td>
</tr>
</tbody>
</table>

Clopidogrel (CLO) was administered in the fodder: standard mice diet AIN93G + clopidogrel at a dose of 15 mg/kg per day (calculated on the basis of mean daily food intake), whereas all other groups received AIN93G food without the drug from the same manufacturer (ZooLab, Sedziszow, Poland). The food was changed every 2 days.

The experiment with the intravenous cell inoculation (models of experimental metastasis) lasted 24 h (model of extravasation) or 14 days (model of lung settlement), whereas the experiment with the orthotopic cell inoculation (model of spontaneous metastasis) lasted 14, 21, and 28 days. Then mice were euthanized (Table 1), and lungs, tumor, aorta, and blood were harvested for analyses.

2.5. Estimation of Antitumor Activity. When tumors became palpable, their maximum length and width were measured three times a week, and the tumor volume was calculated according to the following formula

$$TV = \frac{1}{2} \times a^2 \times b$$

where TV is tumor volume, $a$ is the shorter diameter, and $b$ is the longer diameter.

2.6. Evaluation of Antimetastatic Effect. To determine antimetastatic activity of the compounds, the lungs of tumor-bearing mice were excised and fixed in 4% paraformaldehyde in phosphate buffer, and metastatic foci were counted visually. In the model of experimental metastasis (24 h observation time), ex vivo visualizations of the fluorescence of cancer cells localized in lungs were performed using the in vivo MS FX PRO system (Carestream Health INC., USA) as previously described.

Images were analyzed with Carestream MI SE software (Carestream Health INC., USA). The intensity of the fluorescence signal is shown as the mean intensity of the region of interest and expressed in arbitrary units [AU].

2.7. Tumor Blood Perfusion. Blood flow in the tumor was investigated on day 24 of the experiment using a high-frequency ultrasound Vevo 2100 system for small animal research as previously described. A volume of 50 µL of dissolved contrast MicroMarkerTM Contrast Agent (VisualSonics, Ontario, Canada) required for the visualization was administered to the lateral tail vein of an immobilized mouse. Mice were subjected to general anesthesia by injection of 0.5 mg/kg of medetomidine solution followed by continuous administration of a 2–3% isoflurane mixture (Baxter, Deerfield, Germany) in synthetic air (200 mL/min). The wash-in rate parameter was calculated using the Vevo LAB 1.7.1. Software (VisualSonics, Ontario, Canada).

2.8. Analysis of Protein Expression. 2.8.1. Tissue Lysate Preparation. Tumors taken from mice were frozen in liquid nitrogen and then stored at −80 °C. In total, 300 mg of sample was collected from the frozen tissue and subsequently transferred to cork-sealed homogenizing tubes containing a homogenizing ball (Mp Biomedicals LLC., Santa Ana, USA) and 500 µL of RIPA buffer with phosphatase and protease inhibitor cocktail (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Tissues were homogenized using the Fast Prep-24 MP Bio homogenizer (Mp Biomedicals LLC., Santa Ana, USA) and stored as previously described.

Protein concentration was determined by the Bradford dye-binding method.

2.8.2. Western Blot Analyses. The analysis of markers related to EMT was performed on tumor lysates using the Western blot method. E-cadherin, N-cadherin, and α-SMA expression were evaluated. Briefly, electrophoresis was performed in polyacrylamide gel using 50 µg of protein samples and transferred onto PVDF membrane with a 0.45 µm pore size (Merc Millipore, Billerica, MA, USA). After 1 h of incubation with 5% BSA (Sigma-Aldrich, Saint Louis, USA), membranes were probed with antibody solutions overnight at 4 °C. The antibodies used together with the corresponding dilutions are listed in Table 2.

<table>
<thead>
<tr>
<th>determined protein</th>
<th>type of antibody</th>
<th>dilution</th>
<th>manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td>rabbit polyclonal</td>
<td>1:1000</td>
<td>Proteintech Group Inc.,</td>
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<tr>
<td></td>
<td></td>
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<td>Chicago, IL, USA</td>
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<tr>
<td>N-cadherin</td>
<td>rabbit polyclonal</td>
<td>1:1000</td>
<td>Proteintech Group Inc.,</td>
</tr>
<tr>
<td></td>
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<tr>
<td>α-SMA</td>
<td>rabbit polyclonal</td>
<td>1:1000</td>
<td>Abcam, Cambrige, UK</td>
</tr>
<tr>
<td>β-actin</td>
<td>HRP-conjugated rabbit monoclonal antibody</td>
<td>1:1000</td>
<td>Santa Cruz Biot., Dallas, TX, USA</td>
</tr>
</tbody>
</table>

Finally, the proteins were detected as previously described using an antirabbit IgG–HRP secondary antibody (1:10 000) (Santa Cruz Biotechnology, Dallas, USA) and a reaction mixture for signal detection (ECL method). The chemiluminescence reading was performed using the Image Station 4000MM PRO gel system (Carestream, Rochester, USA). After reading, the membranes were washed three times for 10 min in PBST solution and then incubated for 1 h at room temperature with an anti-β-actin–HRP monoclonal antibody (1:1000). The membranes were rinsed according to a standard scheme, and the detection described above was followed. Densitometric analysis was performed using Carestream MI Software 5.0.6.20 (Carestream, Rochester, USA), and the chemiluminescence intensity results for the tested proteins were presented in relation to the chemiluminescence intensity obtained for β-actin.

