Published in final edited form as:

Toxicon. 2016 December 15; 124: 1–7. doi:10.1016/j.toxicon.2016.11.002.

Functional analysis of four single (RGDWL_L, RGDWM_M, RGDWP_P, RGDMN_N) and two double (RGDNM_{NM}, RGDMP_{MP}) mutants: The importance of methionine (M) in the functional potency of recombinant mojastin (r-Moj)

Daniel A. Gutierrez^a, Ana S. Aranda^a, David A.R. Carrillo^a, Melissa A. Koshlaychuk^a, Elda E. Sanchez^b, Sara E. Lucena^b, and Julio G. Soto^{a,*}

^aBiological Sciences Department, San José State University, One Washington Square, San José, CA 95192-0100, United States

^bNational Natural Toxins Research Center, Texas A&M University, Kingsville, TX 78363, United States

Abstract

We have demonstrated in previous studies that a single amino acid change can alter the activity of the recombinant disintegrin r-Moj. In this study, four r-Moj recombinants containing single mutations (r-Moj-W_L, r-Moj-W_M, r-Moj-W_P, r-Moj-M_N) and two containing double mutations (r-Moj-M_P and r-Moj-N_M) at the binding loop were produced, purified, and tested. All r-Moj-W_L, r-Moj-M_L, and r-Moj-NM mutant peptides inhibited platelet aggregation at higher potency than r-Moj-D_L mutants. Five of the seven r-Moj peptides inhibited angiogenesis at different levels. Two of the mutant peptides with a methionine at the second position carboxyl of the RGD (r-Moj-W_M and r-Moj-N_M) were the strongest angiogenesis inhibitors, with r-Moj-W_M being the most potent. Recombinant r-Moj-M_P and r-Moj-W_N failed to inhibit angiogenesis. Only the r-Moj-M_P mutant peptide induced apoptosis of SK-Mel-28 cells significantly ($p = 0.001$). This was confirmed by chromatin condensation. Proliferation of SK-Mel-28 cells was inhibited at high levels (>70%) by all r-Moj mutant peptides. Recombinant r-Moj-M_N and r-Moj-W_N failed to inhibit cell migration significantly ($p > 0.5$). Recombinant r-Moj-NM was the strongest cell migration inhibitor ($98\% \pm 0.69$), followed by r-Moj-M_P ($80\% \pm 2.87$), and r-Moj-W_M ($61.8\% \pm 5.45$). The lowest inhibitor was r-Moj-W_L ($50\% \pm 12.16$). Our functional data suggest that the most potent r-Moj disintegrins contain a methionine in the first or the second position carboxyl to the RGD.

Keywords

Melanoma cell line; Recombinant disintegrin; Binding-loop mutants; Cell migration; Cell proliferation; Mojastin; Angiogenesis; Apoptosis

*Corresponding author. Julio.Soto@sjsu.edu (J.G. Soto).

Ethical statement

Our manuscript has not been published and is not under consideration for publication in other journals. All co-authors in the manuscript carefully read the manuscript, carried-out the experiments, and agreed on the content presented in the manuscript.

Conflict of interest statement

The authors declare that there is no conflict of interest.

1. Introduction

Several snake venom toxins target different mechanisms that are employed during cancer initiation, promotion, and progression (Chaisakul et al., 2016; Calderon et al., 2014). Targeted mechanisms include activation or repression of genes involved in cell death and survival, activation of apoptosis, angiogenesis inhibition, cell division inhibition, and metastasis inhibition. Several investigators have shown that disintegrins have the ability to induce cellular responses that target the mechanisms listed above (McBride et al., 2016; Ramos et al., 2016; Lucena et al. 2014, 2015; Ribeiro et al., 2014; Carey et al., 2012; Selistre-de-Araujo et al., 2010; Minea et al., 2010; Seoane et al., 2010; Ren et al., 2006; Alimenti et al., 2004; Brassard et al., 1999; Wu et al., 2003; Hong et al., 2003; Zhou et al., 1999; Yeh et al., 1998).

