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ORIGINAL RESEARCH

Aaptamine Alters Vimentin Expression and Migration Capability of Triple-Negative Breast Cancer Cells

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Purpose: This study aimed to explore the effect of Aaptamine, an alkaloid derived from marine sponges, on the vimentin expression in both mRNA and protein levels and the migration capacity of breast cancer cells.

Methods: The triple-negative breast cancer cell line MDA-MB-231 was used for in vitro experiments. Low-cytotoxicity concentrations of Aaptamine (12.5 to 50 μM) were given to MDA-MB-231 cells. The vimentin mRNA and protein expression were evaluated using RT-qPCR and immunofluorescence, respectively, 72 h after Aaptamine treatment. The migration scratch assay was conducted for 48 hours.

Results: Aaptamine treatment in three different doses did not affect the expression of vimentin at the mRNA level while significantly lowering vimentin protein expression at the concentration of 12.5 μM. In addition, Aaptamine significantly inhibited breast cancer cell migration in a dose-dependent manner.

Conclusion: Aaptamine inhibits vimentin protein expression and demonstrates anti-migration activity in the sub-cytotoxic dose.

Keywords: aapatamine, cellular migration, EMT, MDA-MB-231, vimentin

Introduction

Triple-negative breast Cancer (TNBC) is one of the breast cancer subtypes, which is known for its aggressiveness and is relatively more prone to metastasize.[1–4] The absence of three biomarkers in TNBC is making targeted therapy ineffective. Thus, systematic and less specific chemotherapy is becoming the first-line treatment for TNBC patients. However, chemotherapy has various mild to severe side effects, including the chance of developing chemoresistance.[5] Previous studies also reported the impact of one of the chemotherapy drugs, doxorubicin, on increasing the aggressiveness of breast cancer cells by promoting cell migration, invasion, and epithelial-to-mesenchymal transition (EMT), which leads to the cells' inclination to metastasize.[1–4]

EMT is a biological mechanism in which polarized epithelial cells undergo morphological changes and acquire mesenchymal phenotypes, considered the initiation of cell invasion and metastasis. During the EMT process, epithelial cells undergo multiple biochemical modifications to gain the mobility of mesenchymal cells. The biochemical mechanism involves the initiation of EMT by transcription factors, the expression of specific cell-surface proteins, cytoskeletal modification and rearrangement, the secretion of enzymes for extracellular matrix (ECM) degradation, and alterations in the expression of specific microRNAs.[6,7] Thus, those characteristics, as well as transcription factors related to its expression, become biomarkers known to be associated with EMT, including cytoskeletal proteins such as vimentin, actin, and cytokeratin.[8–10]

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As discussed in previous studies, vimentin is a biomarker for EMT that is currently gaining interest as it is also reported to be a potential biomarker for poor-prognosis cancer.[10–12] Vimentin is classified as a type III intermediate filament of the cytoskeleton.[13] According to a recent study, the persistent cells' enhanced vimentin expression after chemotherapy suggested they had become more invasive. Additionally, the same study found that in MDA-MB-231 persistent cells, downregulating vimentin could enhance sphere-forming ability while decreasing the invasive capability of the cancer cells.[4] A recent study reported the role of vimentin as an excellent prognostic biomarker for TNBC patients. In the study, the 48-month overall survival (OS) of vimentin-positive TNBC patients treated with platinum-based chemotherapy is significantly worse compared to the vimentin-negative TNBC patients (80% vs 43.8%).[12]

With all the drawbacks and limited effectiveness of TNBC's current treatment options, more and more research on developing a therapeutic approach for TNBC is still being conducted around the globe, especially research on the anticancer properties of natural ingredients that hope to be relatively safer.[14] Previous research on marine sponges disclosed a tremendous cytotoxic effect on breast cancer in in vitro studies using three-dimensional cell culture.[15,16] Bioactive compounds in marine sponge extract have also been reported to suppress cell migration in breast cancer cell lines.[17] One of the rising bioactive compounds from marine sponges (*Aaptos sp.)* that exhibit favorable antineoplastic properties is Aaptamine.[18,19]

