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The disintegrin tzabcanin inhibits adhesion and migration in melanoma and lung cancer cells

Anthony J. Saviola[a,c], Patrick D. Burns[a], Ashis K. Mukherjee[a,b], Stephen P. Mackessy[a,*]

[a] School of Biological Sciences, University of Northern Colorado, 501 20th St., CB 92, Greeley, CO 80639-0017, USA
[b] Microbial Biotechnology and Protein Research Laboratory, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur 784028, Assam, India
[c] Current address, Department of Pharmacology, Weill Cornell Medicine, 1300 York Avenue, New York, NY 10065-4805, USA

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ABSTRACT

Integrins play an essential role in cancer survival and invasion, and they have been major targets in drug development and design. Disintegrins are small (4–16 kDa) viperid snake venom proteins that exhibit a canonical integrin-binding site (often RGD). These non-enzymatic proteins inhibit integrin-mediated cell–cell and cell-extracellular matrix interactions, making them potential candidates as therapeutics in cancer and numerous other human disorders. The present study examined the cytotoxic, anti-adhesion, and anti-migration effects of a recently characterized disintegrin, tzabcanin, towards melanoma (A-375) and lung (A-549) cancer cell lines. Tzabcanin inhibits adhesion of both cells lines to vitronectin and exhibited very weak cytotoxicity towards A-375 cells; however, it had no effect on cell viability of A-549 cells. Further, tzabcanin significantly inhibited migration of both cell lines in cell scratch/wound healing assays. Flow cytometric analysis indicates that both A-375 and A-549 cell lines express integrin $\alpha_v\beta_3$, a critical integrin in tumor motility and invasion, and a major receptor of the extracellular matrix protein vitronectin. Flow cytometric analysis also identified $\alpha_v\beta_3$ as a binding site of tzabcanin. These results suggest that tzabcanin may have utility in the development of anticancer therapies, or may be used as a biomarker to detect neoplasms that over-express integrin $\alpha_v\beta_3$.

1. Introduction

Integrins comprise an important family of cell surface receptors that mediate cell–cell and cell-extracellular matrix (ECM) interactions [1,2]. To date, 24 distinct integrin heterodimers have been described, based on the appropriate noncovalent pairing of one of 18 α subunits with one of 8 distinct β-subunits [3]. The specific pairing of these subunits regulates the substrates to which a cell will adhere and upon which it will migrate, which subsequently influences the activity of the cell [4]. Typically the α-subunit dictates ligand specificity, whereas the β-subunit associates with the downstream signaling pathway [3,5]. Integrins have the ability to recognize a single, or several, ECM ligands or cell membrane proteins, each contributing to the regulation of an array of cellular functions [6–8]. For example, integrins $\alpha_5\beta_1$, $\alpha_4\beta_1$ and $\alpha_v\beta_3$ recognize fibronectin; in addition $\alpha_v\beta_3$ and $\alpha_v\beta_5$ show high affinity to both vitronectin and fibrinogen. Integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$ bind laminin, and both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ recognize collagen [8]. The integrin $\alpha_{IIb}\beta_3$ is expressed on platelets where it binds to fibrinogen or von Willebrand factor and assists in platelet aggregation [8,9]. Approximately one-third of the 24 integrins recognize these adhesive molecules through the tripeptide Arg-Gly-Asp (RGD) binding sequence, while others bind the triple helical GFOGER amino acid sequence present in collagen [5], or YIGSR in laminin [10]. Integrins are critical to numerous aspects of cell function, and mutations targeting integrin receptors or integrin-related pathways are known to contribute to numerous human disorders [11].

It is well documented that several integrins contribute to cancer progression [12–14] and have a significant role in tumor angiogenic activity, proliferation, survival, and metastasis [15,16]. In addition, expression of these cell membrane proteins may vary significantly between normal and cancerous tissue, increasing their potential as selective targets in cancer therapy [14,17,18]. Whereas integrins $\alpha_6\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_6$ are almost undetectable in normal epithelial tissue [12], they may be highly over-expressed in cancerous cells [19,20]. Integrin $\alpha_v\beta_3$ has been shown to increase 50–100 fold in melanoma (A-375) cells displaying an increased metastatic phenotype, indicating that increased integrin expression

Abbreviations: ECM, extracellular matrix; RGD, arginine-glycine-aspartic acid; EDTA, ethylenediaminetetraacetic acid; FN, fibronectin; VN, vitronectin.

* Corresponding author.
E-mail address: stephen.mackessy@unco.edu (S.P. Mackessy).