Disintegrins are non-enzymatic peptides that are present in viper venom (Calderon et al., 2014). These peptides were first characterized by their ability to inhibit platelet aggregation by binding to the α IIb β 3 integrin present on platelets (McLane et al., 2004). Integrins are cell membrane receptors composed of α and β subunits (Bhuvaneshwari et al., 2014; Gahmberg et al., 2009; Arnaout, 2002). The combination heterogeneity of these subunits provides integrins with specificity of binding (Moschos et al., 2007; Hynes, 2002). Integrins form heterogeneous adhesions in complexes containing 232 molecules called adhesomes (Winograd-Katz et al., 2014). Some of these molecules have been shown to have roles in cancer, cardiovascular diseases, and hematological disorders, among others. Integrins bind to extracellular matrix proteins (*for example*, fibronectin) or soluble peptides (disintegrins), transferring signals that activate or repress transduction pathways that allow cells to migrate, proliferate, or undergo apoptosis (Winograd-Katz et al., 2014; Calderon et al., 2014; Marcinkiewicz, 2013; Gahmberg et al., 2009; Mousa, 2008; Lu et al., 2008).

Our research group has been focusing on the mutation, expression, and testing of anti-cancer activities of truncated recombinant mojastin peptides. Our truncated recombinant mojastin peptides lack the first 12 amino acids at its amino terminus as compared with the wild-type mojastin-1 (Sánchez et al., 2010). The truncated recombinant mojastin cDNA (r-Moj-WN) was cloned from a Mohave rattlesnake venom gland (Soto et al., 2007). In a previous article, we produced and tested six r-Moj peptides with a second aspartate immediately carboxyl to their RGD (Ramos et al., 2016). Our research demonstrated that a second aspartate is not sufficient to induce apoptosis of SK-Mel-28 cells. However, r-Moj-D₂ disintegrins activated anti-cancer activities by antagonizing the α v integrin subunit (Ramos et al., 2016). Furthermore, we demonstrated that 40 genes were differentially expressed in SK-Mel-28 cells after r-Moj-DM treatment (McBride et al., 2016). Functional analysis of these cells suggest that one of these identified targets, TXNIP, is required early in the apoptotic-inducing pathway resulting from r-Moj-DM binding to the α v integrin subunit.

We are interested in producing targeted mutations that will potentially increase anti-cancer activities of r-Moj peptides. In the present study, four r-Moj recombinant peptides containing single mutations (r-Moj-W $\underline{L}$, r-Moj-W $\underline{M}$, r-Moj-W $\underline{P}$, r-Moj-M $\underline{N}$) and two containing double mutations (r-Moj-M $\underline{P}$ and r-Moj-N $\underline{M}$) at the binding loop were produced, purified, and

tested. Our research demonstrates that r-Moj peptides with a methionine at the first or second position immediately carboxyl to their RGD are the most potent.

2. Materials and methods

2.1. Production of six additional mutants from r-Moj-WN cDNA

A truncated *r-Moj-WN* cDNA was previously obtained (Soto et al., 2007). Additional r-Moj mutants were obtained using the methods described in Seoane et al. (2010). Three tryptophan (W) single mutations were produced. These were designated as r-Moj-WL, r-Moj-WM, and r-Moj-WP. Three methionine (M) mutations were produced. These were designated as r-Moj-MN (single mutation), r-Moj-NM, and r-Moj-MP. The designation corresponds to the two amino acids following the RGD tripeptide.

2.2. Expression and purification of r-Moj-WL, r-Moj-WM, r-Moj-WP, r-Moj-WN, r-Moj-MN, r-Moj-MP, and r-Moj-NM peptides

Expression and purification of r-Moj-WL, r-Moj-WM, r-Moj-WP, r-Moj-WN, r-Moj-MN, r-Moj-MP, and r-Moj-NM peptides were performed with a method described earlier (Ramos et al., 2016).

2.3. Structural protein modeling of r-Moj-WL, r-Moj-WM, r-Moj-WP, r-Moj-WN, r-Moj-MN, r-Moj-MP, and r-Moj-NM peptides

Three-dimensional (3D) structural models of r-Moj peptides were produced using the methods described by Seoane et al. (2010). These 3-D structural models of r-Moj peptides were produced by homology with trimestatin. Trimestatin's high resolution X-ray structure coordinates (PDB ID 1J2L) were used as a template. Preliminary models were built for each protein by MODELLER bioinformatics program. Best quality models were chosen after evaluating the Ramachandran plots using PROCHECK. Molecular images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco.

2.4. Cell culture conditions

SK-Mel-28 cells were grown in Eagle's minimum essential medium (ATCC). Media was supplemented with 10% fetal bovine serum (FBS) and penicillin-100 (IU/mL), streptomycin (0.1 mg/mL), and amphotericin B (0.25 µg/mL). HUVECs were obtained from ATCC and grown using Lonza EGM-2 media supplemented with the EGM-2 SingleQuots. Cells were grown at 37 °C in a humidified incubator with 5% CO₂.