Aaptamine is considered a potent marine-derived anti-cancer drug candidate that demonstrates the anti-proliferative, anti-migration, and metastatic effects on various cancers in both in vitro and in vivo studies.[18–23] Aaptamine is reported to inhibit cancer proliferation by directly inhibiting the PI3K/AKT pathway by degrading phosphorylated AKT and suppressing protein MMP7 and MMP9 expression.[20] Aaptamine specifically decreases the ratio of p-AKT/AKT through specific dephosphorylation of AKT. The study reported the synergizing effect of Aaptamine in perifosine, AKT antagonist, mediated dephosphorylation while it counteracted EGF-induced activation of AKT.[20] Furthermore, intriguing reports demonstrate the involvement of PI3K/AKT and MMP9 pathways in regulating vimentin expression.[24–26] Thus, Aaptamine potentially suppresses vimentin expression through the PI3K/AKT pathway. Therefore, this current study aimed to evaluate the effect of Aaptamine on EMT activation through the expression of vimentin and migration capability of triple-negative breast cancer cell line MDA-MB-231, which is related to the EMT mechanisms of the cancer cells.

Materials and Methods

Cell Culture and Dose Determination (MTT Assay)

The human triple-negative breast cancer cell line MDA-MB-231 was cultured in Roswell Park Memorial Institute medium (R8758, Sigma-Aldrich) supplemented by 10% fetal bovine serum (10270106, Gibco) and 1% penicillin-streptomycin at 37°C, 5% CO_2. The MDA-MB-231 cells were obtained from Dr Thordur Oskarsson (DKFZ, Germany) and approved for use by the Ethics Committee of the Universitas Padjadjaran (No. 265/UN6.KEP/EC/2025).[27] The determination of treatment dosage was evaluated using 3-[4,5-dimethylthiazol-2-yl]-2.5 diphenyl tetrazolium bromide (MTT) assay, as described in the previous study.[15] The cells were seeded into 96-well plates with a seeding density of 10^4 cells/well incubated overnight in a complete medium. Following that, the cells were treated with Aaptamine (HY-N4225, MedChemExpress, USA) in a serum-free medium with a concentration range of 12.5 to 150 μM with technical replication n = 4. After 72 hours of treatment, the medium was changed into a fresh medium with 10% MTT stock solution (Invitrogen™) and incubated for three hours. MTT reaction was then stopped by adding 100 μL solubilizing stop solution (DMSO) in which the crystal formazan was diluted. The optical density was measured using a microplate reader at 570 nm wavelength. The absorbance value of samples was used to calculate the cell death percentage. Three concentrations with inhibition lower than 50% were used for experiments, which are 12.5, 25, and 50 μM.

Immunofluorescence Staining

Vimentin protein expression in breast cancer cells was identified using immunofluorescence labeling (n = 2). The samples were fixed with 4% paraformaldehyde for 10 min and permeabilized using 0.1% Triton™ X-100 for 15 min at room temperature (RT). Following that, the samples were blocked with 1% BSA at RT for 1 hour. The specimens were incubated with Vimentin Mouse Monoclonal Antibody (MA511883, Invitrogen) in 0.1% BSA overnight at 4°C with

a dilution rate of 1:100, followed by Goat anti-Mouse IgG (H+L) Superclonal™ Secondary Antibody, Alexa Fluor® 488 conjugated (A27034, Invitrogen) with dilution rate of 1:500 for 60 min at RT. Subsequently, the cells were counterstained with Rhodamine Phalloidin (R415, Invitrogen) at dilution 1:200 and DAPI (62248, Invitrogen). The stained samples were observed using a Confocal Laser Scanning Microscope (LV1200, Olympus). The intensity of protein expression data was analyzed using ImageJ by calculating the fluorescence intensity (n = 5), as described previously.[28]

Quantitative RT-qPCR Analysis

In this study, vimentin's mRNA expression was assessed using quantitative RT-qPCR. Table 1 lists the primers utilized in this investigation. The total mRNA was isolated from the breast cancer cell culture after 72 hours of treatment (n = 3) using the Quick-RNA™ Miniprep Kit (Zymo Research, US). The SensiFast cDNA synthesis kit (Bioline Reagents Ltd., UK) and SensiFast SYBR no-ROX kit (Bioline Reagents Ltd., UK) were then used to perform RT-qPCR (n = 2) in accordance with the manufacturer's instructions. The activation of the polymerase reaction was set at 95°C for 2 minutes for initial denaturation, followed by 40 amplification cycles comprised denaturation of 95°C for 5 seconds and annealing/extension at 55°C for 10 seconds, and elongation at 72°C for 20 seconds. The vimentin mRNA expression was normalized with GAPDH mRNA expression as the housekeeping gene.

