Disintegrins from the Venom of Vipera ammodytes ammodytes Efficiently Inhibit Migration of Breast Cancer Cells

Zorica Latinović,1,2 Adrijana Leonardi,1 Toni Petan,1 Margareta Žlajpah1 and Igor Križaj*

1 Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Jamova cesta 39, Ljubljana, Slovenia
2 Jožef Stefan International Postgraduate School, Jamova cesta 39, Ljubljana, Slovenia

* Corresponding author: E-mail: igor.krizaj@ijs.si
Phone: +386 1 477 3626. Fax: +386 1 477 3984.
Received: 15-09-2016
For Cutting Edge 2017

Abstract

Integrins are plasma membrane proteins, whose dysfunction frequently results in cancer pathology, and therefore they represent important targets of anti-tumour therapy. Snake venoms are a rich source of disintegrins (Dis), proteins that specifically bind integrins and thus interfere with their functions. In an attempt to discover new molecules for treatment of breast cancer, the major type of cancer in women, we isolated a dimeric Dis (Vaa-Dis) from the venom of the nose-horned viper. By cell viability testing we demonstrated that 50 nM and higher concentrations of Vaa-Dis were toxic to highly invasive human breast adenocarcinoma cell line MDA-MB-231. Wound-healing assay revealed that already at one order of magnitude lower concentrations Vaa-Dis efficiently inhibited MDA-MB-231 cell migration. This exposed a promising anti-metastatic potential of Vaa-Dis and a good perspective of these natural snake venom proteins for further research and development towards the application in breast cancer treatment.

Keywords: snake venom, disintegrin, integrin, cancer, metastasis, drug

1. Introduction

Cancer, one of the deadliest diseases worldwide, is caused by inherited or acquired mutations of the genetic material. The main characteristics of cancer include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Such properties enable cancer cells an unlimited growth and spreading, invasion through the organism and finally causing its death.1 Prevention of metastatic growth is an efficient therapy in cancer control. The key roles in the process of migration and cell viability are played by transmembrane proteins called integrins.1,2

Integrins are cell adhesion receptors on the cells’ surface that bind components of the extracellular matrix (ECM), various biological ligands and receptors on adjacent cells. They are heterodimeric proteins consisting of one α- and one β-subunit. 18 different α-subunits and 8 β-subunits are known, which have been found to appear in 24 different α-β combinations, each of them possessing unique binding specificity.2 Recent studies have exposed integrins as important factors in tumour cell survival, tumour growth and metastasis by establishing and breaking bonds between malignant cells and molecules in their surroundings. For this reason, integrins have become important targets of anti-tumour therapy.2 Despite the fact that several therapeutics, integrin antagonists, have already been developed, a search for more efficient integrin-binding substances continues.3–4

Snake venom (SV) proteins are broadly investigated as substances that can be used in diagnosis and treatment of human diseases. Among them, disintegrins (Dis) have been found to inhibit various cell functions by their interaction with different integrins, for example platelet aggregation, angiogenesis, tumour growth and metastasis.5

Latinović et al.: Disintegrins From the Venom of Vipera ammodytes ammodytes ...
Dis are non-enzymatic cysteine-rich polypeptides. Their molecular mass ranges between 4 kDa and 15 kDa. They can be directly synthesized or formed by proteolysis of the P-II class metalloproteinases (MPs). SV Dis can be monomeric or dimeric, hetero- or homodimeric. In dimeric Dis, individual subunits are interconnected by disulfide bonds, which is crucial for stability and maintenance of a distinct globular structure. Dimerization also defines the configuration of the so called inhibitory loop, essential for the interaction with integrin receptor and, consequently, the biological activity.6,7

In this work we focused on Dis from the venom of Vipera ammodytes ammodytes (Vaa-Dis). They were purified and biochemically characterized. We investigated their influence on cell viability and in vitro migration in a model of highly invasive triple-negative breast cancer and found that Vaa-Dis potently inhibited the migration of cancer cells.

2. Material and Methods

2.1. Purification of Vaa-Dis

Raw Vaa venom was obtained from the Institute of Immunology, Zagreb, Croatia. Lyophilized venom was stored at −20 °C and before use dissolved in 50 mM Tris, 2 mM CaCl₂, 300 mM NaCl, pH 7.0 (buffer A). 250 μl of stored at –20 °C and before use dissolved in 50 mM Tris, 2 mM CaCl₂, 300 mM NaCl, pH 7.0 (buffer A). 250 μl of lyophilized venom was 0–45% in 13 min and 45–55% in 10 min. A215 was moni-

tored non-reducing and reducing conditions according to Laemmli.8 Proteins in gels were visualized by PageBlue™ (Thermo Scientific, USA) as instructed by the manufacturer. Molecular mass standards were from Fermentas (Lithuania).

