Functional characterization of six aspartate (D) recombinant mojastin mutants (r-Moj): a second aspartate amino acid carboxyl to the RGD in r-Moj-D_ peptides is not sufficient to induce apoptosis of SK-Mel-28 cells.

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1 ABSTRACT

2 Disintegrins are small peptides produced in viper venom that act as integrin antagonists.
3 When bound to integrins, disintegrins induce altered cellular behaviors, such as apoptotic
4 induction. Disintegrins with RGDDL or RGDDM motifs induce apoptosis of normal and
5 cancer cells. We hypothesized that a second aspartate (D) carboxyl to the RGD is
6 sufficient to induce apoptosis. Five recombinant mojastin D mutants were produced by
7 site-directed mutagenesis (r-Moj-DA, r-Moj-DG, r-Moj-DL, r-Moj-DN, and r-Moj-DV).
8 Stable α_v integrin knockdown and shRNA scrambled control SK-Mel-28 cell lines were
9 produced to test a second hypothesis: r-Moj-D_ peptides bind to α_v integrin. Only r-Moj-
10 DL, r-Moj-DM, and r-Moj-DN induced apoptosis of SK-Mel-28 cells (at 29.4%, 25.6%,
11 and 36.2%, respectively). Apoptotic induction was significantly reduced in SK-Mel-28
12 cells with a stable α_v integrin knockdown (to 2%, 17%, and 2%, respectively), but not in
13 SK-Mel-28 cells with a stable scrambled shRNA. All six r-Moj-D_ peptides inhibited
14 cell proliferation; ranging from 49.56% (r-Moj-DN) to 75.6% (r-Moj-DA). Cell
15 proliferation inhibition by r-Moj-D_ peptides was significantly reduced in SK-Mel-28
16 cells with a stable α_v integrin knockdown. All six r-Moj-D_ peptides inhibited SK-Mel-
17 28 cell migration at high levels (69% to 100%). As a consequence, *rac-1* mRNA
18 expression levels were significantly reduced as early as 1 h after treatment, suggesting
19 that *rac-1* is involved in the cell migration activity of SK-Mel-28.

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22 **Key words:** melanoma cell line, recombinant RGD disintegrin, $\alpha_v\beta_3$ integrin, α_v

23 knockdown, cell migration, cell proliferation, apoptosis induction, *rac-1* expression

24

1. Introduction

Integrins are heterodimeric transmembrane proteins, consisting of one alpha and one beta subunit. Integrins can activate signal transduction pathways that can result in altered cell behaviors such as cell migration, proliferation, and apoptosis (Desgrosellier and Cheresch 2010; Mousa 2008; Ridley et al. 2003). Integrin function is often the result of specific subunit expression. Antagonists, such as disintegrins, are being studied as these molecules can bind and alter integrin signaling and as such hold promise as potential therapeutic agents for the treatment of cancer (Selistre-de-Araujo et al. 2010; McLane et al. 2008).

Disintegrins are non-enzymatic peptides found in viper venom that were initially characterized as being platelet aggregation inhibitors (McLane et al. 2004). Many of the known disintegrins contain an RGD motif, a usual structural characteristic of integrin antagonists (Rouslahti 1996). Seven naturally occurring or recombinant disintegrins with a second aspartate amino acid immediately carboxyl of the RGD induce apoptosis.

Apoptotic-inducing disintegrins with a second aspartate are in two categories: one with an RGDDL and the second with an RGDDM motif. RGDDL disintegrins with reported apoptotic activity include: vicrostatin (Minea et al. 2010), salmosin (Hong et al. 2003), contortrostatin (Zhou et al. 1999), and accutin (Yeh et al. 1998). RGDDM disintegrins with reported apoptotic activity include: DisBa-01 (Ribeiro et al 2014), r-Moj-DM (Seoane et al. 2010), and echistatin (Alimenti et al. 2004; Brassard et al. 1999).

Published reports demonstrate that most of these disintegrins antagonize the $\alpha v \beta 3$ integrin (Minea et al. 2010; Ramos et al. 2008; Kang et al. 1999; Kumar et al. 1997).

This integrin has been studied as a target for therapy development (Mitjans et al. 2000),

1 since it is overexpressed in highly metastatic cancers. Voetseder et al. (2013)
2 demonstrated that $\alpha v \beta 3$ expression increased in several types of cancer cells that
3 metastasize to brain tissue, including melanomas. Melanocytes do not express $\alpha v \beta 3$.
4 Overexpression of this integrin has been linked to tumor progression and metastasis of
5 melanomas (Van Belle et al. 1999; Felding-Habermann et al. 1992).