Scratch Assay

The breast cancer cells (MDA-MB-231) were seeded in a complete medium into 12-well plates with a seeding density of 1.5×10^5 cells/well. The cells were cultured at 37°C temperature for 24 hours in a 5% CO_2 incubator. The following day, change the medium into serum-free medium and add Aaptamine with the subsequent concentration (n = 3): 0, 12.5, 25, or 50 μM. Scratch vertically the cell culture at the well's bottom using yellow pipette tips. The scratched areas were observed, and pictures were taken at 0, 24, and 48 hours. Using ImageJ software, the migration area was calculated by computing the clear-of-cells area of each sample and normalized to the initial wound area. One-way ANOVA was used to examine the data extracted from ImageJ statistically.

Statistical Analysis

The RT-qPCR and migration assay data were analyzed using one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons comparing means to the control group. Meanwhile, the vimentin immunofluorescence intensity data was analyzed using an unpaired *t*-test. Group differences were regarded as statistically significant if they reached $p < 0.05$. The statistical analysis and graphical presentation were produced utilizing GraphPad Prism (version 10.2.3).

Results

Sub-Lethal Dosed of Aaptamine on TNBC Cells

The minimum inhibition concentration (IC_{50}) of Aaptamine on the MDA-MB-231 cell line was 76.86 μM. Three of sub-lethal doses of Aaptamine were used in the experiments, which are 12.5, 25, and 50 μM with inhibition rates of 4.5%, 11.6% and 29.1%, respectively (Figure 1).

Table 1 List of Primers

Marker		Sequence	PCR Size
VIM (NM_003380.5)	Forward Reverse	CTC GTC ACC TTC GTG AAT AC GCA GAG AAA TCC TGC TCT C	111 bp
GAPDH (NM_001289746.2)	Forward Reverse	CAT CAG CAA TGC CTC CTG C ATG GAC TGT GGT CAT GAG TCC	100 bp

Abbreviations: bp, base pairs; VIM, vimentin.

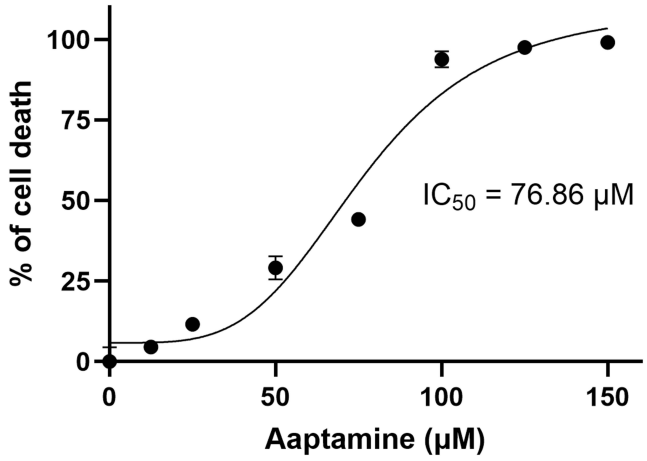

Figure 1 MTT assay result of Aaptamine on MDA-MB-231 cell line.

Aaptamine Suppressed Vimentin Expression at the Protein Level

The immunofluorescence staining was successfully done with the staining of vimentin (AlexaFluor-488), F-actin (rhodamine phalloidin), and nucleus (DAPI). The immunofluorescence staining visualized the vimentin expression in the control and 12.5 µM Aaptamine treated groups. This group was chosen to evaluate the vimentin expression of the cells treated with very low toxicity dose of Aaptamine (4.5% inhibition rate). The cells of the treated group exhibited a relatively lower fluorescence signal than the negative control group, as seen in Figure 2A.

ImageJ software quantified the fluorescence signal intensity to help confirm the morphological observation. A comparison of Vimentin immunofluorescence intensity was done using an unpaired *t*-test. The result demonstrated a significant difference in vimentin expression in MDA-MB-231 cells treated with 12.5 µM Aaptamine compared to the control group, with a p-value of 0.0476. The mean difference between the treated and control groups (Δmean ± SEM) was −0.9540 ± 0.04446 (Figure 2B). Furthermore, statistical analysis of F-actin expression also demonstrated a significant difference with *p*-value <0.0001 (Figure 2C). The findings from this parameter indicate that low-cytotoxic dose Aaptamine treatments downregulated vimentin expression at the protein level.

