Enhanced Platelet Activation Induced by Palbociclib Treatment in MCF-7 Breast Cancer Cells

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ABSTRACT

Background and aim: The crosstalk between platelets and cancer cells is bidirectional. Cancer cells can activate platelets, a process known as "tumor cell-induced platelet aggregation". On the other hand, platelets provide essential assistance to tumor cells by helping them adhere to blood vessels, facilitating their escape from the bloodstream, and enabling their spread to distant tissues. This study investigates the effect of Palbociclib, an FDA-approved cyclin-dependent kinase 4 and 6 inhibitor, on breast cancer-cell-induced platelet activation.

Method: Platelet activation, as indicated by the expression of CD62P (P-selectin), was assessed by flow cytometry. In addition, platelet attachment to MCF-7 cancer cells was studied using confocal microscopy.

Results: Palbociclib's half-maximal concentration (IC_{50}) was found to be 19.54 µM after 72 h. About 36.9% $\pm$ 0.98 of platelets were activated by untreated MCF-7. Pretreatment of MCF-7 cells with Palbociclib (9.75 µM equivalent to 1/2 IC_{50}) increased platelet activation significantly by 63.3% $\pm$ 8.85 ($P<0.01$) while 4.87 µM Palbociclib (1/4 IC_{50}) increased platelet activation by 43.0% $\pm$ 2.83 with no significant difference compared to untreated cells. Confocal microscopy results suggest the presence of a direct interaction between breast cancer cells and platelets.

Conclusion: Palbociclib increased tumor-induced platelet activation in MCF-7 hormone-positive breast cancer cell line.

Keywords: Confocal microscopy; Flow cytometry; MCF-7; Palbociclib; Platelets; P-selectin**.**

INTRODUCTION

The bidirectional interaction between platelets and tumor cells was reported since 1968. Platelets can be activated by cancer cells both *in vitro* and *in vivo*, a process known as "tumor cell-induced platelet aggregation"[(1)]. Beyond regulating tumor growth, platelets play an essential role in metastasis [(2)]. They adhere to the surface of cancerous cells shielding them from shear stress and hindering immune system recognition and attack [(3)]. Furthermore, platelets facilitate the adherence of tumor cells to blood vessels enabling them to escape from the bloodstream and spread to distant organs [(4)]. Moreover, platelet granules contain substances that aid in the metastasis and promote tumor vascularization including P-selectin (CD62P) and CD63 which are exposed on the platelet surface after activation [(5)].

Breast cancer is the most frequent cancer in women and the leading cause of cancer death in females worldwide (6-10). According to World Health Organization (WHO) estimate, one million women are newly diagnosed with breast cancer yearly. In Jordan, breast cancer ranks as the most prevalent malignancy and stands as the third most frequent cause of cancer-related mortality, after lung and colorectal cancers [(11)]. In 2015, food and drug

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administration (FDA) approved the first cyclin-dependent kinase 4 and 6 inhibitor (CDK4/6i), Palbociclib, as a first-line therapy for postmenopausal women with hormone positive/HER2 negative advanced or metastatic breast cancer to be used in combination with letrozole or fulvestrant [12].

A multicenter retrospective study conducted in the USA, which included 266 patients, found that the 1-year incidence of thrombosis in patients taking Palbociclib was 10.9%. Most thrombotic events recorded were venous (72%) and only 34% were arterial. This happened despite the cohort's aspirin use rate of 26%, which may have reduced some of the thrombotic risk [13]. In another study, Palbociclib had the highest 1-year cumulative incidence of thrombosis, followed by Ribociclib and Abemaciclib [13]. A higher proportion of patients undergoing treatment with Palbociclib and Ribociclib exhibited cardiovascular comorbidities, notably hypertension, in contrast to those treated with Abemaciclib. Specifically, 30% of individuals who developed venous thromboembolism while using Palbociclib had cardiovascular conditions[14]. In a retrospective study involving 424 patients, venous thromboembolic events occurred in 9% of subjects (mainly receiving Palbociclib). Deep venous thrombosis alone was found to be the most common presentation (47.4%), followed by visceral vein thrombosis (21.1%) and pulmonary embolism (18.4%) [15]. A single-center audit of 64 individuals treated with Palbociclib over five months in an Irish tertiary referral hospital recorded seven venous thromboembolic events comprising 11% of the studied group [16], whereas no increased risk of pulmonary embolism was found when comparing new users of Palbociclib–fulvestrant with patients using fulvestrant alone [17].

