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Afzelin inhibits migration of MDA-MB-231 cells by suppressing FAK expression and Rac1 activation

Eva Rachmi[1,2*], Basuki Bambang Purnomo[3], Agustina Tri Endharti[4,5], Loeki Enggar Fitri[4,5]

[1]Doctoral Program in Medical Science, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.
[2]Department of Anatomy, Faculty of Medicine, Universitas Mulawarman, Samarinda, Indonesia.
[3]Department of Urology, Faculty of Medicine, Universitas Brawijaya/dr Saiful Anwar General Hospital, Malang, Indonesia.
[4]Department of Parasitology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.
[5]Biomedical Central Laboratory, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.

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ABSTRACT

Triple-negative breast cancer (TNBC) has the worst prognosis and the highest rate of metastasis among other types of breast cancer. These characteristics are supported by the dysregulation of focal adhesion kinase (FAK) and Rac1 which are the key players of mesenchymal cell migration on TNBC. Afzelin is a secondary metabolite that is contained in a variety of plants. This study explored the anti-migration effect of afzelin and its interaction with FAK and Rac1 on the highly invasive TNBC cell line, MDA-MB-231. Cell viability was assessed by 3-(4,5-dimethyl 2-thiazolyl)-2,5-diphenyltetrazolium bromide assay, and cell migration was evaluated using *in vitro* scratch assay. Rac1 activation was analyzed using the colorimetric assay, while vinculin and actin filaments were stained through immunofluorescence. The quantity of total FAK and phosphorylated FAK tyr397 was detected by Western blotting. Afzelin decreased cell viability and inhibited two-dimensional cell migration in a dose-dependent manner. Under confocal laser scanning microscopy, vinculin localization at the cell edge demonstrated a reduction of focal adhesion formation by afzelin. Further exploration showed that afzelin decreased FAK expression but did not affect FAK phosphorylation at tyr397. In addition, afzelin decreased Rac1-GTPase activation, which is a downstream effector of FAK. Taken together, these results suggest that afzelin suppresses TNBC cell migration, through inhibition of FAK expression and Rac1-GTPase activation.

INTRODUCTION

Breast cancer ranks as the second-highest incidence in women and is the leading cause of cancer deaths in 135 countries (Bray *et al*., 2018). The five-year survival rate of breast cancer patients decreases dramatically when distant metastases occur (Howlader *et al*., 2016). Among breast cancer subtypes, triple-negative breast cancer (TNBC) has the worst prognosis, particularly in the first three years after the diagnosis (Dai *et al*., 2015; Ovcaricek *et al*., 2011). TNBC tends to metastasize the visceral organs and the central nervous system (Anders and Carey, 2009), which lead to only 13 months of median overall survival (Bacalbasa and Ionescu, 2016).

The inhibition of metastasis is challenging because it involves a cascade of processes, each of which has its own requirements. However, the ability of metastatic cells to migrate is a common characteristic in almost all steps, which can be targeted in inhibiting metastasis (Mackay, 2008; Wells *et al*., 2013). Cell migration occurs through a cycle consisting of membrane protrusion, focal adhesion formation, extracellular matrix protease secretion, contraction of stress fibers, and rear cell retraction (Friedl and Wolf, 2003).

Focal adhesion kinase (FAK) as an adapter of focal adhesion and Rac1 as a modulator of cytoskeletal rearrangement in the lamellipodia formation play a crucial role in the migration mechanism. Both were found to be hyperactivated in various types of solid cancers (Lou *et al*., 2018; Yoon *et al*., 2015), which could be caused by abnormal upstream inputs, overexpression, or

*Corresponding Author
Eva Rachmi, Doctoral Program in Medical Science, Universitas Brawijaya, Malang, Indonesia; Department of Anatomy, Medical Faculty, Universitas Mulawarman, Samarinda, Indonesia.
E-mail: e.rahmi @ fk.unmul.ac.id

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deregulated degradation (De *et al*., 2019). In TNBC, dysregulation of FAK (Golubovskaya *et al*., 2014) and Rac1 (Tsai *et al*., 2015) is associated with increased invasion, metastasis, and poor prognosis. Inhibition of TNBC invasion could be achieved by suppressing FAK (Taliaferro-Smith *et al*., 2015) or Rac1 (Morimura and Takahashi, 2011) activation.