6 Previously, we demonstrated that apoptosis induction of a human melanoma cell
7 line (SK-Mel-28) resulted when the wild-type binding motif (RGDWN) was mutated to
8 RGDDDM in recombinant mojastin, r-Moj (Seoane et al. 2010). We are interested in
9 understanding the mechanism by which mutated versions of r-Moj antagonize integrins in
10 the induction of apoptosis of cancer cells. In the present study, six mutated versions of r-
11 Moj containing a second aspartate in the binding motif were produced, purified, and used
12 to test two hypotheses. First, we hypothesized that a second aspartate (D) carboxyl of the
13 RGD is sufficient to induce apoptosis. Second, we hypothesized that r-Moj-D_ peptides
14 bind to αv integrin receptors. Three of r-Moj-D_ motifs have naturally occurring
15 counterparts (RGDDL, RGDDM, and RGDDV), and three do not (RGDDA, RGDDG,
16 and RGDDN). Our research demonstrates that a second aspartate is not sufficient to
17 induce apoptosis, but that r-Moj-D_ peptides bind to αv integrin.

19 **2. Materials and methods**

20 *2.1. Production of five additional aspartate mutants from r-Moj-DM cDNA*

21 A mutant r-Moj-DM cDNA was previously obtained (Seoane et al. 2010).
22 Additional r-Moj-D_ mutants were obtained using the methods described in Seoane et al.
23 (2010). The mutant mojastin peptides were designated as r-Moj-DA, r-Moj-DG, r-Moj-

DL, r-Moj-DN, and r-Moj-DV. The designation corresponds to the two amino acids following the RGD tripeptide.

2.2. Expression and purification of r-Moj-DA, r-Moj-DG, r-Moj-DL, r-Moj-DM, r-Moj-DN, and r-Moj-DV peptides

Expression and partial purification of r-Moj-DA, r-Moj-DG, r-Moj-DL, r-Moj-DM, r-Moj-DN, and r-Moj-DV peptides were performed with a method described earlier (Seoane et al. 2010), with the following modifications. Removal of the GST-tagged was performed using a thrombin cleavage capture kit while bound to the Glutathione Sepharose 4B beads using biotinylated thrombin for 16 h at 4°C with no agitation. Streptavidin agarose beads and spin filters (Novagen) were used to remove the thrombin from the supernatant containing the cleaved r-Moj-DA, r-Moj-DG, r-Moj-DL, r-Moj-DM, r-Moj-DN, or r-Moj-DV peptides at room temperature for 30 min with gentle rotation.

2.3. Structural protein modeling of r-Moj-DA, r-Moj-DG, r-Moj-DL, r-Moj-DM, r-Moj-DN, and r-Moj-DV peptides

Three-dimensional structural models of r-Moj-D_ peptides were produced using the methods described by Seoane et al. (2010).

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. *Integrin α v gene expression knockdown*

Inhibition of integrin α v expression was performed in SK-Mel-28 cells using integrin α v shRNA (h) lentiviral particles (Santa Cruz Biotech, sc-29373-v). Control shRNA lentiviral particles (sc-108080) were used as a negative scrambled shRNA sequence control. Cells were plated in 12-well plates, 24 h prior to viral infection with 1 mL of complete EMEM media. Then, the media was changed with complete media containing 5 µg/mL of Polybrene (sc-134220). Integrin α v shRNA (h) lentiviral particles were thawed and added to the cells, mixed gently, and incubated overnight in a humidified 5% CO₂ air incubator at 37 °C. After 24 h, the media was changed with 1 mL of complete media without Polybrene and incubated overnight. To select stable clones, cells were split in the ratio of 1:5 and incubated for 24 h in complete media. After 48 h, stable clones expressing α v shRNA were selected using Puromycin dihydrochloride (sc-108071) at 5 µg/ml. The cells were expanded and grown in puromycin containing complete media until ready for use.