2.5. Inhibition of platelet aggregation

The inhibition of platelet aggregation study was done according to the Sánchez et al. (2010) method using a dual-channel Chronolog-Log Whole-Blood Aggregometer [Ca²⁺] model 560 (Havertown, USA). Briefly, different concentrations of r-Moj peptides (10 µL) were added to 10% citrated whole human blood, and pre-incubated at 37 °C for 2 min. Platelet aggregation was initiated by 10 µL of ADP (10 µM), and percentage of impedance reflecting percentage

of aggregation was measured. The maximal aggregation in the absence of r-Moj was given as 100% aggregation. Three independent measures were performed in triplicate.

2.6. Angiogenesis assay

The *in vitro* angiogenesis assay Kit (Millipore) was used. Briefly, after preparing the gel matrix and allowing it to form a solution, 7.0×10^3 HUVECs, alone or mixed with 2.5 μ M of r-Moj-WL, r-Moj-WM, r-Moj-WP, r-Moj-WN, r-Moj-MN, r-Moj-MP, or r-Moj-NM peptide treatments, were seeded per well onto the surface of the polymerized ECMatrix™ as per the manufacturer's instructions. The plate was incubated at 37 °C for 16 h. Tubule and branch points formation were inspected, photographed using a Olympus inverted light microscope, and counted. Three of the co-authors in this paper counted branch points and number of tubules independently.

2.7. Apoptotic assay

Five hundred thousand SK-Mel-28 cells were seeded in six wells of a 12 well plate and allowed to grow for 24 h. Cells were treated for 24 h with 5 μ M r-Moj-WL, r-Moj-WM, r-Moj-WP, r-Moj-WN, r-Moj-MN, r-Moj-MP, or r-Moj-NM, or no treatment. Then, non-adherent cells were transferred to a flow cytometry tube, followed by the remaining cells detached with 0.05% trypsin EDTA. Cells were centrifuged at 300g for 5 min and fixed in 1% para-formaldehyde for 1 h. Cells were washed twice with 1X PBS and resuspended in 70% ethanol. Cell suspensions were stored at –20 °C for 24 h. Cells were processed using an APO-BRDU™ Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's protocol. Cell suspensions were analyzed using a Becton Dickinson FACSCalibur flow cytometer with CellQuest software. Experiments were performed in triplicate.

2.8. Chromatin condensation

SK-Mel 28 cells were grown on chambered slides. One and a half million cells were grown in 2 mL of media for 24 h. After initial incubation, cells were treated with 5 μ M r-Moj-WL, r-Moj-WM, r-Moj-WP, r-Moj-WN, r-Moj-MN, r-Moj-MP, or r-Moj-NM peptides for 24 h. The media was aspirated and the cells washed twice with 1X PBS. Cells were fixed on the slide with 3% formaldehyde for 1 h, followed by two washes with 1X PBS. Cells were mounted and their nuclei stained with Vectashield mounting medium with DAPI and viewed at 100X, oil immersion, with a Zeiss AXIO fluorescent microscope.

2.9. Cell proliferation assay

The effects of r-Moj-WL, r-Moj-WM, r-Moj-WP, r-Moj-WN, r-Moj-MN, r-Moj-MP, or r-Moj-NM peptides on cell proliferation were determined using the WST-1 assay (GenDEPOT). Fifty thousand SK-Mel-28 cells/well were added to a 96-well plate grown for 24 h at 37 °C in a 5% CO₂ incubator. After 24 h, growth media was aspirated. Then, 100 μ L of culture media containing 3.5 mM of r-Moj-WL, r-Moj-WM, r-Moj-WP, r-Moj-WN, r-Moj-MN, r-Moj-MP, or r-Moj-NM peptides were added to their respective wells. Negative controls consisted of cells treated with equal volume of PBS. After 24 h all wells were then treated with 10 μ L of WST-1 cell proliferation assay reagent for 1 h. The same volume of culture medium and WST-1 reagent used to treat the cells was used as the control blank for

the microplate reader. The formation of the formazan product was measured at 450 nm. The reference wavelength was 630 nm. A dual wavelength reading was used to obtain the results, where the reference wavelength was subtracted from the reading wavelength, 450–630 nm. Percentage of cell proliferation inhibition was calculated using the following formula:

$$(\text{Abs}_{450 \text{ nm}} - \text{Abs}_{630 \text{ nm}} \text{ of treated} / \text{Abs}_{450 \text{ nm}} - \text{Abs}_{630 \text{ nm}} \text{ of untreated}) \times 100.$$

Experiments were performed in triplicate.