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is correlated with advanced cancer states [19]. Tumor dependence on angiogenesis is also well documented [21,22], and the formation of new blood vessels is required for delivering nutrients as well as providing waste removal for tumors. Although numerous integrins are involved in angiogenesis [23], evidence indicates that $\alpha_v\beta_3$ is critical for tumor angiogenic activity [24,25], likely permitting angiogenic endothelial cells to recognize proteins present in the tumor microenvironment [12]. Recently, it has also been shown that the expression of $\alpha_v\beta_3$ is associated with treatment resistance, and this integrin is necessary in the reprograming of tumor cells towards a cancer stem cell-like phenotype [26]. The significance of integrins in cancer biology cannot be overemphasized, so the isolation and characterization of compounds that have integrin-blocking activity may result in novel anti-neoplastic therapies, reveal new approaches to controlling cancer cell proliferation and metastasis, or be used as biomarkers to elucidate disease state [21].

The use of toxins as potential therapeutics has been an increasing emphasis of biomedical research in the last decade, and several novel compounds developed from the poisons and venoms of animals are currently in clinical trials and use [27–33]. Snake venoms in particular have been a promising source of several protein drugs and protein drug leads [27–33] because they consist of a complex mixture of proteins and peptides that exhibit an array of biochemical and pharmacological functions [34]. As many of these proteins often mimic compounds with normal physiological activities, but contain dramatically different pharmacologies, venom components have been subjected to detailed examination for their potential in biomedical or therapeutic use [27,35,36]. One class of venom proteins, the disintegrins, are small, cysteine-rich, non-enzymatic proteins that result from the post-translational proteolytic processing of the enzymatic P-II class of snake venom metalloproteinases [37,38]. Many disintegrins contain an RGD-binding domain in the carboxyl terminal portion of the molecule and were originally characterized due to their ability to inhibit platelet aggregation by binding integrin $\alpha_{IIb}\beta_3$ [39]. RGD disintegrins have also been shown to bind integrins $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, and $\alpha_v\beta_3$, and variants of this tripeptide sequence demonstrate differing levels of selectivity to numerous integrin receptors [40].

Due to their potent integrin binding activity, disintegrins are continuously being explored for their ability to reduce experimental metastasis. Contortrostatin, a homodimeric RGD disintegrin from the venom of the Southern Copperhead (*Agkistrodon contortrix contortrix*), has been shown to inhibit cell adhesion, migration, invasion, and angiogenesis in numerous cancer cell lines [41–43]. In addition, monomeric disintegrins such as obtustatin, which contains a KTS tripeptide sequence, and RGD disintegrins such as crotatroxin 2 and colombistatin have also been shown to exhibit various anti-cancer effects [44–46]. We previously reported the isolation and characterization of a novel 7.1 kDa, RGD-containing disintegrin, tzabcanin, from the venom of the Yucatan Rattlesnake (*Crotalus simus tzabcan*). Tzabcanin was not cytotoxic, but it inhibited colon (Colo-205) and breast (MCF-7) cancer cell adhesion to the ECM proteins fibronectin (FN) and vitronectin (VN) [47]. Analyses of cell adhesion assays suggest that tzabcanin may bind $\alpha_v\beta_5$ and/or $\alpha_v\beta_6$, both of which are expressed in Colo-205 and MCF-7 cell lines [48,49], and they recognize VN and FN, respectively. To continue addressing the pharmacology of tzabcanin, the current study was designed to examine the anti-adhesion, anti-migration, and cytotoxic effects of this disintegrin against two highly metastatic cell lines, human melanoma (A-375) and lung carcinoma (A-549). Flow cytometry analysis was further utilized to identify integrin $\alpha_v\beta_3$ as one of the binding sites for tzabcanin in both A-375 and A-549 cell lines.

2. Materials and methods

2.1. Snakes, venom collection, and biochemicals

Venoms from two adult Middle American Rattlesnakes (*C. simus tzabcan*) housed individually at the University of Northern Colorado Animal Resource Facility were extracted as previously described [50]. Venoms were centrifuged (10,000*g* for 5 min), lyophilized, and stored at −20 °C until use. Matrigel (356234) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). $\alpha_v\beta_3$ antibody (sc-7312 FITC) conjugated with a FITC was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). FN (F0895), VN (V8379), and all buffers and additional reagents (analytical grade) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

2.2. Purification of tzabcanin

Isolation and purification of tzabcanin were conducted as previously described [47] by a combination of low-pressure size exclusion and two steps of C_{18} reverse-phase high-pressure liquid chromatography. Mass determination, purity and identification of tzabcanin were ascertained by both SDS-PAGE and MALDI-TOF mass spectrometry as described [47].

2.3. Cell line and culture conditions

Human malignant melanoma (A-375; ATCC CRL-1619) and human lung adenocarcinoma (A-549; ATCC CCL-185) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). A-375 cells were maintained in 75 cm^2 flasks in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained as a monolayer culture (<80% confluent) in a humidified 5% CO_2/air incubator at 37 °C. A-549 cells were also maintained in 75 cm^2 flasks with ATCC-formulated F-12 K growth medium supplemented with 10% FBS as a monolayer culture under the same conditions mentioned above. Subcultivation of cells was performed according to ATCC instruction, using trypsin-EDTA (0.05% trypsin and 0.02% EDTA). Cells were counted manually with a hemocytometer (4×, averaged) and diluted to appropriate densities.