2.6. *Integrin α v β 3 expression using flow cytometry*

Integrin α v β 3 expression analyses were performed using fluorescently labeled anti- α v β 3 antibody and flow cytometry on untreated cells, shRNA scramble control cells, and α v knockdown cells. The antibody against α v β 3 (α v β 3 sc-7312 FITC) and respective

isotype were purchased from Santa Cruz Biotechnology. Cells were cultured to 90-95% confluency and detached from the flask surface using 0.05% trypsin EDTA. Trypsin was neutralized with complete culture media. Cells were resuspended in 1X PBS to a final concentration of 10^6 cells/mL, and blocked with 1 μ L/mL normal rabbit serum, for 25 min at room temperature. After blocking, cells were washed with 1mL 1X PBS, centrifuged, and resuspended in FCM Wash Buffer (Santa Cruz Biotechnology) to 10^6 cells/mL. One hundred microliters of cells were distributed in three, 12 Å~ 75 mm round bottom glass test tubes and fixed in 1% formaldehyde for 1 h. After fixation, cells were washed three times in 1X PBS. Four hundred microliters of 1X binding buffer were added and the samples analyzed using a Becton Dickinson FACScan flow cytometer and FACSCalibur software. Isotype control samples were used to determine gating parameters. Expression of $\alpha v \beta 3$ on untransduced, shRNA scramble control, and αv knockdown cells was normalized and compared with isotype control samples. Data are presented as means of percentages of $\alpha v \beta 3$ expression for three independent experiments.

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-DA, r- Moj-DG, r-Moj-DL, r-Moj-DM, r-Moj-DN, or r-Moj-DV peptides, 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 300xG for 5 min and fixed in 1% paraformaldehyde 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

1 were processed using an APO-BRDU™ Apoptosis Detection Kit (BD Biosciences)
2 according to the manufacturer's protocol. Cell suspensions were analyzed using a Becton
3 Dickinson FACSCalibur flow cytometer with CellQuest software. Experiments were
4 performed in triplicates.

6 2.8. *Chromatin condensation*

7 SK-Mel 28 cells were grown on chambered slides. One and a half million cells
8 were grown in 2 mL of media for 24 h. After initial incubation, cells were treated with
9 5μM r- Moj-DA, r-Moj-DG, r-Moj-DL, r-Moj-DM, r-Moj-DN, or r-Moj-DV peptides for
10 24 h. The media was aspirated and the cells washed twice with 1X PBS. Cells were fixed
11 on the slide with 3% formaldehyde for 1 h, followed by two washes with 1X PBS. Ten
12 micrograms/mL of Hoechst stain was added to cells and incubated in the dark for 15 min.
13 Excess Hoechst stain was removed by two 1X PBS washes. Slides were mounted with
14 30% glycerol and viewed at 100X, oil immersion, with a Zeiss AXIO fluorescent
15 microscope.

17 2.9. *Cell Proliferation Assay*

18 The effects of r-Moj-D_ peptides on cell proliferation were determined using the
19 WST-1 assay (GenDEPOT). Fifty thousand SK-Mel-28 cells/well were added to a 96-
20 well plate grown for 24 h at 37°C in a 5% CO₂ incubator. After 24 h, growth media was
21 aspirated. Then, 100 μL of culture media containing 3.5 mM of r-Moj-D_ peptide were
22 added to their respective wells. Negative controls consisted of cells treated with equal
23 volume of PBS. After 24 h all wells were then treated with 10 μL 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: $(\text{Abs}_{450\text{nm}-600\text{nm}} \text{ of treated} - \text{Abs}_{450\text{nm}-600\text{nm}} \text{ of untreated}) \times 100$. Experiments were performed in triplicate.

2.10. Wound Healing Assay

The effects of r-Moj-D₁ peptides on cell migration were determined using the method described by Carey et al. (2012).

2.11. Detection of *rac-1* expression levels by QPCR after r-Moj-DA, r-Moj-DL, r-Moj-DM, r-Moj-DN, or r-Moj-DV peptide treatment

One million SK-Mel 28 cells were treated with r-Moj-DA, r-Moj-DL, r-Moj-DM, r-Moj-DN, or r-Moj-DV peptides for 1 h, 2 h, 4 h, and 6 h. Total RNA was extracted from each sample using the RNeasy Mini Kit (Qiagen). Total RNA was quantified by the use the NanoDrop spectrophotometer. One thousand nanograms of total RNA were isolated from any contaminating genomic DNA with the use of gDNA Wipeout Buffer from the QuantiTect Reverse Transcription Kit (Qiagen). The isolated RNA was reverse transcribed at 42°C for 15 min using Quantiscript Reverse Transcriptase (Qiagen). Reactions were prepared for each sample cDNA with correlating primers. Sample