Aaptamine Does Not Alter Vimentin mRNA Expression

According to the real-time PCR result, all treated groups expressed vimentin mRNA, and there was no discernible difference between them and the negative control groups (Figure 3). This finding indicated that Aaptamine has minimal impact on vimentin expression at the messenger level ($p = 0.9451$).

Aaptamine Suppresses the Migration of TNBC Cells

Breast cancer cell migration was observed at 0, 24, and 48 hours upon treatment (Figure 4A). The migration area in this experiment was calculated with the help of ImageJ software. The 48-hour area was normalized to the initial 0-hour area. The quantified data from each picture was then statistically analyzed using One-way ANOVA. The result implied that the Aaptamine significantly suppressed the migration capability (*p*-value = 0.0009) of breast cancer cells MDA-MB-231 in a dose-dependent manner (Figure 4B). The *p*-values of Dunnett's multiple comparison tests of the treatment groups compared to the negative control are 0.0306 (12.5µM), 0.0023 (25µM), and 0.0004 (50µM). Hence, these findings implied that Aaptamine demonstrated an anti-migration effect on breast cancer cells. In addition, Aaptamine displayed a suppressive effect on vimentin protein expression in MDA-MD-231 cells.

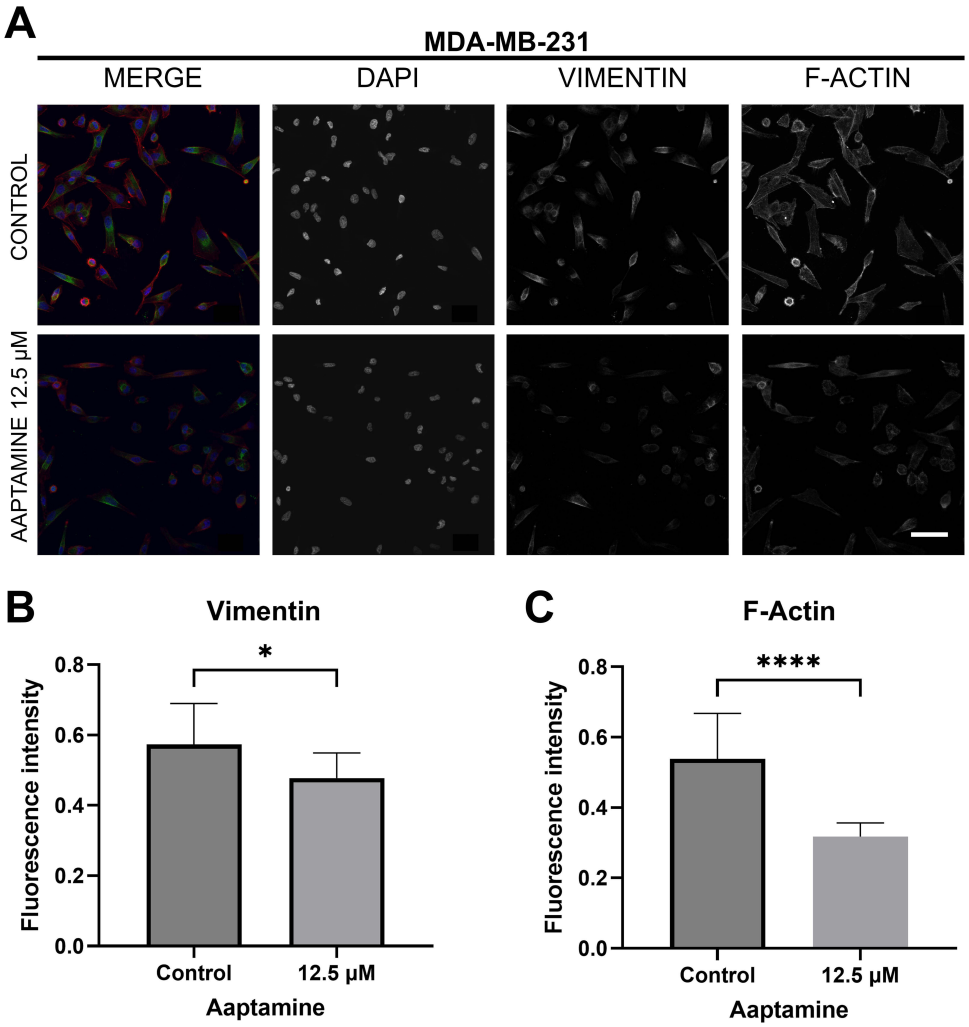

Figure 2 (**A**) Immunofluorescence staining of MDA-MB-231 cells of negative control and Aaptamine-treated groups. Fluorescence staining: Blue, DAPI; Green, Vimentin; Red, F-actin. (**B**) Vimentin and (**C**) F-Actin immunofluorescence intensity of low-dose Aaptamine treatment in breast cancer cell line MDA-MB-231. **p*<0.05; *****p*<0.0001; *Bars* 50 µm.

Discussion

Aaptamine, an alkaloid derived from marine sponges, has demonstrated significant cytotoxic effects on several cancer types.[19–21,23] Meanwhile, this compound was reported not to affect the viability of normal breast cells even in cytotoxic concentrations for cancer cells in vitro. It implies the preferable cancer cytotoxic effect and safety for normal cells.