2.4. A-375 and A-549 cell adhesion assays

Triplicate wells of Immulon-II 96 well microtiter plates were coated with 100 μL of either tzabcanin (2 μg per well), FN (0.5 μg per well), VN (0.3 μg per well) or Matrigel (0.5 μg per well) that was dissolved in 0.01 M PBS, pH 7.2; protein was allowed to incubate overnight at 4 °C. Excess proteins were washed away twice with 1% bovine serum albumin (BSA) in PBS, and unbound sites were blocked with 200 μL 2.5% BSA in PBS and incubated at 37 °C for 1 h. Cells were treated with various concentrations of tzabcanin (7.8 nM–2 μM), or the cation chelator disodium EDTA, and allowed to incubate at 37 °C for 1 h immediately prior to seeding in treated plates. The BSA blocking solution was aspirated, and excess proteins were washed away twice with 1% BSA in PBS. Treated cells (100 μL, 5×10^5/mL) were seeded in the coated microtiter plate wells and returned to 37 °C for 1 h. Unbound cells were washed away 3 times with 1% BSA in PBS by filling and aspirating, and 100 μL of serum-free medium with 1% BSA containing MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-teyrazolium bromide] (5:1 v/v) was added to wells and incubated at 37 °C for 2 h. Detergent reagent (ATCC; 100 μL) was then added to the wells, and cells were incubated overnight in the dark at 21 °C. The plate was gently shaken and the absorbance read at 570 nm using a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA). The percent of cell binding inhibition was calculated by the equation [(absorbance of

control – absorbance of treatment)/absorbance of control] × 100. Assays at each tzabcanin concentration for all cell lines were performed in triplicate, and each assay was repeated 3–6 times.

2.5. Cytotoxicity of tzabcanin towards A-375 and A-549 cells

Cytotoxicity of tzabcanin towards A-375 and A-549 cells was measured by the colorimetric MTT assay [51,52]. Both cell lines were trypsinized and resuspended in complete media at a concentration of 5.0×10^5 cells/ml. Aliquots of 100 μL of A-375, and A-549 cell suspensions were added to 96-well cell culture plates that were treated with tzabcanin ranging from 0.22–14 μM, or 20 μg of crude *C. s. tzabcan* venom and incubated at 37 °C for 24 h. After 24 h, 10 μL of MTT reagent (ATCC) was added to the cells which were then returned to 37 °C for 2 h. Following incubation, 100 μL of detergent reagent (ATCC) were added to cells, which were then incubated overnight in the dark at 21 °C. The plate was gently shaken and the absorbance read at 570 nm using a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA). Assays at each concentration of tzabcanin were performed in triplicate and each assay was repeated 2–3 times.

2.6. Cell migration/Scratch assay

To measure the effects of tzabcanin on cell migration, a modified wound healing/scratch assay was completed as previously described by Liang et al. [53]. Twelve well Immulon-II plates were seeded with 1 mL of A-549 or A-375 cells (5×10^5/mL) and allowed to grow to confluence at 37 °C. The complete media was then discarded and cells were starved in serum-free media for 48 h at 37 °C, followed by 2 h incubation with 10 μg/mL mitomycin C in serum-free medium at 37 °C. Mitomycin C inhibits DNA synthesis and therefore was used to evaluate the contribution of cell migration in the absence of cell proliferation. A scratch in the cell monolayer was created with a 200 μL pipet tip, followed by extensive washing with serum-free medium to remove cell debris. Cells were then incubated with either 10 μL of tzabcanin (1 μg/μL) resuspended in PBS, or PBS alone as a control. Photographs were taken at the same location of the culture well using an Olympus D21 camera attached to an Olympus CKX41 inverted microscope at 4x magnification. Because tzabcanin exhibited very low but demonstrable cytotoxicity (see Results) towards A-375 cells, the migration evaluation for this cell line was conducted at 24 h, with photographs taken at 0 and 24 h. However, tzabcanin was not cytotoxic to A-549 cells, and therefore the migration assay for this cell line was conducted for an extended period, with photographs taken at 0, 24, 48 and 72 h intervals. Migration was determined by taking multiple measures of the width of the scratch for each cell line at three randomly selected locations, and calculated using the equation [(S – F)/S] × 100, where S is the distance (mm) of the cell edge at 0 h, and F is the distance (mm) of the cell edge at 24, 48, and 72 h (when possible). Assays were performed in triplicate and each assay was repeated three times.

2.7. Competitive binding assay

A-375 and A-549 cells were resuspended in 1% BSA in PBS at a density of 1×10^6 cells/mL. Cell aliquots of 100 μL were incubated with 1% BSA in PBS only (control), 0 (antibody positive control) or 2 μg of tzabcanin resuspended in PBS for 30 min at 37 °C, followed by the addition of mouse monoclonal anti-$\alpha_v\beta_3$ antibody conjugated with FITC (10 μg/mL). After 30 min incubation at 21 °C in the dark, cells were gently pelleted and washed 3 times with 1% BSA in PBS to remove unbound antibody, and the fluorescence intensity of the cells was analyzed using flow cytometry (FACscan, Becton Dickinson, Bedford, MA). Tests were performed in triplicate and all experiments were repeated twice.