2.10. Wound healing assay

The effects of r-Moj-WL, r-Moj-WM, r-Moj-WP, r-Moj-WN, r-Moj-MN, r-Moj-MP, or r-Moj-NM peptides on SK-Mel-28 cell migration were determined using the method described by Carey et al. (2012).

3. Results

3.1. Four tryptophan and three methionine r-Moj mutants

Seven r-Moj mutants were produced and purified without the GST tag (Fig. 1A–C). The three-dimensional models showed that amino acid substitutions after the binding motif (RGD) altered the predicted orientations in their RGD loops (Fig. 1B).

3.2. Platelet aggregation activity

All r-Moj mutant peptides tested inhibited platelet aggregation at different potencies (Table 1). Recombinant r-Moj-MN was the strongest inhibitor ($\text{IC}_{50} = 14.2 \text{ nM} \pm 7$) and r-Moj-NM ($\text{IC}_{50} = 84.6 \text{ nM} \pm 4$), the weakest. Overall the r-Moj-W₋, r-Moj-M₋, and r-Moj-NM peptides were stronger platelet aggregation inhibitors than recombinant mojastins with a second aspartate, r-Moj-D₋ peptides (Table 2).

3.3. Angiogenesis inhibition

Five of the seven r-Moj peptides tested inhibited angiogenesis at different levels (Fig. 2). Two of the mutant peptides with a methionine at the second position carboxyl of the RGD (r-Moj-WM and r-Moj-NM) were the strongest angiogenesis inhibitors, with r-Moj-WM being the most potent, as treated HUVECs failed to form tubules. Recombinant r-Moj-MP and r-Moj-WN failed to inhibit angiogenesis, as there was no significant difference between the number of tubules or branch points of untreated compared to r-Moj-MP or r-Moj-WN treated cells ($p > 0.05$).

3.4. Apoptotic inducing activity of r-Moj-MP

Only r-Moj-MP mutant peptide induced apoptosis of SK-Mel-28 cells significantly ($p = 0.001$) (Fig. 3A). The apoptotic-inducing activity of r-Moj-NM was not significant, as there was no difference in the percentage of apoptotic cells between cells treated with r-Moj-NM disintegrin and untreated cells ($p = 0.28$). The other tested r-Moj mutant disintegrin peptides did not induced apoptosis (data not shown).

Chromatin condensation, a morphological characteristic of apoptotic cells, was used to confirm apoptotic activation. Only cells treated with r-Moj-MP showed chromatin condensation (Fig. 3B).

3.5. Cell proliferation inhibition

Proliferation of SK-Mel-28 cells was inhibited at high levels (>70%) by all r-Moj mutant peptides tested (Fig. 4).

3.6. Cell migration inhibition

Recombinant r-Moj-MN and r-Moj-WN failed to inhibit cell migration significantly ($p > 0.5$) (Fig. 5). Recombinant r-Moj-NM was the strongest cell migration inhibitor ($98\% \pm 0.69$), followed by r-Moj-MP ($80\% \pm 2.87$), and r-Moj-WM ($61.8\% \pm 5.45$). The lowest inhibitor was r-Moj-WL ($50\% \pm 12.16$).

4. Discussion

4.1. Activity influence by amino acids carboxyl of the RGD in r-Moj peptides

It has been shown, that amino acids flanking the RGD tripeptide influence the selectivity and integrin inhibitory potency of disintegrins. This influence is exerted by amino acids flanking the amino or carboxyl terminal to the RGD. Rahman et al. (1998) demonstrated that substituting the asparagine for a methionine in elegantin (RGDNP to RGDMP) resulted in higher platelet aggregation inhibition activity of the elegantin RGDMP mutant. In the same study, reduced platelet aggregation inhibitory activity was obtained when the alanine was substituted for a proline (ARGDNP to PRGDNP). Introducing an alanine (A) after the methionine in rhodostomin (RHO) RGD binding loop (RGDMAP) resulted in the loss of binding affinity and platelet aggregation inhibition of the RHO mutant (Chang et al., 2001).

Our research group have demonstrated that substituting one or two of the amino acids immediately carboxyl of the RGD of r-Moj resulted in the elimination of platelet aggregation inhibition for the r-Moj-DM mutant (Table 2), the introduction of apoptotic inducing activity for r-Moj-DM (Ramos et al., 2016) and r-Moj-MP (Fig. 3A), and activity potency differences for mutants produced for the Ramos et al. (2016) study and the current one.