[29] Recent findings discovered that Aptamine's involvement in regulating the PI3K/AKT pathway is associated with its antiproliferative response to cancer cells. This compound was also reported to induce phosphorylated-AKT degradation and suppression of MMP9 and MMP7 under the PI3K/AKT pathway.[20,30] Intriguingly, this particular pathway is also frequently discussed as related to the EMT process and vimentin expression, one of the widely recognized EMT markers.[24,31] Hence, this study explored the effect of Aaptamine on the EMT marker expression, vimentin, and the impact on the capability of TNBC cells to migrate, which is one of the prerequisites for cancer to metastasize.

According to confocal microscope observation, the cells treated with a low-cytotoxic dose of Aaptamine were comparatively smaller than the control group. Furthermore, compared to the control, the stained nucleus of the treated cells seemed smaller and dimmer. This investigation also showed a discernible decrease in the expression of vimentin and actin filament. The shrinkage of the treated cells may be caused by the cleavage of actin filaments, which are one of the target proteins of caspases.[32] Caspase-3, one of the mediators of apoptosis, catalyzes the cleavage of various vital cellular proteins, which causes cells to shrink and ultimately leads to cell death.[33,34] According to a different study, non-

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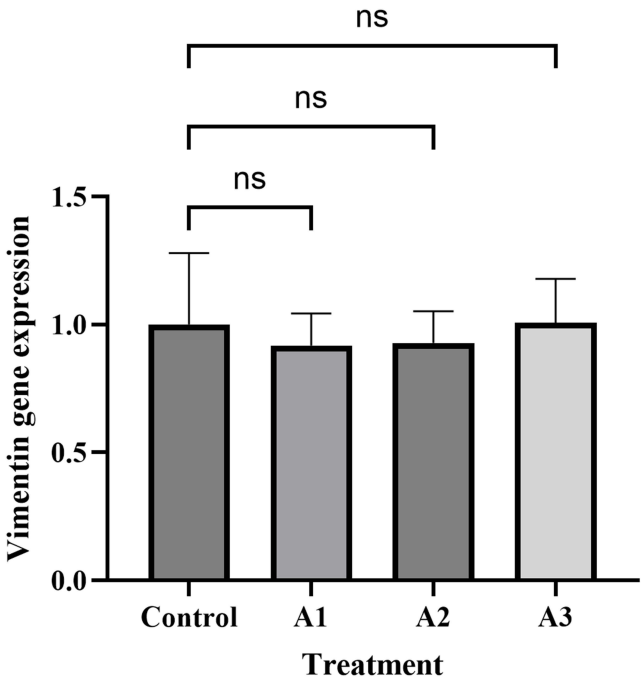

Figure 3 Vimentin mRNA expression in MDA-MB-231 cell line treated with Aaptamine.
Abbreviation: ns, not significant.

small lung cancer cells treated with a sub-lethal dose of Aaptamine (8 μg/mL) showed a substantial rise in the levels of apoptosis markers, including PARP and caspase-3.[20] PARP-1 activation rapidly increased in response to DNA damage.[35] This condition suggests that the unhealthy condition of the cells may cause the dimming intensity of DAPI in this study due to DNA damage. The low cytotoxic dose (12.5 μM) of Aaptamine used in the experiment caused approximately 5% cell death. However, the morphological observation showed the characteristic of unhealthy cells with the possibility of the initiation of cell death. Nevertheless, further evaluation is required to validate this conjecture.