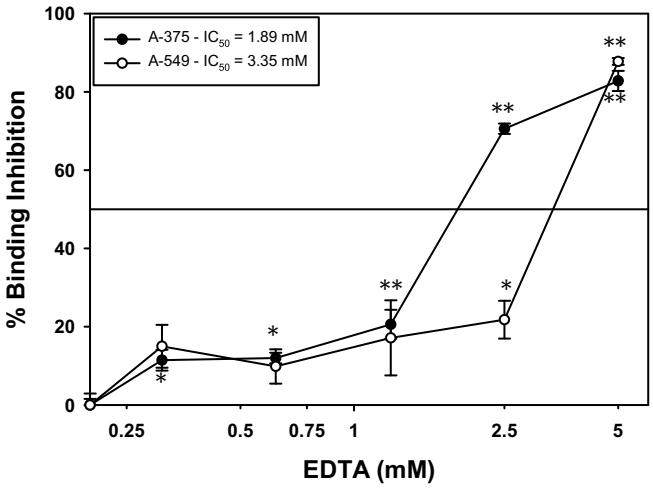

Fig. 1. Percent inhibition of binding of A-375 and A-549 cells to immobilized tzabcanin following cell incubation with the cation chelator EDTA. A-375 and A-549 cells (5.0×10^5 cells/mL) were incubated with various concentrations of EDTA (0.3–5.0 mM) prior to addition to wells containing immobilized tzabcanin. All treatments were significantly different from controls (*, $p < 0.01$; **, $p < 0.001$).

2.8. Statistical analyses

Cytotoxicity and cell adhesion data were analyzed by Analysis of Variance (ANOVA) followed by Tukey's post-hoc test using R version 2.15.2. Cell migration assays were analyzed using a Student's T-test, comparing the percent migration of the treatment to the percent migration for the control for the respective time interval. All p values <0.05 were considered as statistically significant.

3. Results

3.1. Tzabcanin binds to both A-375 and A-549 cells via integrin(s)

Integrin-ligand interactions are cation-dependent [54], and to confirm that cell recognition to tzabcanin in both A-375 and A-549 cells was via integrins, tzabcanin was immobilized in 96-well microtiter plates, and cell adhesion was measured following cell incubation with the cation chelator EDTA. A-375 and A-549 binding to tzabcanin was inhibited by EDTA in a dose-dependent manner (Fig. 1), with IC_{50} values of 1.89 mM and 3.35 mM, respectively. These results strongly indicate that tzabcanin interacts with both cell lines through integrin receptors, likely by cell recognition of the RGD binding region of tzabcanin.

3.2. Cytotoxicity towards A-375 and A-549 cells in vitro

Crude *C. s. tzabcan* venom (20 μg/100 μL) showed significant cytotoxicity toward A-375 cells, with approximately 13% cell viability remaining following 24 h of incubation (Fig. 2; $p < 0.001$). In addition, tzabcanin appeared to exhibit a slight dose-dependent decrease (~2–6% lower) in A-375 cell viability, and results were statistically significant ($p < 0.05$, 0.44 μm to 14 μM) at all concentrations except 0.22 μM. In contrast, both crude *C. s. tzabcan* venom and tzabcanin failed to show any significant decrease in A-549 cell viability (all p's > 0.05).

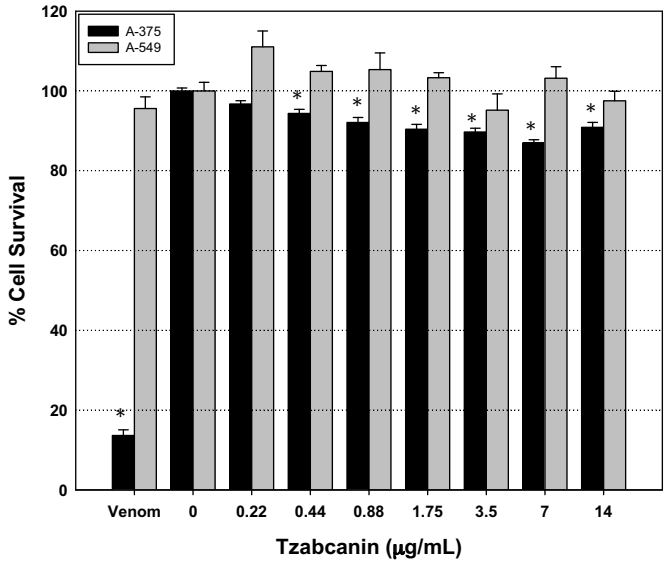

Fig. 2. Percent cell survival of A-375 and A-549 cells following exposure to crude *C. s. tzabcan* venom or purified tzabcanin. *, $p < 0.05$, significantly different compared to controls.