2.8.3. ELISA Test. Appropriate dilutions of the blood plasma samples or tumor lysates were prepared for each test (see Table 3). Analyses were performed according to the manufacturer’s protocols attached to the reagent kits. The read was read using a Synergy H4 Hybrid reader (Bio-Tek Instruments Inc.,

<table>
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<tr>
<td>E-cadherin</td>
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</tr>
<tr>
<td></td>
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<td>Chicago, IL, USA</td>
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<td>β-actin</td>
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</tr>
</tbody>
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Table 3. Markers Evaluated in ELISA Test

<table>
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<th>process</th>
<th>marker</th>
<th>tested material</th>
<th>R²</th>
<th>manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>platelet activation</td>
<td>TXB₂</td>
<td>plasma</td>
<td>35:100</td>
<td>Elabscience, Houston, TX, USA</td>
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<tr>
<td></td>
<td>vWF</td>
<td>plasma</td>
<td>1:100</td>
<td>R&amp;D Systems, Minneapolis, MN, USA</td>
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<tr>
<td></td>
<td>sP-selectin</td>
<td>plasma</td>
<td>5:100</td>
<td>R&amp;D Systems, Minneapolis, MN, USA</td>
</tr>
<tr>
<td></td>
<td>TGF-β</td>
<td>plasma, tumor lysates</td>
<td>35:100</td>
<td>R&amp;D Systems, Minneapolis, MN, USA</td>
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<tr>
<td>endothelial dysfunction</td>
<td>ET-1</td>
<td>plasma, tumor lysates</td>
<td>30:100</td>
<td>Elabscience, Houston, TX, USA; R&amp;D Systems, Minneapolis, MN, USA</td>
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<tr>
<td></td>
<td>s-CAM</td>
<td>plasma</td>
<td>50:100</td>
<td>R&amp;D Systems, Minneapolis, MN, USA</td>
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<tr>
<td></td>
<td>sV-CAM</td>
<td>plasma</td>
<td>10:100</td>
<td>R&amp;D Systems, Minneapolis, MN, USA</td>
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<tr>
<td></td>
<td>PGII</td>
<td>plasma</td>
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<td>R&amp;D Systems, Minneapolis, MN, USA</td>
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<td></td>
<td>sE-selectin</td>
<td>plasma</td>
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<tr>
<td>angiogenesis</td>
<td>VEGF</td>
<td>plasma, tumor lysates</td>
<td>25:100</td>
<td>Thermo Fisher Scientific, Waltham, MA, USA; R&amp;D Systems, Minneapolis, MN, USA</td>
</tr>
<tr>
<td>inflammation in cancer</td>
<td>iNOS</td>
<td>tumor lysates</td>
<td>25:100</td>
<td>Elabscience, Houston, TX, USA</td>
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<tr>
<td>development</td>
<td>COX-2</td>
<td>tumor lysates</td>
<td></td>
<td>Elabscience, Houston, TX, USA</td>
</tr>
</tbody>
</table>

“Dilutions are presented only for plasma samples, whereas for tumor lysate samples, the following quantities were used: TGF-β: 80 μg per well, ET-1: 300 μg per well, VEGF: 100 μg per well, iNOS: 10 μg per well, COX-2: 30 μg per well.

Winooski, USA) with the optical density of the solutions measured at 450 nm. Based on the absorbance of standard solutions of the known concentration, a standard curve was prepared and then used to determine the concentration of test samples.

2.8.4. Immunohistochemical Analyses of Mice Aorta. Immunohistochemical reaction was performed on histological slides of the aorta as previously described, using mouse anti-eNOS antibody and mouse anti-V-CAM/CD106 antibody (both from Abcam, Cambridge, UK). Expression of V-CAM and eNOS was evaluated using the modified semiquantitative IRS scale by Remmele. The method takes into account both the proportion of positively stained cells and the intensity of color reaction, while its final results represent the product of both parameters with values ranging from 0 to 12 points (no reaction = 0 points (−); weak reaction = 1–2 points (+), moderate reaction = 3–5 points (++), intense reaction = 6–12 points (+++)). Microphotographs of all the studied tissues were subjected to a computer-assisted image analysis using a computer coupled to a BX53 optical microscope (Olympus, Tokyo, Japan). The measurements were carried out using the CellA software (Olympus Soft Imaging Solution GmbH, Germany).