To determine whether Aaptamine modulates vimentin expression at the transcriptional level, this study evaluated vimentin mRNA expression using quantitative RT-qPCR. The RT-qPCR result on vimentin expression intriguingly showed a not significant effect of Aaptamine on the expression of vimentin mRNA (Figure 2). Previous findings reported that Aaptamine demonstrated inhibition and degradation effects on phosphorylated AKT, which is one of the upstream of vimentin.[20] However, the suppression of transcription-level vimentin did not occur in this study. It may be due to multiple signaling cascades involved in vimentin expression, including STAT3, Wnt, TGF-β, NOTCH, and RTK pathways.[36]

In this study, Aaptamine demonstrated a substantial suppression effect on vimentin protein expression at a low cytotoxic dose (Figure 2B). This intriguing result, where an insignificant effect is observed on vimentin mRNA, yet considerably lower vimentin protein expression occurs, may be due to the possibility that Aaptamine targets the protein molecule of vimentin. The downregulation of the vimentin can occur due to multiple mechanisms, including translational inhibition, ribosomal dysfunction, and proteasomal degradation. Based on the previous study, we proposed that Aaptamine lowers vimentin expression by inhibiting AKT1 phosphorylation, which leads to vimentin proteasomal degradation. The binding of p-AKT to vimentin results in the phosphorylation of vimentin at Ser39, which enhances vimentin's ability to promote cell motility and invasion. At the same time, phosphorylated vimentin could evade degradation through caspase-induced proteolysis.[24] Furthermore, a study on Aaptamine has shown that it degrades the phosphorylated AKT by dephosphorylating AKT through perifosine-mediated dephosphorylation. Hence, the activity of p-AKT1 was inhibited.[20] However, the specific mechanism by which Aaptamine targets vimentin still needs to be determined through additional evaluation methods in a follow-up study.