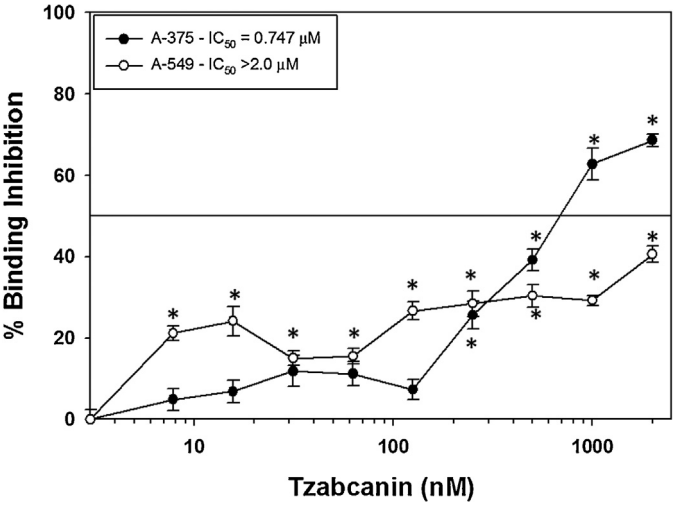

Fig. 3. The effects of tzabcanin on A-375 and A-549 adhesion to immobilized VN. Various concentrations of tzabcanin (7.8 nM–2.0 μM) were incubated with A-375 and A-549 cells (5.0×10^5 cells/mL) prior to addition to 96-well culture plates containing immobilized VN. *$p < 0.001$, significantly different compared to controls.

3.3. Tzabcanin inhibits cell adhesion of A-375 and A-549 to VN

The ECM proteins FN and VN, and membrane matrix Matrigel, all support adhesion to A-375 and A-549 cells. By treating both cell lines with various concentrations of tzabcanin, its ability to inhibit cell adhesion to these matrices was evaluated. Results show that tzabcanin inhibits adhesion of both A-375 and A-549 cells to VN in a dose-dependent manner (Fig. 3); however, this inhibition is much more potent towards A-375 cells. The IC_{50} for A-375 cells was 747 nM, whereas A-549 failed to reach 50% binding inhibition even at 2 μM. Tzabcanin failed to inhibit adhesion of either cell line to FN or Matrigel (data not shown).

3.4. Tzabcanin inhibits cell migration

Cell migration was measured following an *in vitro* scratch/wound healing assay. Tzabcanin (10 μg/mL) inhibited A-375 cell migration by approximately 45% when compared to the untreated control over 24 h (Fig. 4a; $p < 0.01$). Likewise, tzabcanin inhibited cell migration of A-549 cells by 76, 47, and 37% over the 24, 48, and 72 h time intervals, respectively (Fig. 4b; all $p < 0.01$). At 72 h, the scratches for all of the untreated A-549 controls had closed 100%.

3.5. $\alpha_v\beta_3$ is identified as a binding site for tzabcanin on A-375 and A-549 cells

Because tzabcanin inhibits binding of A-375 and A-549 cells to VN, a major ligand of integrin $\alpha_v\beta_3$ [55], we examined if tzabcanin binds to this receptor. Flow cytometry analysis demonstrates that both A-375 and A-549 cells express integrin $\alpha_v\beta_3$ (Fig. 5), consistent with previously published reports [19,56]. When A-375 and A-549 cells were pre-treated with tzabcanin, anti-$\alpha_v\beta_3$ antibody binding was significantly inhibited (Fig. 5), indicating that tzabcanin binds to $\alpha_v\beta_3$ on both cell lines. This inhibition effect was more pronounced in A-375 cells.

4. Discussion

In 2015, it is estimated that over 585,000 deaths are expected from cancer in the United States alone [57]. Although there appears to be declining or stable trends among most cancers, melanoma incidence appears to be increasing, and cancers of the lung and bronchus remain as the most common cause of cancer-related deaths in both men and women [58,59]. The molecular mechanisms involved in metastasis are complex, enabling cancerous cells to disseminate from the primary tumor, invade local tissue, enter into circulation, and ultimately adhere, proliferate, and stimulate angiogenesis at a distant site [59]. This ability to metastasize is a significant cause of treatment failure and death in cancer patients [60]. Therefore, there is a tremendous need to identify compounds that may effectively arrest the numerous factors involved in metastasis, or be utilized as biomarkers to enhance early cancer detection.