Due to the multifactorial etiology of cardiovascular diseases, causative evidence concerning the effect of CDK4/6i(s) in general and Palbociclib in particular on thrombotic events is hard to achieve. Understanding how Palbociclib increases the risk of thrombosis could aid in implementing preventive measures during treatment. The current study examined the impact of Palbociclib on tumor-induced platelet activation in a hormone-positive breast cancer cell line through *in vitro* experiments utilizing flow cytometry and confocal microscopy.

MATERIALS AND METHODS

Ethical consideration

This study was approved by the research ethical committee at Al-Ahliyya Amman University (ethical approval number IRB: AAU/5/14/2021-2022). Written and verbal informed consents were taken from all study participants. Blood (2.5 mL) was collected from 4 healthy females between 19 and 30 years old. To prevent platelet activation during blood drawing, a 21-gauge needle was used. The first 2 mL of blood drawn were discarded to exclude the effect of mechanically activated platelets. Whole blood was collected in sodium citrate tubes. Participants' exclusion criteria included pregnancy, oral contraceptive use, previously identified disease, smoking, and consumption of any medication in the previous week before the experiment. Blood was collected between days 1 and 10 of the menstrual cycle since low levels of estrogen and progesterone in blood exist during this period. To prepare platelets-rich plasma (PRP), whole blood samples were centrifuged at 200x g for 15 minutes.

Cytotoxicity assay

MCF-7 cell lines were kindly provided by The University of Jordan and cultured in accordance with established procedures and techniques. The MTT (3- [4, 5-dimethyl-2-thiazolyl] - 2, 5-diphenyl tetrazolium bromide) kit (Thermo scientific, USA) was used to assess cell cytotoxicity as previously described [18]. MCF-7 (passage 17) cells were cultured in RPMI media to which 10% (v/v) heat-inactivated fetal bovine serum, penicillin (50 mg/mL), and streptomycin (50 mg/mL) were added and maintained at 37 °C under 5% CO_2 and 95% humidity. MCF-7 cells (10^4 cells) were planted into 96-well plates and incubated for 24 hours then treated with Palbociclib

(Tocris Bioscience, UK) dissolved in sterile distilled water for 72 hours. Cells without any drug treatment were used as a control.

Co-culturing platelets with cancer cells

MCF-7 (Passage number: 18) cells were seeded onto 24-well plates at 10^5 cells/well seeding density and incubated in 1 mL media for 24 hours at 37 °C and 5% CO_2 for cell adherence and monolayer formation. After 24 hours, MCF-7 cells were incubated with Palbociclib at 9.75 µM (1/2 IC_{50}) and 4.87 µM (1/4 IC_{50}) of Palbociclib in media for 72 hours at 37 °C. After 72 hours of drug treatment, the media in the wells was removed, and cells were washed twice with phosphate-buffered saline (PBS). The remaining cells were then treated for 20 minutes with 200 µL diluted PRP in PBS (1:1 ratio). Then PBS was used to wash cells after incubation with PRP. The PRP and the PBS used for washing were centrifuged at 200× *g* for 5 minutes and the resulting pellet was re-suspended in 150 µL PBS buffer for double-labeling with mouse monoclonal CD42b antibody conjugated with phycoerythrin (PE) (Biotech/Novus USA-UK) (1:100 dilution) and mouse monoclonal CD62P antibody conjugated with fluorescein isothiocyanate (FITC) (Biotech/Novus USA-UK) using 1:100 dilution in the dark at room temperature for 10 minutes at 37 °C.