4.2. Activity comparisons between r-Moj mutant peptides and other disintegrins

Our r-Moj-mutants exhibited differences in activity and potency with other disintegrins with the same RGD loops. For instance, r-Moj-NM is a more potent platelet aggregation inhibitor than ocellatusin, ocellatin, and pyramidin A, three disintegrins with an RGDNM binding loop (Smith et al., 2002). Recombinant r-Moj-MP induced apoptosis of SK-Mel-28. This r-Moj mutant's RGD loop resembles rhodostomin, a medium-sized disintegrin with an RGDMP motif that induces apoptosis (Wu et al., 2003).

Our study revealed functional differences between r-Moj-WN and r-mojastin-1. Both of these recombinant mojastin peptides contain the wild-type RGDWN, and they only differ in the presence of 12 additional amino acids at the amino terminus (EAGEECDG-SPA) in r-

mojastin-1. In the present study, r-Moj-WN failed to inhibit angiogenesis using a matrigel *in vitro* angiogenesis assay. Recombinant r-mojastin-1 inhibited angiogenesis using a similar assay (Lucena et al., 2014). Both peptides were purified using similar methods. Perhaps, the difference in anti-angiogenesis activity can be attributed to the presence of the additional amino acids at the amino terminus of r-mojastin-1.

4.3. The role of methionine in the functional potency of r-Moj mutant peptides

Our data suggest that methionine has a great effect in increasing different biological activities of r-Moj mutant peptides (Fig. 6). The potential functional role of methionine within the RGD binding loop has been previously shown. Wierzbicka-Patynowski et al. (1999) demonstrated that substituting asparagine for methionine in eristostatin (RGDWN to RGDWM) increased binding inhibition to VNRC3 cells.

Recombinant, r-Moj mutant peptides with a methionine carboxyl to the RGD loop were more potent than the other peptides tested in this study. These include r-Moj-WM, r-Moj-MP, r-Moj-NM, and r-Moj-MN. Furthermore, two of the four r-Moj mutant peptides with apoptotic-inducing activities on SK-Mel-28 cells contain a methionine. These are r-Moj-MP (this study) and r-Moj-DM (Ramos et al., 2016).

The r-Moj-NM and r-Moj-MN mutant peptides were not functionally equivalent, suggesting the possibility of different binding interactions of these r-Moj mutants with the integrin. Recombinant r-Moj-MN was more potent than r-Moj-NM in the anti-angiogenesis ($p < 0.01$) and 5.9 times more potent in the platelet aggregation assays. While r-Moj-NM was a potent cell migration inhibitor and r-Moj-MN did not significantly inhibit cell migration.

4.4. Conclusions

This study provides further evidence of the functional importance of the two amino acids immediately carboxyl of the RGD. Our study demonstrated that the inclusion of a methionine in either position (RGDM_ or RGD_M) increases the potency of most of the r-Moj mutants produced. However, it was also evident that the methionine position makes a difference, as r-Moj-NM and r-Moj-MN were not functionally identical. The anti-angiogenesis activity of the r-Moj mutant peptides was not due to apoptotic activity of r-Moj mutant disintegrins, as none of the r-Moj with anti-angiogenesis activity induced apoptosis. In this study, only r-Moj-MP has apoptotic inducing activity, but it failed to block the formation of tubules or branches. Our data also provided indirect evidence suggesting that the first 12 amino acids of the amino terminus of medium-sized disintegrins may have functional significance. This interesting possibility should be further studied.

Acknowledgments

We thank Stephanie Mandal for the preparation and labeling of composite figures. Funding for this project was provided by NSF-REU Grant # DBI 1004350, NIH Grant # R25GM071381, NIH Grant # 5R25GM71381, NIH Grant # 5T34GM008253-27, NCRR/Viper # 2P40RR018300, and NIH/ORIP Grant # 5P40OD010960.