Previous studies supported afzelin potential as anti-cancer. In breast cancer cells, estrogen receptor-positive subtype, afzelin increases apoptosis through caspase cascade activation (Diantini *et al*., 2012). The ability of afzelin to activate caspase cascade is also shown in androgen-sensitive prostate cancer (Halimah *et al*., 2015). In addition, afzelin inhibits proliferation and cell cycle of prostate cancer cells that are both androgen-sensitive or independent (Zhu *et al*., 2015). Afzelin shares characteristics with other flavonols in terms of structural similarity with adenine. Therefore, afzelin is predicted to compete with ATP in the ATP-binding enzyme. The addition of rhamnoside groups makes afzelin structure unique, hence it might inhibit kinases more selectively (Smith *et al*., 2005; Utepbergenov *et al*., 2012). For this reason, it is necessary to prove the ability of afzelin to inhibit migration in TNBC, through the suppression of FAK expression and Rac1 activation. The effects will be proven in M.D. Anderson-Metastatic Breast-231 (MDA-MB 231) cells, as the TNBC model whose characteristics are most explored and most frequently used in research (Volk-Draper and Rajput, 2012).

MATERIALS AND METHODS

Treatment material

Afzelin was purchased from ChemFaces® (CFN98757). Afzelin was prepared in dimethyl sulfoxide (DMSO) prior to each treatment and diluted with appropriate cell culture medium to desired concentration (100, 200, 400, and 800 µg/ml).

Cell culture

Human MDA-MB-231 breast cancer cells (ATCC® HTB-26™) were maintained in Dulbecco's modified Eagle's medium—high glucose supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, and 1% penicillin-streptomycin (GIBCO™). The cells were incubated at 37 °C in an incubator with 5% CO_2 at 90% confluence.

Cell viability assay

Cell viability was assayed by the 3-(4,5-dimethyl 2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reduction method. Briefly, MDA-MB-231 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured for 48 hours, followed by treatment with various afzelin concentrations (0, 100, 200, 400, and 800 µg/ml) for overnight. Next, 15 µl MTT solution (0.5 mg/ml) was added to the culture for another 4 hours, and the medium was subsequently removed. The formed formazan crystals were dissolved by adding 100 µl of DMSO. The absorbance of each well was measured at 570 nm by a microplate reader (Bio-Rad Laboratories, USA).

Migration assay

Cell migration was evaluated using *in vitro* scratch assay. For this random cell migration, MDA-MB 231 cells were plated in 24-well plates at 80%–90% confluence. The culture monolayers were wounded with a p200 pipette tip, washed twice with complete medium to remove cell debris, and then treated with serum-starved media (0.5% FBS) containing afzelin. The wells were re-evaluated within 24 and 48 hours. The wounded area was re-photographed in two areas, under an inverted microscope at 400×. Cell migration was determined by examining the area occupied by the cells (Lewis *et al*., 2019) and the number of cells in the wounded area (Tantivejkul *et al*., 2003) assisted by ImageJ software. The area measurement was done through a Montpellier Ressources Imagerie Wound Healing plugin. The cell-occupied area was a cell-free area at 0-hour subtracted by a cell-free area at the time of observation (24 or 48 hours) as a percentage of cell-free area at 0-hour.

Rac1 activation assay

Afzelin inhibition of Rac1 activation in MDA-MB-231 cells was analyzed using the colorimetric-based Rac1 G-LISA™ Activation Assay Kit (CytoskeletonInc; Denver, CO). MDA-MB-231 cells were plated in a 24-well plate with complete media. After reaching 60%–70% confluence, the media was replaced with starvation media (FBS 0.5%) containing different concentrations of afzelin for 24 hours. Cells were washed with ice-cold phosphate-buffered saline (PBS) and then added with serum-free media containing epidermal growth factor (EGF) 50 ng/ml for 5 minutes (Zhang *et al*., 2015). Subsequently, the cells were lysed and active-Rac-1 levels of the lysate were quantified according to the manufacturer procedure. Equal amounts of protein per sample lysate were determined by the UV-VIS spectrophotometer (NanoDrop™, Thermofisher). Active Rac1 levels were expressed as a fold increase over the active Rac1 levels in control conditions.