Flow cytometry

PRP alone was used to measure the auto-fluorescence of the plasma. Adenosine diphosphate (ADP, HART/UK) was incubated with PRP for 20 minutes and used as a positive control for platelet activation. Compensation controls used in platelets' population gating were prepared by incubating platelets in PRP with a single antibody (either anti-CD42 or anti-CD62P). The samples were then fixed with 1% paraformaldehyde for 10 minutes before being centrifuged at 200×*g* for 5 minutes and the supernatant was discarded. The pellet was gently re-suspended in 500 µL of PBS buffer, transferred into flow cytometry tubes, and kept at 4°C before data analysis.

Data was collected on the flow cytometer FACSCanto TM II (BD Biosciences, USA) at Cell Therapy Center (CTC), Jordan. All experiments were repeated in triplicates, each from a different donor of blood. A gating strategy was used to detect the percentage of positivity of CD62P expressed on platelets using a single histogram gated on CD42b-positive cells, a specific marker for platelets, and CD62P positive cells (activated platelets). Mean fluorescence intensity was measured and the percentage activation of platelets was calculated using the formula:

% activation = Q2/(Q1+Q2) where Q1 is the population of platelets stained with PE-labeled CD42 antibody and Q2: is the population stained with both FITC-labeled CD62P and PE-labeled CD42 antibody.

Study of platelet-cancer cell interaction using confocal microscopy

Coverslips were fixed in wells by adding alcohol for 20 minutes. Then, MCF-7 cells (passage: 25) were seeded onto 6-well plates at $60*10^3$ cells/well and incubated in 1ml media for 24 hours at 37 °C and 5% CO_2 for cell adherence and monolayer formation. After 24 hours, MCF-7 cells were incubated with 19.5 µM of Palbociclib (IC_{50}) at 37 °C. After 72 hours of Palbociclib treatment, the media in the wells was removed and cells were washed with PBS. The remaining cells were then treated with 400 µL of PRP for 20 minutes after dilution of PRP with PBS in 1:1 ratio. PRP was then removed and the two antibodies (FITC-labeled CD62P (1:100 dilution) and PE-labeled CD42 (1:100 dilution) were added to wells. The cells were then fixed with 4% paraformaldehyde for 10 minutes before adding ammonium chloride washing buffer for 10 minutes at room temperature. DAPI (4′,6-diamidino-2-phenylindole), a fluorescent stain that binds strongly to adenine–thymine-rich regions in DNA, was added in 1:1500 dilution for 10 minutes. After removing DAPI, the wells were washed. Finally, slides were examined using a confocal immunofluorescent microscope LSM 780 microscopy ZEISS (Germany) at CTC using 63x objective lens.

Statistical analysis

GraphPad Prism version 8 was used to perform

statistical analysis. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used. Results were considered statistically significant at p <0.05

RESULTS

Cell cytotoxicity assay

The half maximal concentration (IC_{50}) value for Palbociclib against MCF-7 cell line after 72 hours was 19.54 ± 4.97 µM.