Integrins mediate cell adhesion, migration, invasion, proliferation and angiogenesis, and their roles in metastasis and tumor survival are now apparent [12,13]. Further, integrin expression levels may vary significantly between normal and cancerous tissues [12], and they are correlated with advanced stages of disease progression. In addition, it has been strongly implicated that in cancer cells possessing $\alpha_v\beta_3$, tumor invasiveness and resistance to cancer treatments are proportional to the expression levels of this integrin [19,26,61]. However, numerous other integrin subfamilies, such as α_3, α_5, α_6, α_v, β_1 and β_4, also enhance tumorigenesis [13]. Our prior results demonstrated that tzabcanin, a 7.1 kDa, RGDN monomeric disintegrin, inhibited adhesion of MCF-7 and Colo-205 cells to FN and VN through competitive binding to integrins [47], though this inhibition was not as potent as has been previously reported for other disintegrins [43,44]. Since the RGDN binding region of disintegrins exhibits higher affinity towards $\alpha_v\beta_3$ [62], an integrin not expressed on either Colo-205 or MCF-7 cells, it was postulated that tzabcanin may demonstrate significantly higher anti-adhesion properties towards cell lines expressing this receptor. Our results here, particularly with inhibiting adhesion of A-375 cells to VN, support this hypothesis.

Immobilized tzabcanin supports adhesion of A-375 and A-549 cells, demonstrating that these cells bind to this disintegrin. Because a cation chelator (EDTA) significantly inhibited adhesion of both cell lines to tzabcanin, it is likely that tzabcanin-cell binding is primarily mediated via integrin receptors. Integrin-mediated cell adhesion to the ECM proteins FN and VN is largely due to the presence of the RGD region found in these proteins [63,64], with roughly one-third of all identified integrins recognizing this binding sequence [5]. Tzabcanin, an RGD disintegrin, inhibited binding

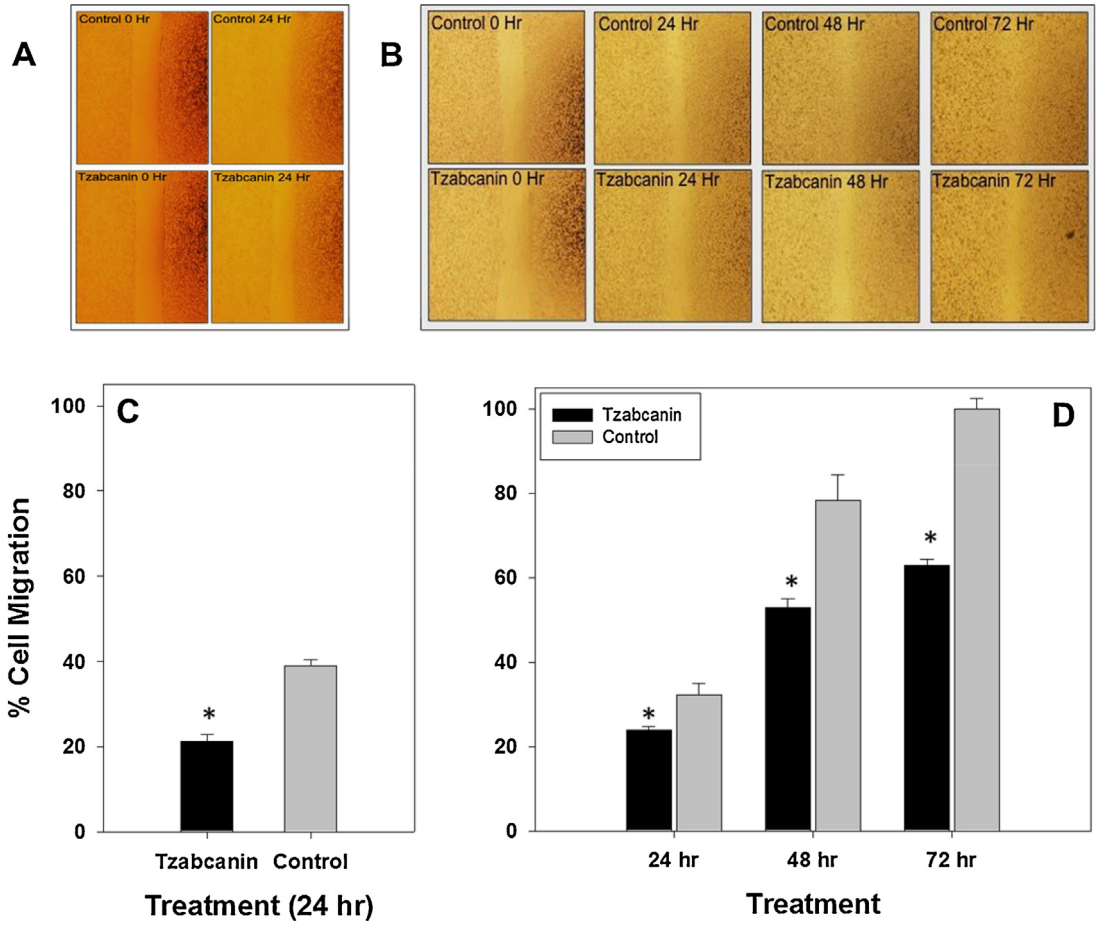

Fig. 4. Inhibition of A-375 and A-549 cell migration. Cells were maintained as a monolayer in serum free medium for 48 h before a 2 h incubation with mitomycin C. A line was scratched through the cell monolayer (0 h), and cultures ((A) A-375; (B) A-549) were allowed to migrate at 37 °C in the presence of tzabcanin or a PBS control. Multiple measurements of the width of the scratch were made for each treatment. (C) Percent migration of A-375 cells after 24 h. (D) Percent migration of A-549 cells at 24, 48, and 72 h. * $p < 0.05$, significantly different compared to controls.