Focal adhesion formation

MDA-MB-231 were seeded on fibronectin-coated glass coverslips in a 24-well plate with complete media for 24 hours. The cells treated with various concentrations of afzelin for 14 hours. After removing the media, the cells were fixed with paraformaldehyde 4% and then washed two times with PBS. Vinculin and actin filaments were stained with actin cytoskeleton and focal adhesion staining kit FAK100 (Merck-Millipore, USA), according to the manufacturer's instructions. Focal adhesion and actin were visualized by a confocal laser scanning microscope FV 1000 (Olympus, USA) with 400× magnification.

Western blot analysis

MDA-MB-231 were cultured at 90%–95% confluence, washed with ice-cold PBS, and scrapped in radioimmunoprecipitation assay buffer (G-Biosciences, USA) containing phosphatase inhibitor (Medchemexpress, USA) and protease inhibitor (Sigma-Aldrich, USA). The total protein concentration of each treatment was measured utilizing a UV-VIS spectrophotometer (NanoDrop™, Thermofisher). Equal amounts of protein were then subjected to electrophoretic separation in sodium dodecyl sulfate polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane, using a semi-dry transblot apparatus at 20 volts and 300 mA for 2 hours. Membranes were blocked overnight with 5% bovine serum albumin (BSA) at 4°C,

followed by probing with primary antibodies such as anti-FAK1 monoclonal (1:500) (bs-3159R) and anti-FAK tyr397 polyclonal (1:500) (bsm 50324M, Bioss Inc, USA) for 2 hours. Afterward, a secondary antibody was added for 2 hours and subsequently Strepavidin-Horse Radish Peroxidase for 1 hour. Bound proteins were detected using 3,3′,5,5′-tetramethylbenzidine substrate in the darkroom. Gel quantifications were performed using ImageJ (Alečković *et al*., 2017).

Statistical analysis

For statistical analyses, results were reported as average ± standard error mean. Analysis of variance followed post hoc analysis was used to explore possible pair-wise comparisons of means between different treatments. A p-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Afzelin reduced MDA-MB-231 cells viability

Effect of afzelin on cell viability was estimated based on the premise that viable cells with active metabolism convert MTT into a purple-colored formazan product (Riss *et al*., 2016). The MTT assay showed a decrease of formazan formation along with the increase of afzelin dose. This result meant that afzelin reduced cell viability in a dose-dependent manner (Fig. 1). Through regression linear analysis, the the IC50 of afzelin on MDA-MB-231 cells was predicted to be 992 µg/ml. Afzelin cytotoxicity on other cancer cell lines was also proven in previous publications such as estrogen-sensitive breast cancer cells (MCF-7), hepatocarcinoma cells (HC-04) (Diantini *et al*., 2012), and both androgen-dependent (LNCaP) and independent (PC-3) prostate cancer cells (Zhu *et al*., 2015).

Afzelin inhibited MDA-MB-231 cells migration

In the scratch monolayer assay model, afzelin inhibited two-dimensional cell migration in a dose-dependent manner. This result was demonstrated by the decrease in the number of cells that migrated into the wounded area. Afzelin treatment at concentrations of 400 and 800 µg/ml suppressed the number of migrated cells, which was demonstrated by non-significant differences between observations at 24 and 48 hours (Fig. 2B).