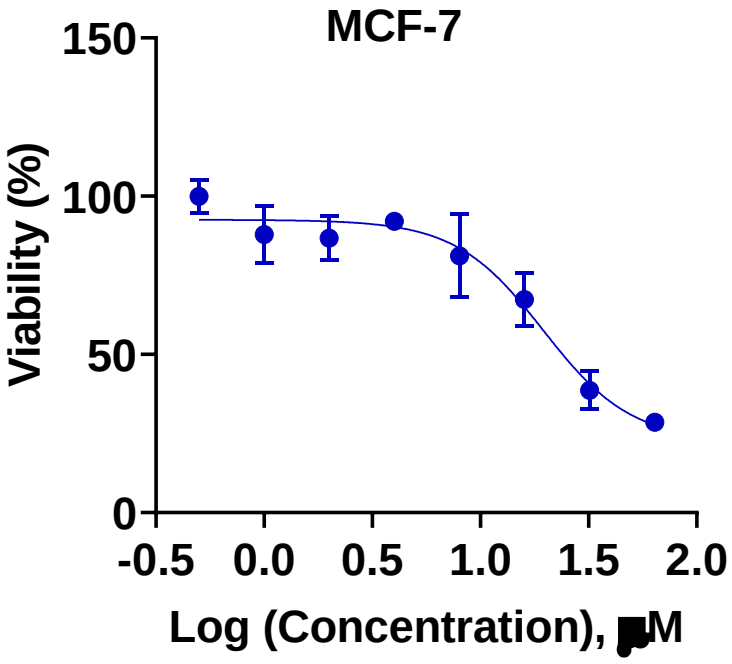

Figure 1: Dose-dependent cytotoxicity assay of Palbociclib on MCF-7 cell line at 72 hours. Each concentration was performed in triplicate.

Percentage change in CD62P expression on platelets co-cultured with MCF-7 cell line.

ADP-treated platelets (positive control) had 84.4% ± 0.14 expression of CD62P (Figure 2A). About 36.9% ± 0.98 of platelets co-cultured with untreated MCF-7 were activated (Figure 2B). Platelets exposed to MCF-7 cells treated with 9.75 µM (1/2 IC_{50}) or 4.87 µM (1/4 IC_{50}) Palbociclib showed 63.3% ± 8.85 and 43.0% ± 2.83 expression of CD62P on their surface, respectively (Figure 2C-D).