References

- Alimenti E, Tafuri S, Scibelli A, D'Angelo D, Manna L, Pavone LM, Belisario MA, Staiano NN. Pro-apoptotic signaling pathway activated by echistatin in GD25 cells. *Biochim Biophys Acta*. 2004; 1693:73–80. [PubMed: 15276326]
- Arnaout MA. Integrin structure: new twists and turns in dynamic cell adhesion. *Immunol Rev*. 2002; 186:125–140. [PubMed: 12234368]
- Bhuvaneshwari M, Van Agthoven JF, Xiong JP, Alonso JL, Adair BD, Rui X, Anand S, Mehrbod M, Mofrad MRK, Burger C, Goodman SL, Arnaout MA. Atomic basis for the species-specific inhibition of αV integrins by monoclonal antibody 17E6 is revealed by the crystal structure of $\alpha v\beta 3$ ectodomain-17E6 fab complex. *JBC*. 2014; 289:13801–13809.
- Brassard DL, Maxwell E, Malkowski M, Nagabhushan TL, Kumar CC, Armstrong L. Integrin $\alpha(v)\beta(3)$ -mediated activation of apoptosis. *Exp Cell Res*. 1999; 251:33–45. [PubMed: 10438569]
- Calderon, LA.; Sobrinho, JC.; Zaqueo, KD.; de Moura, AA.; Grabner, AN.; Mazzi, MV.; Marcussi, S.; Nomizo, A.; Fernandes, CFC.; Zuliani, JP.; Carvalho, BMA.; da Silva, SL.; Stabeli, RG.; Soares, AM. Antitumoral activity of snake venom proteins: new trends in cancer therapy. *Biomed Res Int*. 2014. <http://dx.doi.org/10.1155/2014/203639>
- Carey C, Bueno R, Gutierrez DA, Petro C, Lucena SA, Sanchez EE, Soto JG. Recombinant rubistatin (r-Rub), an MVD disintegrin, inhibits cell migration, proliferation, and it is a strong apoptotic inducer of SK-Mel-28. *Toxicon*. 2012; 59:241–248. [PubMed: 22192732]
- Chaisakul J, Hodgson WC, Kuruppu S, Prasongook N. Effects of animal venoms and toxins on hallmarks of cancer. *J Cancer*. 2016; 7:1571–1578. [PubMed: 27471574]
- Chang CP, Chang JC, Chang HH, Tsai WJ, Lo SJ. Positional importance of Pro⁵³ adjacent to the Arg⁴⁹-Gly⁵⁹-Asp⁵¹ sequence of rhodostomin in binding to integrin $\alpha IIb\beta 3$. *Biochem J*. 2001; 367:57–64.
- Gahmberg CG, Fagerholm SC, Nurmi SM, Chavakis T, Marchesan S, Gronholm M. Regulation of integrin activity and signaling. *Biochim Biophys Acta*. 2009; 1790:431–444. [PubMed: 19289150]
- Hong SY, Lee H, You WK, Chung KH, Kim DS, Song K. The snake venom disintegrin salmosin induces apoptosis by disassembly of focal adhesions in bovine capillary endothelial cells. *BBRC*. 2003; 302:502–508. [PubMed: 12615062]
- Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell*. 2002; 110:673–687. [PubMed: 12297042]
- Lu X, Lu D, Scully MF, Kakkar VV. The role of integrin-mediated cell adhesion in atherosclerosis: pathophysiology and clinical opportunities. *Curr Pharm Des*. 2008; 14:2140–2158. [PubMed: 18781968]
- Lucena SE, Castro R, Lundin C, Hofstetter A, Alaniz A, Suntravat M, Sanchez EE. Inhibition of pancreatic tumoral cells by snake venom disintegrins. *Toxicon*. 2015; 93:136–143. [PubMed: 25450798]
- Lucena SE, Romo K, Suntravat M, Sanchez EE. Anti-angiogenic activities of two recombinant disintegrins derived from the Mohave and Prairie rattlesnakes. *Toxicon*. 2014; 78:10–17. [PubMed: 24269784]
- Marcinkiewicz C. Applications of snake venom components to modulate integrin activities in cell-matrix interactions. *Int J Biochem Cell Biol*. 2013; 45:1974–1986. [PubMed: 23811033]
- McBride TD, Andrew U, Ly N, Soto JG. RNA sequence analyses of r-Moj-DM treated cells: TXNIP is required to induce apoptosis of SK-Mel-28. *Toxicon*. 2016; 121:1–9. [PubMed: 27567705]
- McLane MA, Sanchez EE, Wong A, Paquette-Straub C, Perez JC. Disintegrins. *Curr Drug Targets Cardiovasc Haematol Disord*. 2004; 4:327–355. [PubMed: 15578957]
- Minea RO, Helchowski CM, Zidovetzki SJ, Costa FK, Swenson SD, Markland FS. Vicrostatin e an anti-invasive multi-integrin targeting chimeric disintegrin with tumor anti-angiogenic and pro-apoptotic activities. *PLoS ONE*. 2010; 5(6):e10929. <http://dx.doi.org/10.1371/journal.pone.0010929>. [PubMed: 20532165]
- Moschos SJ, Drogowski LM, Reppert SI, Kirkwood JM. Integrins and cancer. *Oncology*. 2007:21.