2.9. Platelet Surface Antigens. Whole blood was collected from experimental mice into Eppendorf tubes (with 100 μL of heparin at 5000 IU/mL, Polfa Tarchomin, Warsaw, Poland). Blood samples were analyzed using a Mythic 18 hematology analyzer (PZ Cormay S.A., Lomianki, Poland). Next, blood samples were centrifuged at 80 °C for 15 min at 2000 g to obtain the plasma that was subsequently stored at −80 °C and used for further investigations. A 10 μL aliquot of whole blood was used for the evaluation of platelet activation by flow cytometry, and a 10 μL aliquot of whole blood was also diluted in a mixture of 1 mL of Tyrode buffer (PChO, Wrocław, Poland) and 50 μL of TBS (PChO, Wrocław, Poland). Subsequently, the diluted whole blood was centrifuged at 900g for 5 min at room temperature (RT). The pellet was resuspended in 1 mL of Tyrode’s buffer, and CaCl₂ was added to a final concentration of 1 mM. Washed 100 μL samples were incubated with 1 μL of the following antibodies: DyLight649-labeled antimouse CD41/61; PE-labeled antimouse GPIbalpha (CD42b), PE-labeled antimouse GPIIIb/IIa (CD41/61) JON/A, and PE-labeled antimouse P-selectin (CD62P) (EMFRET Analytics GmbH Co. KG, Eibelstadt, Germany). After 30 min of incubation in the dark, 100 μL of PBS was added. Samples were centrifuged at 1100g for 7 min at RT, followed by pellet dilution in 250 μL of PBS. The reading was carried out in the LSR Fortessa cytometer (BD Biosciences, San Jose, USA). Data analysis was carried out using FACSDiva software.

2.10. Patient Characteristics. All procedures were carried out in accordance with the ethical standards of the institutional and national research committee and with the principles of the Helsinki Declaration of 1964, as amended. The study protocol was approved by the Institutional Review Board (IRB approvals no KB 71/2017 and 286/2017), Wrocław, Poland. Informed consent was obtained from all participants of the study. Blood was collected from 48 breast cancer patients diagnosed from July to October 2017 at the Lower Silesian Oncology Center (Wrocław, Poland) into 3.2% buffered sodium citrate solution-containing tubes (BD Biosciences, San Jose, USA). The patients were separated into three groups according to breast cancer severity: patients without metastasis, patients with metastasis in lymph nodes, and patients with distant metastasis. The blood from 14 healthy donors was used as control. (See Table 4 for all patient characteristics.)

Table 4. Patient Characteristics

<table>
<thead>
<tr>
<th>no</th>
<th>average tumor size [mm]</th>
<th>thyroid disease</th>
<th>diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>25 ± 9°</td>
<td>22%</td>
<td>7%</td>
</tr>
<tr>
<td>13</td>
<td>23 ± 6°</td>
<td>25%</td>
<td>7.7%</td>
</tr>
<tr>
<td>8</td>
<td>53 ± 18</td>
<td>0%</td>
<td>12.5%</td>
</tr>
</tbody>
</table>

*p < 0.05 as compared to group with metastatic cancer. Kruskal–Wallis test.

2.10.1. Platelet Aggregation Test. Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 150g for 15 min. Supernatant was harvested for further investigation, and the remaining blood was centrifuged at 2000g for 15 min to obtain platelet-poor plasma (PPP). Next, the number of platelets in PRP was measured by hematological analyzer Mythic 18 (PZ Cormay S.A., Lomianki, Poland). PRP samples were diluted with 0.9% NaCl (PChO, Wrocław, Poland) to achieve the final concentration of 100 μL of PBS.
concentration of $180 \times 10^3$ platelets/$\mu$L. DETA/NO and clopidogrel were added to PRP samples at the concentration of 100 $\mu$M followed by 15 min of incubation in the incubator (NuAir, USA) at 37 $^\circ$C in a humidified atmosphere with 5% CO$_2$. Aggregation of platelets in PRP was induced with collagen (1 $\mu$g/mL). The sample chamber was heated to 37 $^\circ$C. PPP was used as an optical control and considered to be 100% of light transmission, whereas PRP alone is considered to be 0% of light transmission. Platelet aggregation test was performed using the Chrono-Log Model 700 Agregometer (Crono-Log, Havertown, USA) by optical method.

### 2.10.2. Platelet Activation Marker Assessment

The effect of DETA/NO and clopidogrel on platelet activation was investigated on blood samples acquired from breast cancer patients as described above. Seven samples from the group with distant metastasis and metastasis in lymph nodes were taken for the analysis, and six samples of blood from healthy donors were used as control. PRP obtained as described above was incubated with 100 $\mu$M DETA/NO and a combination of DETA/NO with clopidogrel in the incubator (NuAir, USA) at 37 $^\circ$C in a humidified atmosphere with 5% CO$_2$ for 15 min. Then samples were centrifuged at 2000g for 15 min to obtain platelet-poor plasma (PPP), which was used for further analysis of platelet activation. Platelet activation was estimated through the quantitative evaluation of TGF-$\beta$ and TXB$_2$ in the plasma by ELISA test (Elabscience, Houston, USA).

### 2.11. Statistical Analysis

Statistical analysis was carried out using the GraphPad Prism 6 software. The analysis of the normality of data distribution was carried out using the Shapiro-Wilk data normality test with the assumed significance of the test of $p < 0.05$. The statistical analysis applied to individual data, depending on their distribution, was presented in figure legends. Differences between groups for which $p < 0.05$ were considered statistically significant.