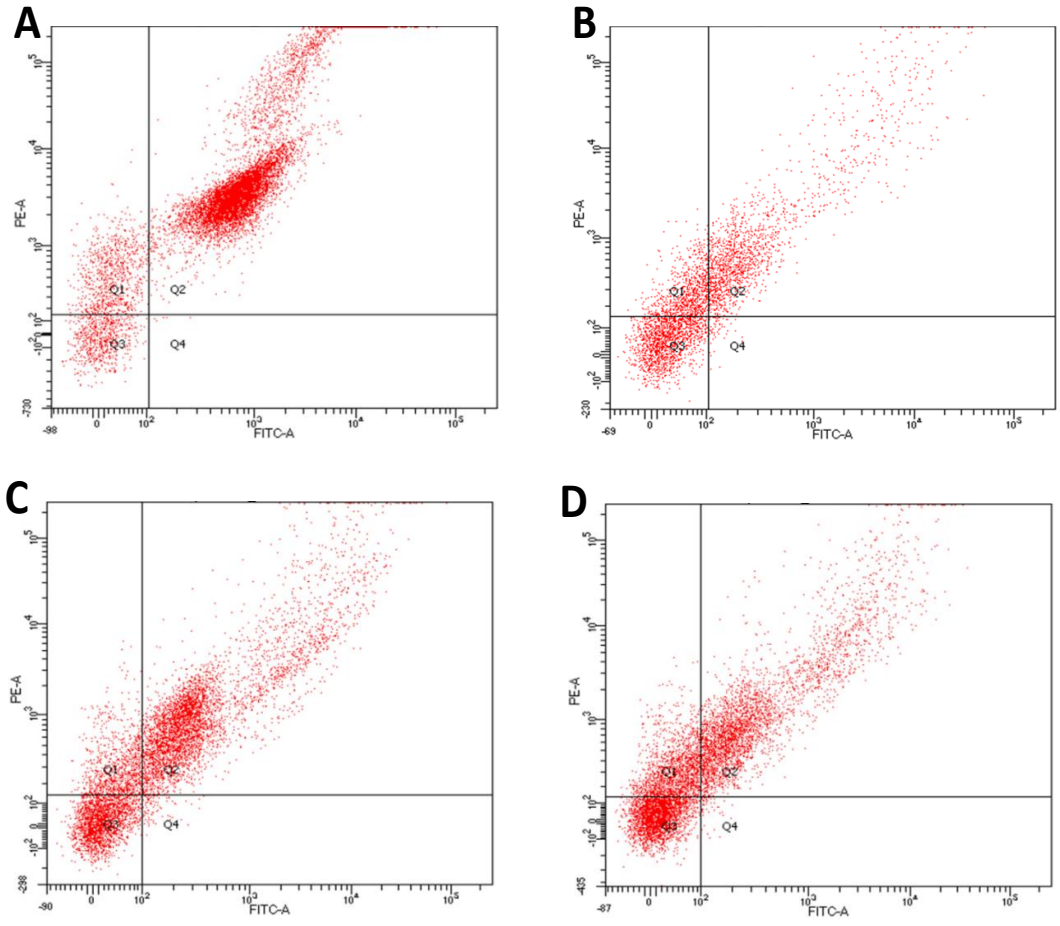

Figure 2: Expression of the activation marker P-selectin (CD62P) on platelets co-cultured with MCF-7 as studied by flow cytometry. The Y-axis represents fluorescence intensity of PE-labeled CD42 while X-axis represents FITC-labeled CD62P. The percentage activation of platelets was calculated using the formula: % activation = Q2/(Q1+Q2) where Q1 is the population of platelets stained with PE-labeled CD42 antibody and Q2 is the population stained with both FITC-labeled CD62P and PE-labeled CD42 antibodies. (A) Platelets exposed to ADP (positive control). (B) Platelets exposed to untreated MCF-7. (C) Platelets exposed to MCF-7 treated with 1/2 IC_{50} (9.75µM) of Palbociclib, (D) Platelets exposed to MCF-7 treated with 1/4 IC_{50} (4.87µM) of Palbociclib.

A significant difference in platelets' activation was observed between platelets co-cultured with untreated MCF-7 cells and platelets co-cultured with MCF-7 treated with Palbociclib (9.75 µM doses) ($P<0.01$) but not with the lower dose of Palbociclib (4.87µM). Also, a significant difference was found between platelets incubated with untreated MCF-7 and ADP-treated platelets ($P<0.001$) (Figure 3).

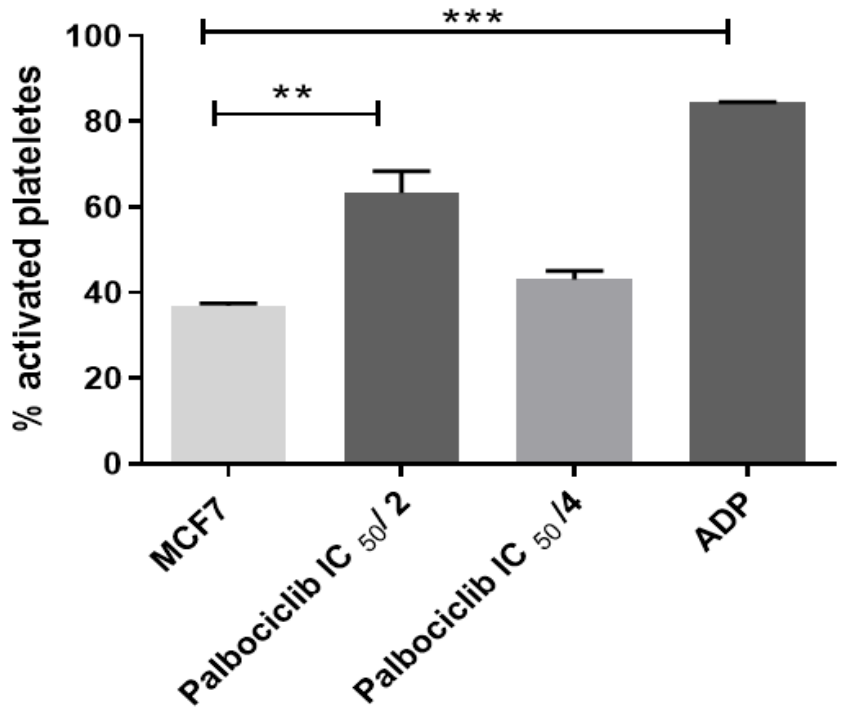

Figure 3: Platelet activation percent in flow cytometry upon co-culture of platelets with untreated MCF-7 cells, Palbociclib-treated cells or ADP (20 µM), used as a positive control, for 20 minutes. * P≤0.05, ** P≤0.01, ***P≤0.001