of both A-375 and A-549 cell lines to VN, but this inhibitory effect was significantly more potent for A-375 cells (IC_{50} = 747 nM). Cell adhesion to VN is mediated by integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_8$ [5]. Using an antibody against integrin $\alpha_v\beta_3$, our flow cytometric analysis indicates that A-375 and A-549 cell lines are $\alpha_v\beta_3$ positive, results that are supported by previous reports [19,26,56]. However, A-375 cells appear to express a higher percentage (29%, S.D. = 3.14%) of this receptor when compared to A-549 cells (16%, S.D. = 3.7%), explaining in part the weak inhibitory effect of tzabcanin on A-549 cell binding to VN. Flow cytometric analysis also indicated that tzabcanin binds to this receptor, inhibiting the binding of the FITC labeled anti-$\alpha_v\beta_3$ antibody and therefore also inhibiting cell adhesion to immobilized VN during adhesion assays. Despite this, it is hypothesized that because tzabcanin (at 2 μM) did not inhibit 100% adhesion of either A-375 or A-549 cells to VN, other integrin receptors may participate in anchoring cells to this ECM protein. Therefore, complete inhibition of attachment of A-375 and A-549 cells to VN would require blocking multiple integrins, and the possibility of other RGD-dependent integrins exhibiting affinity towards VN in A-375 and A-549 cell lines cannot be excluded.

Tzabcanin did not disrupt adhesion of either cell line to FN or Matrigel at concentrations as high as 14 μM. FN is a relatively promiscuous ligand that is recognized by numerous integrins including $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_4\beta_7$. Even though tzabcanin clearly binds to $\alpha_v\beta_3$, the expression of additional FN receptors not recognized by tzabcanin could explain why this disintegrin failed to inhibit cell adhesion to this ECM protein [5]. In addition, the major components of Matrigel are laminin and type IV collagen [65], which are recognized by the β_1 subclass of integrins [5]. Therefore, the results presented here suggest that tzabcanin does not bind to the β_1 subclass of integrins, or a vast majority of integrins that recognize FN. These results further indicate that tzabcanin shows some specificity towards $\alpha_v\beta_3$, which likely accounts for the inhibitory effects of adhesion of both cell lines to VN but not to FN or laminin.

Crude *C. s. tzabcan* venom was significantly cytotoxic to A-375 cells, results that confirm those of Bradshaw et al. [52] while, crude venom failed to exhibit any cytotoxic effects towards A-549 cells. This outcome was surprising due to the potent toxicity of *C. s. tzabcan* venom toward several immortal cell lines and whole mice (LD_{50} = 0.74 μg/g; [66]). Phenotypic differences between cell lines could account for the drastic differences in toxic effects of crude venom. Although purified tzabcanin failed to show a decrease in A-549 viability, there was a dose-dependent small decrease in A-375 cell viability. It is currently unknown if this decrease is the result of apoptosis or necrosis. However, it has recently been shown that the recombinant disintegrins r-mojastin 1 and *r*-viridistatin 2 induced apoptosis in approximately 20% of human pancreatic adenocarcinoma (BXPC-3) cells at concentrations of 5 μM following 24 h incubation [67].

Migration is a critical step in metastasis, and cancer cells express various adhesion molecules facilitating movement from the primary tumor site to remote tissues or organs. Expression and activation of $\alpha_v\beta_3$ has been shown to enhance migration