### 3. RESULTS

#### 3.1. Antimetastatic Activity of DETA/NO Combined with Clopidogrel (CLO) in Intravenous Experimental Model of Mammary Gland Cancer Metastasis

The intravenous model was performed in order to exclude tumor impact during the investigation of the antimetastatic effect of the compounds. Mice were treated with LPS 5 h before 4T1-luc2-tdTomato i.v. injection to induce inflammation, which is typical for 4T1 tumor progression. Subsequently, the experiment followed the scheme presented in Figure 1A. A statistically significant inhibition of lung metastases was observed in mice treated with a lower dose of DETA/NO (1.6 mg/kg/24h) combined with CLO (Figure 1A). In these mice, the plasma level of endothelin 1 (ET-1) was the lowest (Figure 1A). A higher dose of DETA/NO (2.4 mg/kg/24h) was ineffective. In this experimental condition, the ET-1 level was diminished, but in the group of animals treated with a combination of compounds, it remained at a level similar to that observed in mice treated with both agents alone (Figure 1B). Similar results were observed in mice pretreated with CLO and DETA/NO (2.4 mg/kg/24h) for 7 days before tumor cell inoculation, in which cell extravasation in the lung as well as the ET-1 level (decreased by all treatments) were measured 24 h after tumor cell inoculation (Supplemental Figure 1).

#### 3.2. Antimetastatic Activity of DAETA/NO Combined with Clopidogrel (CLO) in Orthotopic Model of Spontaneous Mammary Gland Cancer Metastasis

On the basis of the above results, a lower dose of DETA/NO was chosen for further investigation in the 4T1 mouse mammary gland cancer metastasis model.
Neither compound showed any influence on primary tumor growth, and the mice body weight did not change significantly during the treatment (maximum decrease did not exceed 4%) (Supplemental Figure 2). Nevertheless, we observed the transitory inhibition of the formation of lung metastases on day 21 by CLO alone and CLO combined with DETA/NO ($p < 0.05$) (Figure 2A). Summarizing these results, we have observed statistically significant inhibition of lung metastasis by the combination of DETA/NO + CLO in the both intravenous (4T1-luc2-tdTomato) and orthotopic (4T1) model of mammary gland cancer.

### 3.3. Analysis of Plasma Markers of Endothelial Dysfunction (ET-1, sICAM, sVCAM, sE-selectin), Angiogenesis (VEGF), and Markers of Platelet Activation (vWF, PGI2, TXB2, sP-selectin, TGF-$\beta$) in Orthotopic Model

Quantitative analysis of endothelial dysfunction markers (ET-1, sICAM, sVCAM, sE-selectin) showed significant diminishing of the ET-1 plasma level on days 14 and 28 as a result of using DETA/NO combined with CLO. At both time points, the level of ET-1 was the lowest in the group treated with the combination compared to other investigated groups (Figure 2B). Soluble E-selectin was diminished by DETA/NO on day 21, whereas on day 28, its plasma level was the lowest in the combined therapy group (Figure 3A,B). The sICAM plasma level was diminished by the combined therapy on day 28 (Figure 3C). The sVCAM level was not affected by the treatment applied (Supplemental Figure 3A).

Markers of platelet activation (vWF, PGI2, TXB2, sP-selectin, TGF-$\beta$) and angiogenesis (VEGF) were also analyzed in mice plasma (Figure 3 and Supplemental Figure 3). The vWF plasma level was diminished in the control group (tumor-bearing mice) as compared to healthy animals (day 28, Supplemental Figure 3B); however, only DETA/NO downregulated the vWF level compared to the control group on day 21 of the experiment ($p < 0.05$) (Figure 3D). The TXB2 plasma level was significantly increased in control mice as compared to healthy mice on all days of measurement (Supplemental Figure 3C). CLO alone, but not in combination with DETA/NO, diminished the level of TXB2.
this protein (day 21, \( p < 0.05 \)) (Figure 3E). The level of PG\(_I_2\) was downregulated in the plasma from tumor-bearing control mice, while the combination of DETA/NO with CLO significantly increased the protein level on day 21 of the experiment (Figure 3F). The soluble P-selectin level was significantly decreased in tumor-bearing mice, but the treatment did not change its level (Supplemental Figure 3D). The TGF-\( \beta \) plasma level increased during 4T1 tumor progression as compared to healthy mice (Supplemental Figure 3E). CLO alone and combined with DETA/NO significantly diminished its level on days 14 and 28 (Figure 3G,H). The plasma level of VEGF was almost unchanged by tumor progression or by the treatment used (Supplemental Figure 3F). Only on day 14 the tendency to decrease VEGF level by the combination of both compounds was observed (\( p < 0.05 \) as compared to DETA/NO alone) (Figure 3I).