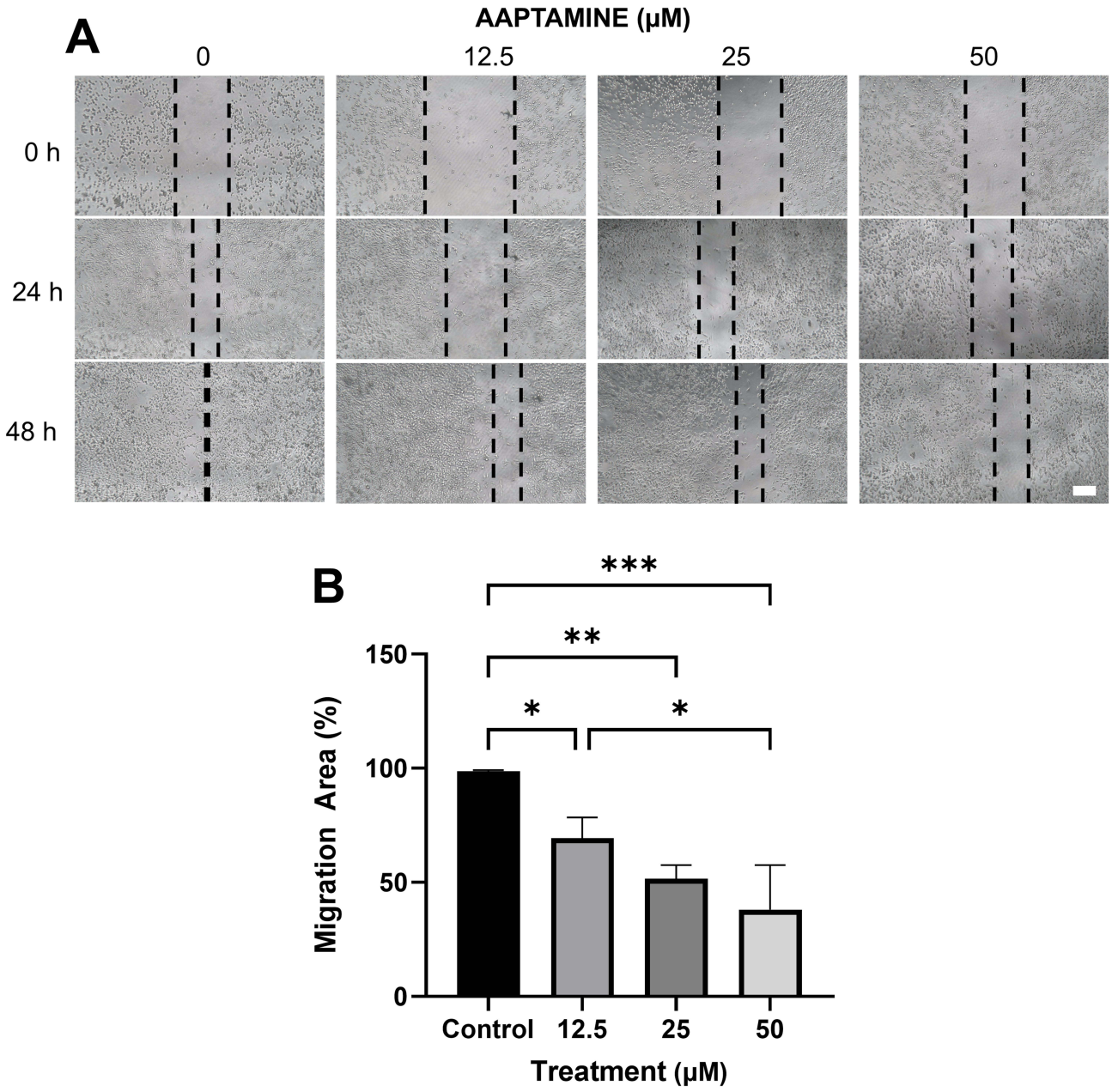

Figure 4 (**A**) Migration assay of breast cancer cells MDA-MB-231 treated with Aaptamine. (**B**) Migration assay statistical analysis of breast cancer cells (MDA-MB-231) treated with Aaptamine compared to the control group after 48 hours. *p<0.05; **p<0.01; ***p<0.001. *Bars* 200 μm.

This current study demonstrated the statistically significant suppression of breast cancer cell migration by Aaptamine intervention (Figure 4). This finding aligns with the earlier research on Aaptamine, which reported its impact in reducing the motility and invasiveness of non-small lung cancer cells.[20] In addition, the rearrangement of actin, as observed in Figure 2, suggests that the low-dose Aaptamine suppressed the migration of the cells as phenotypically observed that the treated group formed less protrusion of filopodia formed by the rearrangement of F-actin during migration. Furthermore, in the study of another anticancer compound, WFA, breast cancer cells' migration capability attenuated was correlated with the vimentin degradation and rearrangement in sub-cytotoxic doses.[37] Another research on WFA described that the WFA treatment in vivo greatly lowers the vimentin protein expression. Furthermore, in the in vivo mouse model, the intervention also prevented the spread of breast cancer to the lungs.[37,38] Moreover, this current study showed significant inhibition in both vimentin protein expression and cellular migration in the lowest cytotoxic dose of the Aaptamine treatment group compared to the control. This outcome is in line with earlier research on other vimentin-targeting compounds.[37]

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Conclusion

Aaptamine is a bioactive compound with antiproliferative properties against a variety of malignancies.[18–23] The effect of Aaptamine on vimentin expression and rearrangement has yet to be reported; hence, this work is the first to be published on the subject. In this current study, Aaptamine has a negligible impact on the expression of vimentin mRNA in breast cancer cells, suggesting that its mode of action in inhibiting vimentin may not be at the messenger level. However, the immunofluorescence labeling of the vimentin protein in breast cancer cells revealed that a low-toxicity dose of Aaptamine moderately reduced the vimentin expression at the protein level. Furthermore, Aaptamine showed a substantial dose-dependent anti-migration effect on breast cancer cells.

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This study was executed at Molecular Genetic and Cell Culture and Cytogenetic Laboratories, Faculty of Medicine, Universitas Padjadjaran, Indonesia.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest for this work.

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