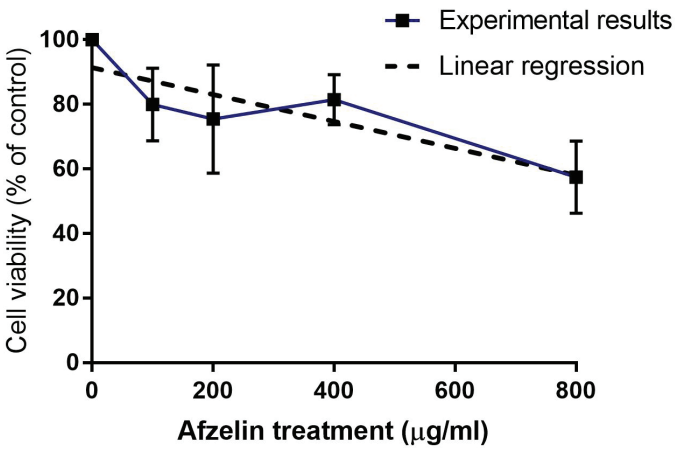

Figure 1. Cell viability assay in MDA-MB-231 cancer cell after overnight treatment of afzelin. The relative amount of viable cells was estimated by measuring cell suspension absorbance after MTT assay. Calculation of IC50 value was predicted through the trend line equation (Y = −0.0417.X + 91.38) based on graph of cell viability versus afzelin concentration. Data were shown as mean + standard error mean (SEM).

The quantitative degree of migration was verified further by measuring the cell-covered area, which showed a similar trend with the decline of migrated cells (Fig. 2C). Compared to the number of cells, the analysis of the cell-covered area gave different results at 400 µg/ml afzelin-treatment group, while observation after 48 hours showed a significant increase in migration compared to that of 24 hours. Afzelin treatment at 400 µg/ml was not inducing a drastic change in MDA-MB-231 cell morphology compared to 800 µg/ml where the cells became thinner and elongated (Fig. 3A). This event might explain why the increase of migrated cells could increase the occupied area significantly in 400 µg/ml afzelin-treatment group, even though the difference of migrated cell numbers between 24 and 48 hours was not significant. Afzelin inhibition of MDA-MB-231 cell migration in scratch assay might predict anti-metastatic potential *in vivo* (Adams *et al*., 2010; Choi *et al*., 2014; Li *et al*., 2017).

To explore the mechanism of the afzelin-mediated inhibition of cell migration, the focal adhesion formation was analyzed based on the vinculin localization in the edge of the cells. As compared to the control negative group, the focal adhesion formation was reduced following the increase of afzelin concentration (Fig. 3A). This result was in line with the decrease of two-dimensional migration that was induced by afzelin treatment. Although, in this study, we did not determine afzelin effect on the descriptor of focal adhesion (size, number, surface density, and shape), the decrease of migrated cell number and cell-covered area might be related with the diminishing of the focal adhesion size (reducing cell speed) or change of focal adhesion shape (reducing cell speed, final distance traveled, and persistence distance) (Kim and Wirtz, 2013).

Afzelin inhibited FAK expression and Rac1 activation

Afzelin effectively inhibited EGF-stimulated activation of Rac1 GTPase at 400 and 800 µg/ml, which was diminished by 60% compared to the negative control (Fig. 3C). The formation of membrane protrusion as the initial step of migration is controlled by the Rho-GTPase family, with Rac-1 playing a crucial role. Rac1 regulates lamellipodia formation in response to growth factor receptors stimulation or integrin activation (Nobes and Hall, 1999). Lamellipodia provides forward cell traction. To achieve this function, focal adhesion (FA) needs to be formed in lamellipodia, which will connect integrins with extracellular matrix (outward) and with actin cytoskeleton (inward). Actin when connected with focal adhesion will contract and then produce stress fibers that pull the cell forward (Mack *et al*., 2011).

Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase and the main adaptor protein of FA that modulates adhesive interaction (Horton *et al*., 2016). FAK regulates cell motility, survival, and proliferation through the integration of growth factors and integrin-mediated signaling (Horton *et al*., 2016; Sieg *et al*., 2000). FAK activation by Src stimulates Rac1 activity through breast cancer anti-estrogen resistance (BCAR)1/ downstream of Crk