Thus, the plasma concentration of the main marker of endothelial dysfunction, ET-1, was significantly downregulated by the DETA/NO + CLO combination in both models of metastasis. In the orthotopic model, the combination of DETA/NO + CLO significantly decreased the sI-CAM and sE-selectin levels in mice plasma on day 28. The level of TGF-\( \beta \) was significantly diminished by CLO and DETA/NO + CLO on day 14 and 28.

### 3.4. Characteristics of Endothelial Cells from the Aorta

Immunohistochemical analysis of aortal endothelium showed that the combination therapy with DETA/NO and CLO abolished the expression of V-CAM. DETA/NO or CLO used alone also have a tendency to diminish V-CAM staining of the endothelium (Figure 4A,D). eNOS expression was significantly increased after the therapy with DETA/NO, and CLO did not affect its level (Figure 4B,C).

### 3.5. Analysis of Resting Platelet Activation Markers

The number of platelets was increased by CLO on days 14 and 28, while DETA/NO did not reveal any effect on their number...
at these time points, downregulating platelet count only on day 21 (Supplemental Figure 4A). The presence of activated platelets was estimated by flow cytometry. Upregulated CD42b on the platelet surface was observed in combination therapy group on day 14, but at the end of the experiment (day 28), the percentage of CD42b positive platelets was diminished by CLO and CLO + DETA/NO (Figure 5A−C). P-selectin positive platelets increased in mice treated with CLO alone or combined with DETA/NO on days 21 and 28 (Figure 5B,C). Platelet surface fibrinogen was significantly diminished by combination therapy on day 14 (Figure 5A), but on the next days, this effect was not visible. The active form of CD41/61 (JON/A antibody) was decreased by CLO and DETA/NO used alone on day 21, but then, its level significantly increased in the combination therapy group (Figure 5B,C). vWf positive platelets were decreased by CLO therapy on day 14 (Figure 5A).

In summary, the combination therapy with DETA/NO and CLO abolished the expression of V-CAM in mice aortas, while eNOS expression was significantly elevated only by DETA/NO. We also observed the effect of combined therapy on platelet activation. In the beginning of the orthotopic experiment (day 14), we determined the upregulation of CD42b and the downregulation of fibrinogen as well as vWf. However, on the later stages of experiments (day 21, day 28) the percentage of CD42b positive platelets was diminished, and P-selectin increased.

3.6. Tumor Epithelial to Mesenchymal Transition (EMT) Markers. E-cadherin expression in the tumors was significantly increased by combination therapy on day 21 as compared to control group and groups treated with compounds alone. E-cadherin expression remained increased on day 28 (Figure 6A). The expression of N-cadherin was decreased on day 14 only by DETA/NO but on day 21 by CLO (p < 0.05), and combination therapy elevated the level of this protein, whereas the combination had a less pronounced effect (Figure 6B). However, the ratio of E/N-cadherin was significantly increased only by DETA/NO compared to the group treated with the combination of compounds on day 14, and CLO abrogated this upregulation significantly in the combination therapy group on day 14, while on day 21, DETA/NO elevated the ratio of E/N-cadherin in the combination therapy group, previously decreased by CLO (Figure 6D). CLO significantly decreased the level of α-SMA on day 14, but DETA/NO seemed to reverse the action of CLO. At the next time point, both applied compounds increased the level of α-SMA, but only combination therapy elevated it significantly. CLO and CLO + DETA/NO decreased the level of this protein on day 28 of the experiment (Figure 6C).

Expression of ET-1, VEGF, iNOS, TGF-β, and COX-2 in tumor tissue was also analyzed. The ET-1 level was the lowest in the combination therapy group on day 21, while on day 28 in tumors from all therapy groups, it was decreased as compared to that of the control tumor-bearing mice (Figure 6C). On day 21, all treatment regimens diminished the level of TGF-β in tumor tissue. On day 21, only DETA/NO still revealed the same effect, but on day 28, CLO and DETA/NO alone tended to increase the level of TGF-β, whereas in tumors from mice treated with both compounds, its level was significantly lower as compared to the agents alone (Figure 6E). The iNOS tumor tissue level was decreased significantly by CLO on day 14 (Figure 6F). Data for all analyses of iNOS, VEGF, and COX-2, whose level did not change significantly in tumor tissue, are shown in Supplemental Figure 5.

3.7. Tumor Blood Perfusion Analysis. Blood flow in the tumor was investigated on day 24 of the experiment. DETA/NO...
and CLO alone upregulated tumor blood perfusion, but CLO in combination therapy with DETA/NO reduced tumor blood flow as compared to DETA/NO alone (Figure 6G).

Thus, the combination of DETA/NO + CLO upregulated the E-cadherin level on days 21 and 28, and the N-cadherin level was also elevated on day 21 by the combination therapy. On day 21, the combination increased the α-SMA level, while on day 28, the level of this protein was decreased by CLO and the combination of DETA/NO + CLO. Moreover, all investigated compounds significantly decreased TGF-β in tumor lysates on day 14. The ET-1 level in tumor lysates was diminished by the combination therapy on day 21 and by all investigated compounds on day 28 of experiment. However, only DETA/NO and CLO alone but not the combination upregulated tumor blood perfusion on 24 day of experiment.