Confocal microscopy

Confocal microscopic examination revealed that platelets were attached to untreated MCF-7 cells and can be seen as red dots after staining with PE-labelled CD42 antibody (Figure 4C). However, this antibody can't distinguish activated platelets from inactivated platelets. Therefore, FITC-labelled CD62P antibody was used to stain activated platelets as depicted in (Figure 4A) where a clear expression of CD62P on the platelets' surface attached to MCF-7 was seen as green dots, indicating that platelets were activated.

When MCF-7 cells were treated with Palbociclib and then incubated with platelets, a pronounced increase in the intensity of green color was observed indicating that CD62P is expressed on platelets' surface (Figure 4E). Attachment of platelets to MCF7 cancer cells was evident as indicated by the presence of intense red and co-localization with MCF7 cells (Figure 4F, G).

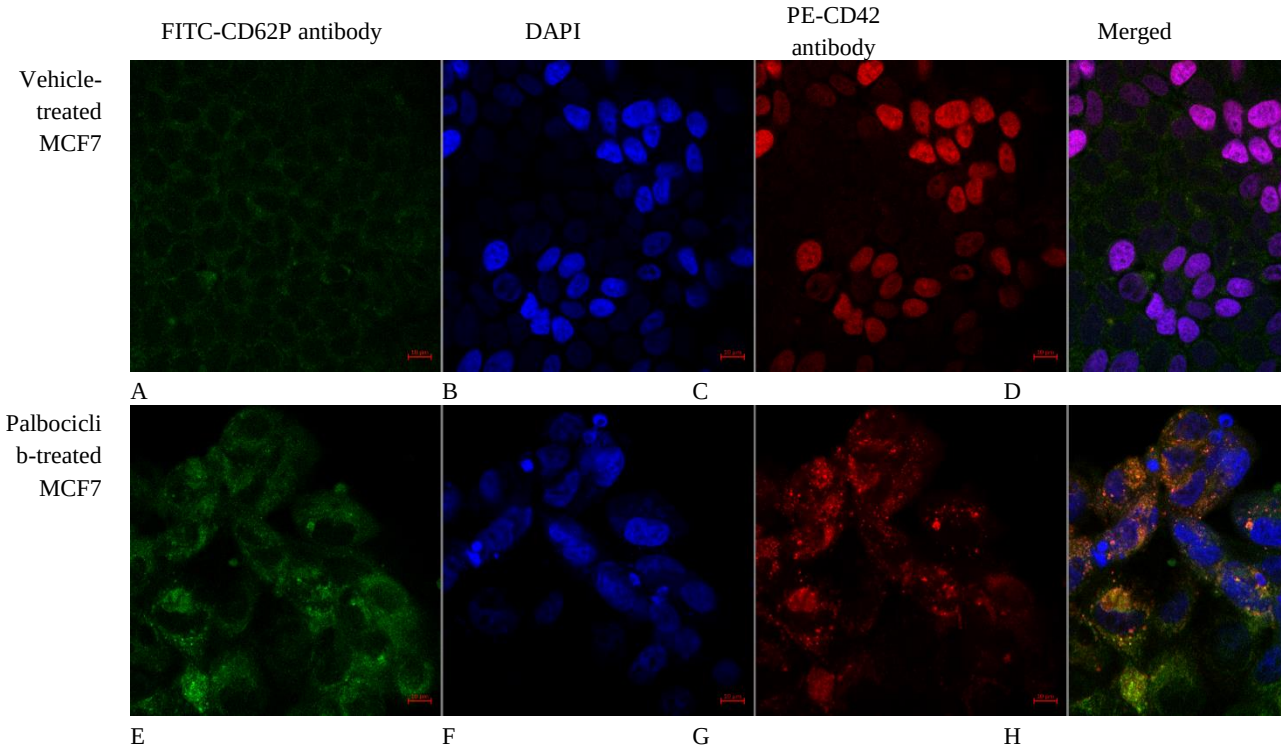

Figure 4: Attachment of platelets to MCF-7 breast cancer cells. (A-D) Platelets incubated with untreated MCF-7 cells stained with FITC-CD62P antibody (A), DAPI (B) or PE-CD42 antibody (C). (D) Represents merged A-C images. (E-H) Platelets incubated with 19.54 µM Palbociclib-treated MCF-7 stained with FITC-CD62P antibody (E), DAPI (F), or PE-CD42 antibody (G). (H) Represents merged E-G images (scale bar: 10µM).