- Mousa SA. Cell adhesion molecules: potential therapeutics & diagnostic implications. *Mol Biotechnol*. 2008; 38:33–40. [PubMed: 18095189]
- Rahman S, Aitken A, Flynn G, Formstone C, Savidge GF. Modulation of RGD sequence motifs regulates disintegrin recognition of α IIb β 3 and α 5 β 1 integrin complexes. Replacement of elegantin alanine-50 with proline, N-terminal to the RGD sequence, diminishes recognition of the α 5 β 1 complex with restoration induced by Mn²⁺ cation. *Biochem J*. 1998; 335:247–257. [PubMed: 9761721]
- Ramos CJ, Gutierrez DA, Aranda AS, Koshlaychuk MA, Carrillo DA, Medrano R, McBride TD, UA, Medina SM, Lombardo MC, Lucena SE, Sanchez EE, Soto JG. Functional characterization of six aspartate (D) recombinant mojastin mutants (r-Moj): a second aspartate amino acid carboxyl to the RGD in r-Moj-D₂ peptide is not sufficient to induce apoptosis in SK-Mel-28 cells. *Toxicon*. 2016; 118:36–42. [PubMed: 27105671]
- Ren A, Wang S, Cai W, Yang G, Zhu Y, Wu X, Zhang Y. Agkistins, a disintegrin domain, inhibits angiogenesis and induces BAECs apoptosis. *J Cell Biochem*. 2006; 99:1517–1523. [PubMed: 16924676]
- Ribeiro LCA, Massimino LC, Durante AC, Tansini A, Urbaczek A, Selistre-de Araujo HS, Carlos IZ. Recombinant disintegrin targets α (v) β (3) integrin and leads to mediator production. *Cell Adhes Migr*. 2014; 8:60–65.
- Sánchez EE, Lucena SE, Reyes S, Soto JG, Cantu E, Lopez-Johnston JC, Guerrero B, Salazar AM, Rodríguez-Acosta A, Galán JA, Tao WA, Pérez JC. Cloning, expression, and hemostatic activities of a disintegrin, r-mojastin 1, from the Mohave rattlesnake (*Crotalus scutulatus scutulatus*). *Thromb Res*. 2010; 126:211–219. [PubMed: 20667584]
- Selistre-de-Araujo HS, Pontes CLS, Montenegro CF, Martin ACBM. Snake venom disintegrins and cell migration. *Toxins*. 2010; 2:2606–2621. [PubMed: 22069567]
- Seoane AI, Tran VL, Sanchez EE, White SA, Choi JL, Gaytan B, Chavez N, Reyes SR, Ramos CJ, Tran LH, Lucena SE, Sugarrek M, Perez JC, Mandal SA, Ghorab S, Rodriguez-Acosta A, Fung BK, Soto JG. The mojastin mutant Moj-DM induces apoptosis of the human melanoma Sk-Mel-28, but not the mutant Moj-NN nor the non-mutated recombinant Moj-WN. *Toxicon*. 2010; 56:391–401. [PubMed: 20398687]
- Smith JB, Theakston DG, Coelho ALJ, Barja-Fidalgo C, Calvete JJ, Marcinkiewicz C. Characterization of a monomeric disintegrin, ocellatusin, present in the venom of the Nigerian carpet viper, *Echis ocellatus*. *FEBS Lett*. 2002; 512:111–115. [PubMed: 11852062]
- Soto JG, White SA, Reyes SR, Regalado R, Sanchez EE, Perez JC. Molecular evolution of PIII-SVMP and RGD disintegrin genes from the genus *Crotalus*. *Gene*. 2007; 389:66–72. [PubMed: 17112685]
- Wierzbicka-Patynowski I, Niewiarowski S, Marcinkiewicz C, Calvete JJ, Marcinkiewicz MM, McLane MA. Structural requirements of echistatin for the recognition of α (v) β (3) and α (5) β (1) integrins. *J Biol Chem*. 1999; 274:37809–37814. [PubMed: 10608843]
- Winograd-Katz SE, Fassier R, Geiger B, Legate KR. The integrin adhesome: from genes and proteins to human disease. *Nat Rev Mol Cell Biol*. 2014; 15:273–288. [PubMed: 24651544]
- Wu WB, Peng HC, Huang TF. Disintegrin causes proteolysis of β -catenin and apoptosis of endothelial cells. Involvement of cell-cell and cell-ECM interactions in regulating cell viability. *Exp Cell Res*. 2003; 15:115–127.
- Yeh CH, Peng HC, Huang TF. Accutin, a new disintegrin, inhibits angiogenesis in vitro and in vivo by acting as integrin α v β 3 antagonist and inducing apoptosis. *Blood*. 1998; 92:3268–3276. [PubMed: 9787163]
- Zhou Q, Nakada MT, Arnold C, Shieh KY, Markland FS. Contortrostatin, a dimeric disintegrin from Agkistrodon contortrix contortrix, inhibits angiogenesis. *Angiogenesis*. 1999; 3:259–269. [PubMed: 14517425]