3.8. Effect of DETA/NO Combined with CLO on Platelet Aggregation and TGF-β and TXB2 Release from Platelets in Blood Samples Obtained from Breast Cancer Patients and Healthy Donors.

Collagen-induced aggregation of platelets was investigated in platelet-rich plasma (PRP). DETA/NO and the combination of DETA/NO with CLO successfully inhibited platelet aggregation induced by collagen (Figure 7A,B). The most pronounced effect was observed in breast cancer patients with distant metastases and lymph node metastases as compared to control (Figure 7B). DETA/NO alone did not reveal any inhibitory effect on platelet aggregation compared to the control in preliminary investigations and therefore was not included in the further analyses (Supplemental Figure 6A).

Resting platelets from breast cancer patients with distant metastases released a significantly lower level of TGF-β when incubated with DETA/NO as compared to control (Figure 7C). DETA/NO or DETA/NO combined with CLO did not influence TXB2 release from breast cancer patients or healthy donors’ platelets (Figure 7D).

Platelets from healthy donors stimulated with collagen and incubated with DETA/NO or DETA/NO combined with CLO released similar levels of TGF-β and TXB2 to control platelets stimulated with collagen (Supplemental Figure 6B and C).

In summary, DETA/NO alone and combined with CLO significantly inhibited platelet aggregation in the PRP of donors from all investigated groups compared to the control group. DETA/NO significantly inhibited TGF-β release in the PRP of patients with metastatic cancer.

4. DISCUSSION

Taking into consideration the known clopidogrel’s antiplatelet and vasoprotective potential and its other well-known role of an agent improving endothelial NO bioavailability, we assumed possible advantages from combined usage of both clopidogrel and an NO donor in antimetastatic therapy. It is
important in the light of the report that during 4T1 mouse mammary gland cancer progression, the availability of NO decreased and the impairment of the NO-dependent endothelium function was observed. In fact, we observed antimetastatic activity of combination therapy with DETA/NO and CLO in the experimental model of lung metastasis of 4T1-luc2-tdTomato cells, whereas such an effect was observed neither for DETA/NO nor for CLO used alone. Interestingly, only combination therapy with a lower dose of DETA/NO significantly affected the metastatic foci formation. McQuaid and Keenan claimed that low concentrations of NO protect the endothelial layer, while a higher amount of NO in the combination with ROS might cause endothelial injury as well as the loss of its barrier function. Therefore, proper selection of doses of NO donors appears to have enormous significance. Moreover, only when a lower dose of DETA/NO was applied concomitantly with CLO was the synergistic diminution of the ET-1 level noticed. Therefore, we performed further studies with the application of a lower dose of DETA/NO combined with CLO in the orthotopic model of 4T1 mammary gland cancer.

Figure 6. Expression of proteins related to EMT in tumor tissue and tumor blood perfusion. (A) E-cadherin, (B) N-cadherin, (C) α-SMA, (D) E-cadherin/N-cadherin ratio, (E) TGF-β level, (F) iNOS level on day 14; all time point measurements shown in Supplemental Figure S5C. (G) Blood perfusion in tumor tissue presented as wash-in rate (WIR) parameter with representative images of tumor perfusion taken on day 24. (A–C) Graphs represent relative density of blots (ratio of measured protein to β-actin), and the representative blots are presented below graphs; Western blot analysis was performed on specimens from 3 to 4 mice. Data presented as mean with SD and with data of individual measurements. Statistical analysis: (A–D and F–G) Dunnett’s; (E) Dunn’s tests for multiple comparisons. *p < 0.05 as compared to control or as indicated.
In the orthotopic model we observed only a transient anti-metastatic effect of CLO, but this effect was not improved by DETA/NO. Nevertheless, the impact of the combination therapy on the endothelial dysfunction and platelet activation markers was significant.

The endothelium contributes to the development of metastasis and exerts a strong influence on migrating tumor cells. The endothelium’s high reactivity and strong secretory ability enable it to control blood coagulation, platelet activation, vessel repair, and cell transmigration through blood vessels.

Endothelium dysfunction develops when endothelial cells stay in an activated stage for a prolonged period of time. This occurs, among other times, during cancer progression. In our orthotopic experiments, the ET-1 plasma level, like in the intravenous metastatic model, was the lowest in the combination therapy group. ET-1 is upregulated during endothelial dysfunction and is considered to be one of the main molecules produced only by endothelial cells that regulate vascular functions, and consequently, it is a significant marker of endothelial dysfunction and is associated with metastatic progression. It was also reported that ET-1 may induce endothelial dysfunction by decreasing NO bioavailability.

On the other hand, we observed an increased eNOS expression in the endothelium from the aorta of mice treated with DETA/NO alone and in combination with CLO. Thus, a decreased ET-1 level due to the therapy used (DETA/NO + CLO) combined with increased eNOS expression observed in our studies and with elevated NO bioavailability by CLO reported by other authors seem to create a favorable activity profile aimed at normalizing the function of the vascular endothelium. Moreover, the combination therapy used in our studies significantly diminished the level of other markers of endothelial dysfunction, such as intercellular adhesion molecule-1 (sI-CAM) and sE-selectin in mice plasma. In addition, immunohistochemical analysis of the aorta endothelium further confirmed decreased endothelial activation, indicating the lowest level of vascular cell adhesion molecule (V-CAM) positive endothelium in mice treated concurrently with DETA/NO and CLO.