2.3. N-terminal Amino Acid Sequence Analysis

Proteins were N-terminally sequenced by automated Edman degradation on a Procise 492A protein sequencing system (Applied Biosystems, USA).

2.4. Culturing of MDA-MB-231 Cells

To carry out the migration assays the highly invasive breast cancer cell line MDA-MB-231 (ATCC, USA) was used. Cells were grown in RPMI-1640 medium (Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in an atmosphere of 5% (v/v) CO₂. Adherent cell monolayers were routinely cultured in T25 and T75 tissue culture flasks (Corning, USA) and passaged in ratios of 1 : 3 to 1 : 4.

2.5. Cell Viability Testing

The in vitro cytotoxic potency of Vaa-Dis was evaluated using the PrestoBlue™ viability assay (Invitrogen, USA) essentially as described previously.9 Cells were trypsinized and counted by Trypan blue (Thermo Scientific, USA) exclusion assay10 using a hemocytometer (Fon-tana, Germany). Cells were seeded in 96-well plates (TPP, Switzerland) at a density of 5000 cells/well in 100 μL RPMI-1640 medium and left to attach overnight. Cells were then treated with different concentrations of Vaa-Dis (0.005; 0.05; 0.5; 5; 50; 500 nM) for 0, 24, 48, and 72 h. After the treatment, 10 μL of PrestoBlue™ was added to each well, and the plates were incubated for 30 min at 37 °C. Cell supernatants were transferred to black microtiter plates (Corning, USA) and fluorescence was measured at an excitation of 560 nm and emission of 590 nm on an Infinite M1000 microplate reader (Tecan, Switzerland) at a density of 5000 cells/well in 100 μL RPMI-1640 medium and left to attach overnight. Cells were then treated with different concentrations of Vaa-Dis (0.005; 0.05; 0.5; 5; 50; 500 nM) for 0, 24, 48, and 72 h. After the treatment, 10 μL of PrestoBlue™ was added to each well, and the plates were incubated for 30 min at 37 °C. Cell supernatants were transferred to black microtiter plates (Corning, USA) and fluorescence was measured at an excitation of 560 nm and emission of 590 nm on an Infinite M1000 microplate reader (Tecan, Switzerland) to determine metabolic activity of the cells. Wells containing only the cell culturing medium and PrestoBlue™ were used as blank reference standards. Experiments were performed in triplicate. The normalization of cell viability was calculated as the ratio of sample absorbance to control absorbance (cells in media without Vaa-Dis).

2.6. Wound-healing Assay

The wound-healing assay (WHA) was used to estimate the potency of Vaa-Dis to inhibit cell migration (anti-migratory potency). MDA-MB-231 cells were trypsinized, counted as specified above and plated in 48-well cell culture dishes (TPP, Switzerland) at a density of 2 × 10⁵
cells/well. Wounds (millimetre gaps) were then scratched in each cell monolayer using a pipette tip. Dead cells were removed by washing with D-PBS buffer and solutions of Vaa-Dis in RPMI-1640/10% FBS at different concentrations (2.5, 5.0, 7.5 and 15.0 nM) were added. Control samples contained only media. The influence of Vaa-Dis on cell migration was determined by observing the width of the gap under a CKX41 inverted microscope equipped with an E-450 camera (Olympus, Japan) after 1 h, 6 h, 12 h and 24 h of incubation. The gap was analysed using the ImageJ software (Softonic International S.A., Spain). The width of the gap was measured as specified (Suppl. Fig. 1) in three different wells for each concentration and incubation time.

2.7. Statistical Analysis

Each experiment was performed in at least three independent repeats. If not stated otherwise, data are presented as a mean percent difference from control with corresponding standard error of the mean. Statistical tests were run by GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) using two-way ANOVA followed by Dunett’s multiple comparisons test. Statistical significance is as follows: not significant for \( p > 0.05 \) (no mark in Fig. 2); significant for \( p \leq 0.05 \) (* in Fig. 2), \( p \leq 0.001 \) (** in Fig. 2) and \( p \leq 0.0001 \) (***) in Fig. 2).