Fig. 1.

(A) Alignment of amino acid sequences for r-Moj mutant peptides. In bold and shaded in gray are shown the two amino acids carboxyl to the RGD motif. (B) r-Moj-peptide models displayed in surface style. The RGD is shown in red. The two amino acids carboxyl to the RGD are colored. The other amino acids are shown in gray. (C) SDS-PAGE of expressed protein resolved in a 4–12% NuPAGE Bis–Tris gel under reducing conditions. Lane 1: Protein marker. Lane 2: r-Moj-MN. Lane 3: r-Moj-NM. Lane 4: r-Moj-WL. Lane 5: r-Moj-MP. Lane 6: r-Moj-WM. Lane 7: r-Moj-WP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. r-Moj-WM and r-Moj-MN mutant peptides were the most potent inhibitors of in-vitro angiogenesis. (A) Number of branch points. (B) Number of tubules. $p = 0.01$ (**), $p < 0.001$ (***). Three of the co-authors in this paper counted branch points and number of tubules independently.

Fig. 3.

(A) r-Moj-MP peptides induced apoptosis of SK-Mel-28 cells ($p = 0.001$). SK-Mel-28 cells were treated for 24 h with 5 µM r-Moj peptides as described in the methods section. Cells were processed using an APO-BRDU™ Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's protocol. Cell suspensions were analyzed using a Becton Dickinson FACSCalibur flow cytometer with CellQuest software. (B) r-Moj-MP induced chromatin fragmentation of SK-Mel-28 cells (white arrows). Representative cells are shown in each panel. SK-Mel 28 cells were grown on chambered slides. Cells were treated with 5 µM r-Moj-MP peptide as described in the methods section. Cells were fixed and their nuclei stained with Vectashield mounting medium with DAPI and viewed at 100×, oil immersion, with a Zeiss AXIO fluorescent microscope.

Fig. 4.

All r-Moj mutant peptides inhibited proliferation of SK-Mel-28 cells. The effects of r-Moj peptides on cell proliferation were determined using the WST-1 assay (GenDEPOT) as described in the methods section. After peptide treatment cells were incubated with WST-1 cell proliferation assay. Percentage of cell proliferation inhibition was calculated using the following formula presented in the methods section. Experiments were performed in triplicate.

Fig. 5.

r-Moj mutant peptides with a methionine were the strongest cell migration inhibitors of SK-Mel-28. A Cell BioLabWound Healing Assay was used to measure cell migration inhibition as described by Carey et al. (2012). Cells were incubated for 24 h with their respective r-Moj treatments. Then pictures were taken of each well and the percentage closure was calculated following the manufacturer's instructions.

Fig. 6. Comparison of the most potent r-Moj mutant peptides in this study. The highest two most active of the r-Moj disintegrins tested are included.

Table 1

Platelet aggregation inhibition by r-Moj_ mutant peptides.

Treatment	IC ₅₀ (nM) ± SD
r-Moj-MN	14.2 ± 7
r-Moj-MP	19.1 ± 7
r-Moj-NM	84.6 ± 4
r-Moj-WL	74.6 ± 6
r-Moj-WM	18.8 ± 2
r-Moj-WP	19.8 ± 5

SD = standard deviation. Three independent measures were performed in triplicate.

Table 2

Platelet aggregation inhibition results of r-Moj-D__ peptides.

Treatment	IC ₅₀ (nM) ± SD
r-Moj-DA	353.9 ± 9
r-Moj-DG	170.4 ± 11
r-Moj-DL	78.4 ± 10
r-Moj-DN	139.2 ± 9
r-Moj-DM	*
r-Moj-DV	91 ± 5

* r-Moj-DM failed to inhibit platelet aggregation. Three independent measures were performed in triplicate. SD = standard deviation.