And again, NO itself and its bioavailability increasing as a result of using CLO may be responsible for this effect, since it was documented that NO inhibits V-CAM, I-CAM, and E-selectin expression on the endothelial surface. Moreover, upregulation of PGI2 by the combination of DETA/NO with CLO was observed on day 21 of the experiment, although the same effect was observed for DETA/NO alone, indicating that CLO cannot enhance the effect of DETA/NO. Thus, the results show that exogenous NO upregulates PGI2, which correlates with the findings of Pierre et al. Authors reported that NO donors increased PGI2 synthesis by endothelial cells in ocular vasculature. Buczek et al. have shown, that in advanced metastasis of 4T1 tumors, the NO-dependent response in the aorta was impaired. This impairment was compensated by an increase in COX-2-mediated synthesis of PGI2. So, the use of the combination of an NO donor and CLO in 4T1 orthotopic model, despite signs of normalization of NO-dependent mechanisms in endothelium, acts also through PGI2, a molecule with a vasoprotective and antimetastatic potential. It is in agreement with the results described earlier, where CLO, in addition to improving endothelial nitric oxide bioavailability, can also activate defensive mechanisms of endothelium involving NO- and PGI2-mediated actions.

Analysis carried out on mice plasma revealed that the investigated compounds alone had a modulating effect on the selected molecules produced by the endothelium, megakaryocytes, and activated platelets, whereas combination therapy did not change their level. However, its diminished level might contribute to the final result concerning metastasis. TXB2 (TXA2 metabolite) and TGF-β were decreased by CLO, and vWF was downregulated by DETA/NO. Whereas TXB2 diminution was restored by DETA/NO in the group treated with combination therapy, the TGF-β level was not affected by the NO donor as compared to CLO alone. Also, in tumor tissue, we observed a decreased level of TGF-β in all treated mice on day 14, which continued also on day 21 in mice treated with DETA/NO alone. Moreover, platelets from patients with...
metastatic breast cancer released lower levels of TGF-β when incubated ex vivo with DETA/NO as compared to vehicle incubated platelets. Activated platelets are considered to be an important source of TGF-β.37 However, a diminished plasma level of TGF-β by CLO alone or combined with DETA/NO correlated with decreased platelet activation only at the early stage of tumor progression (day 14), while at the next stages, both CLO and its combination with DETA/NO upregulated platelet activation markers. Nevertheless, platelets are not the only source of TGF-β. Also cancer cells itself, as well as macrophages and other tumor stroma cells, are an important source of this cytokine.38,39 Taking into consideration the fact that activated endothelial cells are also characterized by increased TGF-β production,40 we can conclude that the applied therapy affected the TGF-β level also by the reduction of endothelial activation.

The elevated TGF-β plasma level is described as a possible marker of metastatic breast cancer with a poor prognosis.41 TGF-β is also one of the master factors in the induction of epithelial to mesenchymal transition (EMT) of cancer cells. Moreover, it is described as a pro or antiangiogenic factor.39

In our previous studies, CLO combined with cyclophosphamide or with 5-fluorouracil in the 4T1 tumor model increased the E/N-cadherin ratio, which correlated with increased antitumor and antimetastatic potency of the combination therapy.22 However, in current studies, the decrease of TGF-β in mice plasma or in tumor tissue correlated with the increase of E-cadherin, but unfortunately, CLO alone or combined with DETA/NO increased the N-cadherin level as well. As a result, the ratio of E/N-cadherin was significantly increased only in DETA/NO treated mice as compared to the combination therapy group (day 14) and in the group treated with the combination therapy as compared to CLO alone treated mice (day 21). It suggests some opposite interactions in the activity of CLO and DETA/NO according to the EMT process. Also, the level of α-SMA, which is another EMT marker,42 diminished at the beginning of tumor growth (day 14) as a result of CLO therapy but was increased in the combination therapy group.

In this study, we observed that both agents (DETA/NO and CLO) used alone increase tumor blood perfusion, and this effect was repressed in the combination therapy group. Improved blood perfusion may be a symptom of blood vessel normalization and may be beneficial in anticancer drug delivery into the tumor.43 Such an effect was previously described for CLO combined with cisplatin,44 and in our studies, it may be related to decreased endothelial dysfunction also of the blood vessels in tumor tissue. It was confirmed in our research by iNOS evaluation in tumor tissue. Nagareddy et al. previously showed that upregulated iNOS expression resulted in endothelial dysfunction development,45 and moreover, iNOS inhibition reduces tumor angiogenesis.45 CLO in our current studies decreased the iNOS level in tumor tissue. However, in the combination, DETA/NO abrogated that effect of CLO. Despite that, CLO alone also diminished the TXB2 plasma level, which was restored by combination therapy as well. TXA2 was reported to be involved in tumor angiogenesis and metastasis,46 and its diminishing induced by CLO may also contribute to blood vessel normalization. On the other hand, improved blood perfusion by DETA/NO was accompanied by the decrease of the vWF plasma level. vWF is able to mediate platelet–tumor cell interactions. Moreover, this molecule has a regulatory role in angiogenesis.47 The effects of single drugs mentioned above may be responsible for improved blood perfusion in tumor tissue, and the abolition of these effects in a group with combination therapy is responsible for the lack of influence on blood perfusion.