DISCUSSION

Platelets can be activated *in vitro* and *in vivo* by different cancer cell types, a process known as "tumor cell-induced platelet aggregation" [(1)]. Important granular membrane molecules, including CD62P and CD63, become exposed on the platelet's cell surface after activation, aiding tumor development and metastasis [(19)]. In the present study, co-culturing MCF-7 with platelets activated them. This activation is indicated by the expression of CD62P on their surface. Earlier studies reported similar findings [(20)]. In a recent study, both MDA-MB-231 and MCF-7 cells increased platelets activation through the secretion of ATP from cancer cells. When platelets and MCF-7 cells were co-incubated, there was a four-fold increase in ATP release from platelet's granules in the case of MCF-7 cells [(21)]. In the present study, ATP release from cancer cells was not investigated. However, activation by direct contact was evident using confocal microscopy.

In our study, Palbociclib pre-treatment of MCF-7 cells increased their activation to platelets, as indicated by the presence of the activation marker CD62P on their surface. Similar results were obtained by other studies in which CD62P increased on the surface of platelets stimulated by cancer cells compared with resting platelets [(19, 22)].

According to reports, patients treated with Palbociclib had a relative risk for thromboembolic events (13). The pathophysiology of thrombotic events in cancer is complicated, and the individual patient's risk for

thrombotic events is affected by a multifactorial interplay between the person's cardiovascular risk and comorbidities, the specific neoplasm, and the treatment regimen [23]. The exact mechanisms underlying cancer therapy-related thrombosis remain uncertain. It is believed that anticancer medications have the potential to activate or disturb the endothelium, which could be a primary mechanism [24]. Additionally, these drugs may reduce the levels of anticoagulant substances or increase the levels of procoagulant substances, activating the coagulation process. Furthermore, anticancer drugs can directly or indirectly stimulate platelets [25]. The findings of this study confirm that Palbociclib-treated, hormone-positive MCF-7 cells exhibit increased platelet activation due to direct interactions between the cancer cells and platelets.

In the present study, ADP was used as a positive control. The percentage of platelets expressing the activation marker CD62P measured by flow cytometry was 84.4% after platelet incubation for 20 minutes with ADP (20 µM) at room temperature. When PRP was incubated with 10 µM ADP for 8 minutes at room temperature, CD62P expression, measured by flow cytometry, was about 59% (26). Another study reported a significant increase in platelet activation after incubation with ADP for 15 minutes, with 54.7% activation [27]. The degree of platelet activation by ADP can be influenced by varying ADP concentrations and different incubation periods.

It is well-known that platelets and cancer cells interact bi-directionally to promote tumor development and metastasis (6). The direct platelet-tumor cell contact depends on many factors facilitating interaction, including adhesion molecules, selectins and integrins (28). High platelet activation was reported by direct contact assessed by confocal microscopy as indicated by the increase in platelet activation markers PAC-1 and P-selectin (29). In the present study, platelet activation was clear as seen using confocal microscopy where platelets incubated with MCF-7 expressed the activation marker CD62P. In a previous study, co-incubation of platelets with MCF-7 showed that platelets promoted the invasion of these cells after direct contact with platelets. Labeling platelets with P-selectin antibody and studying them with flow cytometry, confirmed the enhancement of contacting ability between MCF-7 cells and platelets via surface integrin α2β1 (30). In another study, platelet shape change was visible after co-culturing with MCF-7, indicating at least partially activated platelets when platelets were stained by CD42a and PKH67 antibodies and studied with a confocal microscope [31]. This agrees with our results in which platelets were adherent to MCF-7 and activated.

