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

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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.

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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