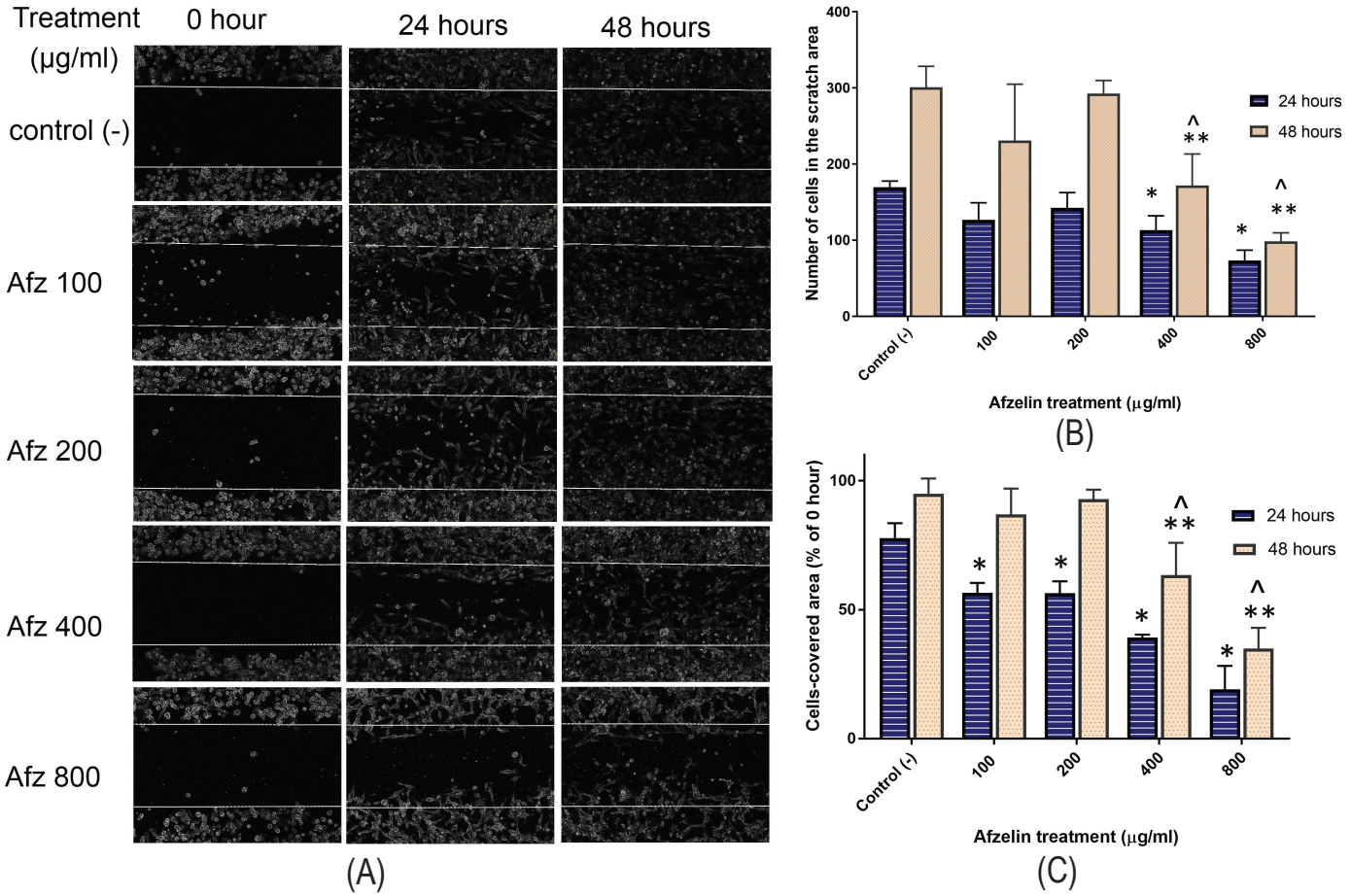

Figure 2. Effect of afzelin on MDA-MB-231 cell migration. (A) The migration was inhibited after afzelin treatment, determined by wound scratch assay. Afzelin suppression on cell migration was quantified based on the number of cells that migrated to the cell-nude area (B) and percentage of cells-covered area inside the cell-nude area (C). *Significantly different than negative-control (24-hours), **Significantly different than negative-control (48-hours), ^Not significantly different than 24-hours treatment. Data were shown as mean + SEM.