1 reactions containing 2.5ul of cDNA and 1.0uM of forward
2 (AAGAGAAAATGCCTGCTGTTGTAA) and reverse
3 (GCGTACAAAGGTTCCAAGGG) primers were prepared in triplicate wells with the
4 use of QuantiTect SYBR Green PCR kit (Qiagen). The PCR conditions were 1 cycle of
5 95°C for 5 min, followed by 40 cycles of amplification (95°C for 10 sec and 60°C for 30
6 sec), and a final extension cycle at 95°C for 15 sec and 60°C for 1 min. A melting curve
7 was used to verify the presence of one specific peak for the gene. Average Ct was used to
8 calculate relative fold change. Normalization of the data was performed by the use an
9 endogenous control, HPRT primers (forward: ATGACCAGTCAACAGGGGAC, and
10 reverse: GGTCCCTTTTCACCAGCAAGC), and an untreated control sample.

12 **3. Results**

13 *3.1. Six aspartate mojastin mutants*

14 Six aspartate mojastin mutants were produced and purified without the GST tag
15 (Figs. 1A-1B). Three-dimensional models showed that single amino acid substitutions
16 after the second aspartate (RGDD_) altered the predicted orientations in their RGD loops
17 and C-termini (Fig. 1C).

19 *3.2. Production of stable SK-Mel-28 α v knockdown and scrambled control cell lines*

20 Expression of α v β 3 integrin was significantly reduced in cells that were
21 transduced with α v shRNA lentiviral particles ($p < 0.001$), as compared to untransduced
22 cells or cells that were transduced with a scrambled shRNA control sequence (Fig. 2).

3.3. Apoptotic inducing activity of *r-Moj-DL*, *r-Moj-DM*, and *r-Moj-DN*

Only three *r-Moj-D_* mutant peptides (*r-Moj-DL*, *r-Moj-DM*, and *r-Moj-DN*) induced apoptosis of SK-Mel-28 cells (Fig. 3). The strongest apoptotic inducer was *r-Moj-DN*, a novel peptide. All three peptides induced apoptosis by binding to αv integrin, as apoptotic induction was inhibited in the treated stable αv knocked down cells.

Apoptotic activation was confirmed by examining chromatin condensation. Only cells treated with *r-Moj-DL*, *r-Moj-DM*, or *r-Moj-DN* showed chromatin condensation, a morphological characteristic of apoptotic cells (Fig. 4).

3.4. Cell proliferation inhibition

All *r-Moj-D_* peptides inhibited cell proliferation significantly ($p < 0.001$, Fig 5). Recombinant *Moj-DA* was the strongest inhibitor (79%) and *r-Moj-DN* was the weakest (50%). All *r-Moj-D_* mutant peptides tested inhibited cell proliferation by binding to αv , as inhibition was reduced or abolished (*r-Moj-DL* and *r-Moj-DN*) in the treated stable αv knocked down cells.

3.5. Cell migration inhibition and *rac-1* mRNA expression

Cell migration of untransduced SK-Mel-28 cells was inhibited by all *r-Moj-D_* mutant peptides (Fig. 6A). Recombinant *r-Moj-DL* was the most potent cell migration inhibitor (100%). Whether *r-Moj-D* peptides induce cell migration inhibition through αv integrin could not be determined directly, as untreated stable αv knocked down cells failed to migrate. *Rac-1* mRNA expression was downregulated in five of the six treatments (Fig 6B). The effect on *rac-1* gene expression was fast and detected 1 h after

1 treatment. The r-Moj-DA effect on *rac-1* gene expression was short lived. In contrast,
2 gene expression effects were longer lasting in r-Moj-DL and r-Moj-DN treated cells.
3 Recombinant r-Moj-DL also induced the highest down regulation at 2 h (13 fold change).
4 Recombinant r-Moj-DV had no effect on *rac-1* mRNA expression.

6 **4. DISCUSSION**

7 *4.1. Apoptosis induction by r-Moj-DL, r-Moj-DM, and r-Moj-DN peptides*

8 Apoptosis can prevent cells attached to either the ECM or adjacent cells from
9 beginning to migrate by activating either intrinsic or extrinsic cell death pathways
10 (Gilmore 2005). Apoptotic avoidance is one of the established hallmarks of cancer
11 development (Hanahan and Weinberg 2011; Reed 1999). Others include: induction of
12 angiogenesis, activation of invasion and metastasis, evasion of tumor suppression,
13 reproductive immortality, and sustaining proliferative signaling (Hanahan and Weinberg
14 2011). An approach for the development of innovative chemotherapies is the screening,
15 selection, modification, and synthesis of compounds that can inhibit multiple landmarks
16 of cancer development (for example, angiogenesis, cell migration, metastasis, and cell
17 proliferation) while inducing apoptosis. Our previous research with two recombinant
18 disintegrins (r-Rub and r-Moj-DM) provided us with good models to investigate this
19 approach (Carey et al. 2012; Seoane et al. 2010).