During the metastatic progression of the disease, the interaction of cancer cells with platelets is of great importance, as it facilitates the survival of cancer cells and their adhesion to the endothelium.48 The analysis of the activation markers on the surface of resting platelets revealed that combination therapy impacted platelets differently during short and prolonged drug administration, or from the point of view of the disease progression at the beginning and at the later stages of cancer progression. On day 14, we observed the signs of diminished platelet activation as a result of combination therapy, with examples such as an increased CD42b level and decreased platelet bound fibrinogen level. Surface antigen CD42b (GPIbα) is known to be responsible for the adhesion of an activated platelet to the vessel wall through the binding of vWF. Therefore, the presence of vWF on the platelet surface can be an indicator of platelet activation, whereas antibody binding to CD42b shows the presence of antigens free of vWF. Consequently, the presence of CD42b indicates a reverse correlation with platelet activation.50 Moreover, we observed significant diminishing of breast cancer patients’ platelet aggregation by DETA/NO alone or in combination with CLO. However, during the progression of the disease in the mouse orthotopic model, continued concomitant therapy with DETA/NO and CLO led to upregulated platelet activation: increased fibrinogen and vWF bond, increased active form of CD41/CD61 (JON/A antibody), and increased P-selectin expression (two last markers are mediated mainly by CLO). In addition, the platelet antiaggregatory potential of combination therapy was more intensified in breast cancer patients without metastases. Increased platelet reactivity and activity following coronary stent implantation in spite of using thienopyridine and aspirin therapy was reported previously, and authors suggest that such therapy may by insufficient in some patients.51 Moreover, Smeda et al. showed that in mice bearing 4T1 cells and treated with CLO combined with aspirin (unfortunately, they do not provide data for each compound used alone), platelet phenotype was more proangiogenic, and the number of large “young” platelets in these mice was higher when compared with control 4T1 mice, indicating thrombopoiesis in response to platelet inhibition with this therapy.52 Such combination therapy applied by Smeda et al. led to decreased survival of the treated mice, although it diminished metastatic spread.52

Our findings suggest that the combined use of an NO donor with CLO may act as an effective antimetastatic regime normalizing endothelial function. However, opposite effects observed in the orthotopic model and the lack of improvement of the antimetastatic potency of both compounds in this model may result from the high impact of growing 4T1 tumors on the endothelium and platelets confirmed by the data of Buczek et al.57 and Smeda et al.,52 which the compounds used in combination are unable to overcome. Based on our previous research and studies by other authors, it may be assumed that the combination of DETA/NO and CLO may have a beneficial effect and support the treatment with traditional anticancer drugs. However, further studies are necessary to confirm this hypothesis.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharma-ceut.8b00781.
Figure S1. Effect of DETA/NO combined with CLO on cancer cell extravasation of 4T1-luc2-tdTomato cells injected intravenously; Figure S2. Kinetics of tumor growth and body weight changes; Figure S3. Selected markers of endothelial dysfunction, platelet activation, and angiogenesis in the plasma of mice bearing 4T1 tumors (orthotopic model)—all time points of measurements; Figure S4. Platelet count (A) and representative dot-plots of platelet phenotype analysis (B). Orthotopic model; Figure S5. Estimation of the influence of DETA/NO, CLO, and their combination on the concentration of crucial markers of the metastatic process in tumor lysates; Figure S6. Influence of CLO on the ex vivo platelet aggregation. Collagen stimulated platelets from healthy donors: release of TGF-β and TXB2 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: wietrzyk@iitd.pan.wroc.pl.

ORCID

Joanna Wietrzyk: 0000-0003-4980-6606

Author Contributions

All authors contributed to the work presented in this paper. All authors have given approval to the final version of the manuscript. K.P. was responsible for the design and execution of the experiments, data analysis and interpretation, as well as drafting the manuscript; D.P., M.P., M.N., R.M., M.E., M.M., and J.B. performed experiments, data analysis and revision of the manuscript; J.W. was responsible for the conception of the study, results interpretation, and writing the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

α-SMA, alpha-smooth muscle actin; CLO, clopidogrel; COX-2, cyclooxygenase-2; EMT, epithelial mesenchymal-like transition; ET-1, endothelin 1; ICAM, intercellular adhesion molecule; IFN-γ, interferon γ; NO, nitric oxide; NOS, nitric oxide synthase; PGI2, prostacyclin; PPI, platelet-poor plasma; PRP, platelet-rich plasma; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; TX, thrombocyte; V-CAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor;

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