3. Results and Discussion

Using gel-filtration chromatography we separated in the first step the raw venom of Vaa into seven fractions (A–F in Fig. 1a). The efficient inhibition of platelet aggregation induced by ADP or collagen by the gel filtration fraction B2 has been suggested to be due to the presence of Dis, which may obstruct this process by binding to \( \alpha_{IIb}\beta_3 \) fibrinogen receptor. For this reason, we decided to analyse the fraction B2 further and split it on a strong cation exchanger into a fraction retained by the exchanger...
Disintegrins From the Venom of Vipera ammodytes ammodytes   ...
6. References

1. D. Hanahan, R. A. Weinberg, *Cell*. 2011, 144, 646–674. https://doi.org/10.1016/j.cell.2011.02.013

2. L. Seguin, J. S. Desgrosellier, S. M. Weis, D. A. Cheresh, *Trends Cell Biol.* 2015, 25, 234–40. https://doi.org/10.1016/j.tcb.2014.12.006

3. A. M. Alizadeh, S. Shiri, S. Farsinejad, *Tumour Biol.* 2014, 35, 8483–8523. https://doi.org/10.1007/s13277-014-2421-z

4. J. S. Desgrosellier, D. A. Cheresh, *Nat. Rev. Cancer* 2010, 10, 9–22. https://doi.org/10.1038/nrc2748

5. J. K. Arruda Macêdo, J. W. Fox, M. de Souza Castro, *Curr. Protein Pept. Sci.* 2015, 16, 532–548. https://doi.org/10.2174/138920371666150515125002

6. J. J. Calvete, *Toxicon* 2013, 62, 40–49. https://doi.org/10.1016/j.toxicon.2012.09.005

7. J. J. Calvete, L. Sanz, P. Cid, P. de la Torre, M. Flores-Díaz, M. C. Dos Santos, A. Borges, A. Brema, Y. Angulo, B. Lomonte, A. Alape-Girón, J. M. Gutiérrez, *J. Proteome Res.* 2010, 9, 528–544. https://doi.org/10.1021/pr0908749

8. U. K. Laemmli, *Nature* 1970, 227, 680–685. https://doi.org/10.1038/227680a0

9. A. Pucer, V. Brglez, C. Payré, J. Pungerčar, G. Lambeau, T. Petan, *Mol. Cancer*. 2013, 12, 111. https://doi.org/10.1186/s1476-4598-12-111

10. W. Strober, *Curr. Protoc. Immunol.* 2001, Appendix 3, Appendix 3B.

11. T. Sajevic, A. Leonardi, I. Križaj, *Toxin Rev.* 2014, 33, 33–36. https://doi.org/10.3109/15569543.2013.835827

12. J. J. Calvete, M. P. Moreno-Murciano, R. D. G. Theakston, D. G. Kisiel, C. Marcinkiewicz, *Biochem. J.* 2003, 372, 725–734. https://doi.org/10.1042/bj20021739

13. H. S. Selistre-de-Araujo, C. L. S. Pontes, C. F. Montenegro, A. C. B. M. Martin, *Toxins (Basel)*, 2010, 2, 2606–2621. https://doi.org/10.3390/toxins2112606

14. R. D. Baird, C. Caldas, *BMC Med.* 2013, 11, 151. https://doi.org/10.1186/1741-7015-11-151

15. R. S. Yang, H. S. Chiang, C. H. Tang, C. S. Yeh, T. F. Huang, *Toxicon* 2005, 46, 387–393. https://doi.org/10.1016/j.toxicon.2005.05.002

Povzetek

Integrini so proteinji v plazemski membrani celic. Nepravilno delovanje teh proteinov lahko vodi v nastanek tumorjev, zato so pomembna tera protitumorskih terapij. Disintegrini (Dis) so proteinji iz kačjega strupa, ki se specifično vežejo na integrin in s tem ovirajo njihovo normalno delovanje. Z namenom odkrivanja novih molekul za terapijo raka dojke, najbolj razširjene vrste raka pri ženskah, smo iz strupa modrasa izolirali dimerni Dis (Vaa-Dis). Izmerili smo vpliv Vaa-Dis na viabilnost celic celične linije visoko invazivnega adenokarcinoma dojke MDA-MB-231 in ugotovili, da je bil Vaa-Dis v koncentracijah, višjih od 50 mM, za celice toksičen. Test celjenja celične rane, s katerim smo preverili vpliv Vaa-Dis na migracijo celic, pa je pokazal, da je Vaa-Dis učinkovito upočasnil migracijo rakavih celic že pri koncentracijah, ki so bile za en velikostni red nizje. Dobljeni rezultati potrjujejo antimetastatski potencial Vaa-Dis in predstavljajo dober obet za nadaljnji razvoj teh molekul iz kačjega strupa v smeri priprave novih učinkovin za zdravljenje raka dojke.