Clinical trials have reported an increased incidence of thrombotic events associated with Palbociclib therapy [9]. This study elucidates a mechanism by which Palbociclib enhances platelet activation by increasing tumor-cell enhanced platelet activation. Whether this effect translates to in vivo settings remains to be verified. Understanding the mechanisms through which Palbociclib heightens thrombotic risks could facilitate the implementation of preventive measures during Palbociclib treatment, potentially involving the prescription of anti-platelet medications for patients. Further clinical investigations are warranted to explore this issue.

CONCLUSION

Treating hormone-positive breast cancer cell line MCF-7 with Palbociclib increased the activation of platelets. The effect of Palbociclib was confirmed by confocal microscopy in which platelets directly interacted with cancer cells. This direct interaction is responsible, at least partially, for platelet activation by cancer cells. Understanding how Palbociclib increases the risk of thrombosis could aid in implementing preventive measures during treatment and the use of antiplatelet medications for patients.

Conflicts of interest statement

The authors declared no conflict of interest in this study.

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زيادة تنشيط الصفائح الدموية الناتج عن العلاج بالبالبوسيكليب في خلايا سرطان الثدي MCF-7

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ملخص

الخلفية والهدف: ان التواصل المتبادل بين الصفائح الدموية وخلايا السرطان ثنائي الاتجاه. إذ يمكن للخلايا السرطانية تنشيط الصفائح الدموية، وهي عملية تُعرف باسم "تراص الصفائح الدموية الناجم عن الخلايا السرطانية". من ناحية أخرى، توفر الصفائح الدموية دعمًا أساسيًا للخلايا السرطانية من خلال مساعدتها على الالتصاق بالأوعية الدموية، مما يسهل هروبها من مجرى الدم وانتشارها إلى الأنسجة البعيدة. تبحث هذه الدراسة في تأثير عقار بالبالبوسيكليب، وهو مثبط لإنزيمات كيناز المعتمدة على السيكلين 4 و6 معتمد من قِبل إدارة الغذاء والدواء (FDA)، على تنشيط الصفائح الدموية الناجم عن خلايا سرطان الثدي.

الطريقة : تم تقييم تنشيط الصفائح الدموية من خلال قياس تعبير CD62P (P-selectin) باستخدام جهاز التدفق الخلوي. بالإضافة إلى ذلك، تم دراسة التصاق الصفائح الدموية بخلايا سرطان الثدي MCF-7 باستخدام المجهر متحد البؤر.

النتائج: تم تحديد التركيز المثبط للنصف (IC_{50}) لعقار البالبوسيكليب بقيمة 19.54 ميكرومول بعد 72 ساعة. تم تنشيط حوالي 36.9% ± 0.98 من الصفائح الدموية بواسطة خلايا MCF-7 غير المعالجة. المعالجة المسبقة لخلايا MCF-7بتركيز 9.75 ميكرومول من البالبوسيكليب ما يعادل (1/2 IC_{50}) أدى إلى زيادة تنشيط الصفائح الدموية بشكل ملحوظ بنسبة 63.3% ± 8.85 (P<0.01) بينما زاد تركيز 4.87 ميكرومول (1/4 IC_{50}) من تنشيط الصفائح الدموية بنسبة 43.0% ± 2.83 دون فرق معنوي مقارنة بالخلايا غير المعالجة. تشير نتائج المجهر متحد البؤر إلى وجود تفاعل مباشر بين خلايا سرطان الثدي والصفائح الدموية.

الاستنتاج: عزز عقار بالبوسيكليب تنشيط الصفائح الدموية الناجم بواسطة خلايا سرطان الثدي الإيجابيه للهرمونات MCF-7.

الكلمات الدالة: المجهر متحد البؤر، جهاز التدفق الخلوي، MCF-7، بالبوسيكليب، الصفائح الدموية، .P-selectin.

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