(DOCK)180/engulfment and cell motility (ELMO)1 signaling, with the DOCK180-ELMO1 complex serving as a guanine nucleotide exchange factor (GEF) for Rac1. The FAK-Src complex also activates the Pak-interacting exchange factor-beta, which is the GEF for Cdc42 and Rac1. Recruitment and activation of Rac1, in turn, will promote the formation of membrane cell protrusions (Danen, 2013; Huveneers and Danen, 2009). To evaluate the afzelin effect on FAK, the quantity of total FAK and phosphorylated FAK tyr397 was detected by Western blotting. During afzelin treatment, FAK expression was down-regulated, particularly at a dose of 800 µg/ml. Meanwhile, afzelin suppression on FAK tyr397 phosphorylation might be due to its inhibition of FAK expression (Fig. 3B). If afzelin inhibits FAK tyr397 phosphorylation directly, the increase of afzelin concentration should have more effect on the reduction of phosphorylated FAK tyr397 than on the reduction of the total FAK.

Our results demonstrated that afzelin had the potential as the inhibitor of TNBC migration, through suppression of FAK expression and Rac1 activation. However, still there are questions that are needed to be further elucidated. In this study, afzelin affected the focal adhesion formation and Rac-1 activation at 400 µg/ml; on the other hand, afzelin affected FAK expression and FAK tyr397 phosphorylation at 800 µg/ml.

These phenomena could be explained through some possibilities. First, considering that FAK activation is a sequential event involving several domains and amino acid phosphorylation following tyr397 activation, afzelin at a dose of 400 µg/ml probably inhibited FAK directly at other sites, such as phosphorylation of tyr576/577 at kinase domain or tyr925 at binding-site of Grb2. Second, afzelin possibly inhibited signal transduction downstream of FAK. Cell adhesion is formed through a dynamic process in the form of focal complexes, FA, or fibrillar adhesion. Focal complexes are small (less than 1 μm^2) at the edges of spreading cells or leading edge of migrating cells, the components of which include vinculin and FAK. To be able to effectively migrate, focal complexes develop into FAs that involve other protein tyrosinases (Rikitake and Takai, 2011). At a concentration of 400 ug/ml, afzelin might be able to downregulate signaling which contributed to focal complexes maturation into FA. Afzelin could possibly downregulate signaling protein downstream of the FAK-Src complex, including p130Cas, paxillin, BCAR, DOCK180, and ELMO1, or even directly inhibited Rac-1 activation. These hypotheses need further investigations.