20 Seven disintegrins with a second aspartate carboxyl to their RGD induce apoptosis
21 (Ribeiro et al 2014; Minea et al. 2010; Seoane et al. 2010; Alimenti et al. 2004; Hong et
22 al. 2003; Brassard et al. 1999; Zhou et al. 1999; Yeh et al. 1998). Therefore, we
23 hypothesized that the second aspartate is sufficient to induce apoptosis. The results of

our present study refute this hypothesis. Of the six r-Moj-D₂ mutant peptides tested, only three induced apoptosis of SK-Mel-28 cells (r-Moj-DL, r-Moj-DM, and r-Moj-DN). Two of these mutants have naturally occurring counterparts (RGDDL and RGDDM) and one does not (RGDDN). Our results suggest that the amino acid immediately carboxyl of the second aspartate is important. Two of the apoptotic inducing r-Moj-D₂ mutant peptides contain a non-polar amino acid after the second aspartate. But the strongest apoptotic inducer, r-Moj-DN has a polar amino acid in that position instead.

4.2. Knockdown of α v integrin revealed its significance in signal transduction pathways induced by r-Moj-D₂ mutant peptides

Several integrins involved in tumor progression are not expressed, or are expressed at low levels in normal epithelial tissue (Desgrosseilier and Cheresh 2010). Among these, the α v β 3 integrin is expressed at high levels on many types melanomas (Vogetseder et al. 2013; Mitjans et al. 2000). In addition, α v β 3 activates transduction signals that target the P13K-PKB/AKT and the Lyn pathways, resulting in cell survival and apoptosis avoidance (Chudakova et al. 2008; Hullinger et al. 1998).

Alpha v subunits can form heterodimers with β 1, β 3, β 5, β 6, and β 8 integrin subunits (Koistinen and Heino 2002). We have previously shown that SK-Mel-28 cells express α v, β 1, and β 3, but not β 5 or β 6 (Seoane et al. 2010). Expression of β 8 subunits was not determined in our previous study. Since SK-Mel-28 cells express α v β 3 at high levels (Seoane et al. 2010), it is possible that α v β 1 is expressed at low levels or not at all. Koistinen and Heino (2002) demonstrated that in melanomas, α v β 1 is only expressed after the other α v heterodimers are formed, such as α v β 3. Our data suggest that r-Moj-

D₂ peptides antagonize $\alpha v\beta 3$ based on the significant reduction of expression of this integrin on the αv knocked down cells. However, we cannot discard the possibility that these cells express $\alpha v\beta 1$ or $\alpha v\beta 8$ and that the r-Moj-D₂ peptides also antagonize these integrins. These possibilities remain to be studied.

Stable knockdowns of αv integrin subunit and scrambled shRNA controls were used to examine the role of r-Moj-D₂ peptide antagonism to αv integrin because of the importance of $\alpha v\beta 3$ in regulating survival/apoptosis, cell migration, and angiogenesis. The results from knockdown experiments allowed us to make some interesting conclusions. The apoptotic inhibition reduction was not equivalent in the r-Moj-D₂ treatments of αv knocked down cells, suggesting that these peptides do not antagonize the receptor with the same strength. It is also possible that in the case of r-Moj-DM, another integrin receptor is more important for apoptotic induction. Our results suggest that r-Moj-DM is not as efficient at antagonizing αv when all of the functional assays we performed with αv knocked down cells are considered. Perhaps, r-Moj-DM exerts most of the apoptotic activity through another integrin receptor, possibly containing a $\beta 1$ subunit. The $\alpha 5\beta 1$ integrin is not a possibility since $\alpha 5$ is not expressed on SK-Mel-28 cells (Seoane et al. 2010).