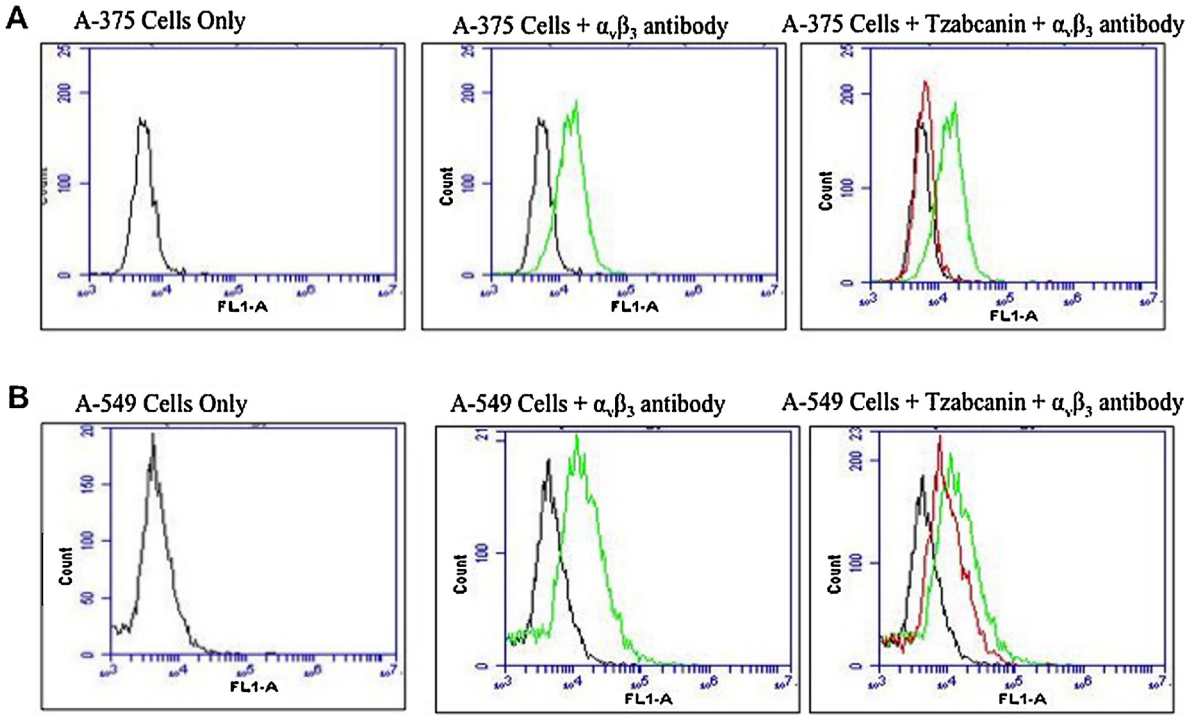

Fig. 5. Tzabcanin inhibits binding of anti-$\alpha_v\beta_3$ to A-375 and A-549 cells. The natural fluorescence of (A) A-375 melanoma cells and (B) A-549 lung cancer cells is shown (black line), and the fluorescence following incubation with $\alpha_v\beta_3$ antibody (green line) indicates antibody-integrin binding. Tzabcanin added to cells prior to addition of the $\alpha_v\beta_3$ antibody effectively inhibits antibody binding (red line), as demonstrated by a shift of cell fluorescence back toward controls (black lines). This effect is particularly striking with A-375 cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly in numerous cancer cell lines [68–70]. Upon ligation, $\alpha_v\beta_3$ induces the myosin light chain kinase through Ras-MAP kinase pathways, causing an increase in phosphorylation of the myosin light chain kinase and leading to the phosphorylation of myosin light chains, thereby influencing cell locomotion [71]. Although tzabcanin (10 μg/mL) effectively inhibited cell migration in both A-375 and A-549 cell lines, it is uncertain if this inhibition was solely due to blocking $\alpha_v\beta_3$. It is hypothesized, however, that by binding to $\alpha_v\beta_3$, tzabcanin may inhibit this cell-signaling pathway and further reduce cell motility in A-375 and A-549 cells. Cell adhesion to FN and Matrigel was not inhibited, but inhibition of cell adhesion is not always correlated with inhibition of cell migration [45,72]. For example, the disintegrin crotatroxin 2 from *Crotalus atrox* significantly inhibited migration of murine mammary breast carcinoma cells (by 66%) yet failed to inhibit adhesion of this cell line to FN or collagen IV and VI [45].

5. Conclusions

In the current study, we have shown that tzabcanin disrupts adhesion of both A-375 and A-549 cell lines to the ECM protein VN. This inhibition is likely due to tzabcanin binding to integrin $\alpha_v\beta_3$, a major VN receptor, and also a binding site for tzabcanin, as confirmed by flow cytometry analysis. Although tzabcanin resulted in a slight decrease in A-375 cell viability, there were no observable cytotoxic effects of tzabcanin or crude *C. s. tzabcan* venom towards A-549 cells. In addition, tzabcanin inhibited migration of both cell lines over 24 (A-375) and 72 (A-549) hr periods.

The RGDN binding region present in tzabcanin may demonstrate higher affinity to integrin $\alpha_v\beta_3$, and not toward other receptors, which explains why tzabcanin failed to inhibit adhesion of either cell line to FN or Matrigel. Tzabcanin therefore appears to be more specific in its integrin-binding capacities than other snake venom disintegrins, suggesting that it might have utility for selective blocking of integrin $\alpha_v\beta_3$ in order to dissect the molecular mechanisms controlling $\alpha_v\beta_3$-mediated metastasis. Tzabcanin may be further utilized as a biomarker for detection of $\alpha_v\beta_3$ to predict disease state and progression. Future studies will assess the effects of tzabcanin *in vivo* and against several other cancer cell lines.

Conflict of interest

The authors declare that they have no conflict of interest.

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