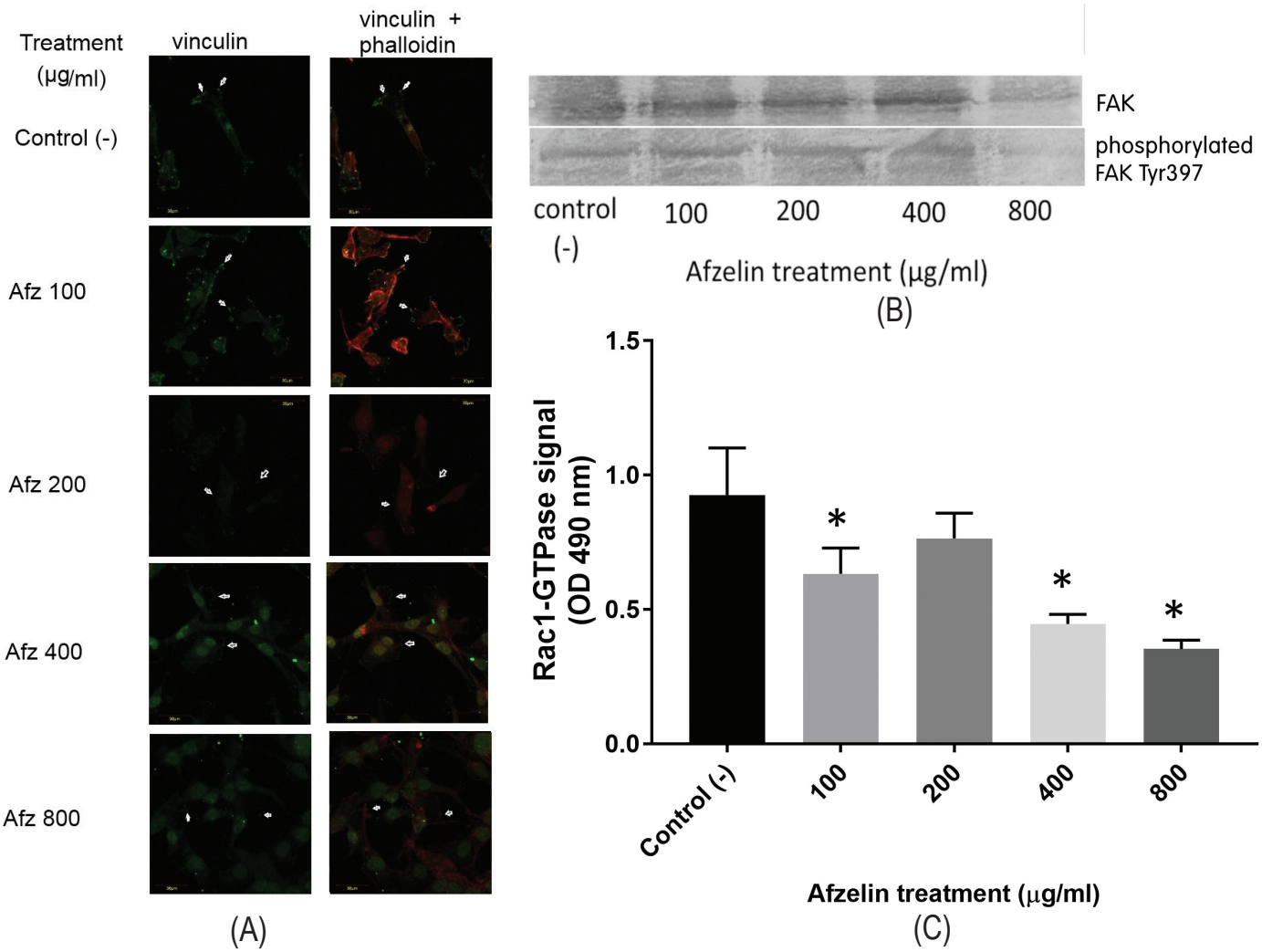

Figure 3. Afzelin inhibited FA formation, FAK expression, and Rac1 activation in MDA-MB-231 cells. (A) After treated with various doses of afzelin, MDA-MB-231 cells were stained with anti-vinculin antibody (green) and TRITC-conjugated phalloidin (red). Focal adhesion was indicated by the arrow. Bar equals approximately 30 µm. (B) The effect of afzelin on FAK expression and FAK tyr397 phosphorylation was assessed qualitatively by Western blotting. (C) Quantification of Rac1 activation demonstrated downregulation by afzelin. * Significantly different than negative-control. Data were presented as mean + SEM.

CONCLUSION

In conclusion, afzelin exposures suppressed cell migration by reducing focal adhesion formation. Afzelin reduced FAK expression and subsequently FAK phosphorylation at tyr397. Besides, afzelin downregulates the activation of Rac1 GTPase. The inhibition of two-dimensional migration *in vitro* on MDA-MB-231 supports the prediction of anti-metastatic ability *in vivo*. These results firmly suggest that afzelin presents a potential anti-metastatic property and a good candidate for further testing as a potential compound or a bioisosteric template for drug development for TNBC metastasis inhibition.

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CONFLICTS OF INTEREST

The authors declared that they have no conflict of interest.

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None.

AUTHORS' CONTRIBUTIONS

Study concept and design: Eva Rachmi (ER), Basuki Bambang Purnomo (BBP), Agustina Tri Endharti (ATE), and Loeki Enggar Fitri (LEF). Acquisition, analysis, and interpretation of data: ER and LEF Drafting of the manuscript: ER Critical revision of the manuscript for intellectual content: ER, BBP, ATE, and LEF Agreement to be accountable for all aspects of the work: ER, BBP, ATE, LEF.

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