4.3. Potential relationship between cell proliferation inhibition and apoptotic induction

Cell proliferation was inhibited in all treated cells. The αv knockdown results for the cell proliferation experiment suggest that not all the cell proliferation inhibition is due to cell death. Recombinants r-Moj-DL and r-Moj-DN failed to inhibit cell proliferation of αv knocked down cells, but apoptosis induction was not completely abolished in these

1 cells. However, it is possible that the difference in cell proliferation and apoptotic results
2 is due to the sensitivity of the tunnel assay used to analyze apoptosis and the indirect
3 measurement of cell proliferation obtained using the WST-1 assay. Furthermore, cell
4 proliferation reduction results caused by r-Moj-DA, r-Moj-DG, and r-Moj-DV are not
5 due to cell death, since these do not induce apoptosis.

6 7 *4.4. Downregulation of rac-1 mRNA expression as a result of r-Moj-D_ peptide* 8 *treatments*

9 Integrins' role in regulating cell migration and metastasis is well studied
10 (Desgrosellier and Cheresch 2010; Selistre-de-Araujo et al. 2010). Our study suggests that
11 αv integrin is required for SK-Mel-28 cell migration, as a knockdown of this subunit
12 renders these cells unable to migrate in the absence of treatment. It has been
13 demonstrated that $\alpha v\beta 3$ and $\alpha v\beta 5$ are expressed on highly malignant cells that can
14 initiate cell migration (Ummanni et al. 2008). It has also been demonstrated that $\alpha v\beta 3$ is
15 critical for transendothelial migration of cancer cells during metastasis (Bauer et al.
16 2007).

17 Cross-talk between several growth factor receptors involved in cell migration and
18 certain integrins result in the activation of cell migration and tumor progression of cells
19 that can move away from the ECM while avoiding apoptosis (Brunton et al. 2004). This
20 mechanistic connection between cell migration and apoptosis provides investigators with
21 the drive to find potential single therapies that block the pathways that connect apoptosis
22 avoidance and cell migration activation of malignant cells.

Rac1, a Rho-GTPase, is involved in cell migration activation and exerts its function downstream of integrin signaling by altering actin-based cytoskeleton dynamics (Lawson and Burridge 2014; Oberoi et al 2012; Yang et al. 2012; Brunton et al. 2004). Rac1 activation also leads to the activation of survival signals such as NF κ b (Tong and Tergaonkar 2014). We investigated the expression of *rac-1* mRNA in order to begin elucidating the signal transduction pathways that result in the cell migration inhibition exerted by r-Moj-D_ peptide treatments. All six r-Moj-D_ peptides inhibited SK-Mel-28 cell migration at high levels (69% to 100%). Our research demonstrated that as a consequence, *rac-1* mRNA expression levels were significantly reduced as early as 1 h after treatment, suggesting that *rac-1* mRNA downregulation is an outcome of most r-Moj-D_ peptide treatments. Of all six r-Moj-D_ peptides tested, r-Moj-DN has the strongest apoptotic-inducer activity and it induced a long lasting downregulation of *rac-1* mRNA expression. Therefore, r-Moj-DN provides us a good model to investigate the possibility of developing a single therapy that targets both apoptotic avoidance and cell migration activation of cancer cells.

4.5. Conclusions

Recombinant disintegrins with mutations in the binding motif are sources of integrin antagonists with potentially different biological activities. Our data demonstrate that a second aspartate immediately carboxyl to the RGD is not sufficient to induce apoptosis. However our second hypothesis was supported, as all of the tested r-Moj-D_ peptides failed to induce or induced lower activities of α v knocked down SK-Mel-28 cells. Our research demonstrates that recombinant disintegrins with non-naturally found

1 binding motifs (RGDDA, RGDDG, RGDDN) are more potent in most of the functional
2 assays we used in our study. It would be of interest to determine if substituting a
3 different polar amino acid immediately carboxyl to the second aspartate is sufficient to
4 render the resulting disintegrin similarly potent as r-Moj-DN. The altered binding motif
5 predicted by the 3-D peptide modeling may provide a testable hypothesis about strength
6 of signal and altered activities of r-Moj-D_ mutant peptides. Recombinant Moj-D_
7 peptide binding to soluble or bound $\alpha v \beta 3$ integrins and structural analysis are interesting
8 possibilities for future studies.

10 **Ethical statement**

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

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Fig. 1. (A) Alignment of amino acid sequences for r-Moj-D_ mutant peptides. The RGD tripeptide is shaded in gray. In bold are shown the two amino acids carboxyl to the RGD motif. (B) 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-DA. Lane 3: r-Moj-DG. Lane 4: r-Moj-DL. Lane 5: r-Moj-DN. Lane 6: r-Moj-DV. (C) r-Moj-D_ 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.

Fig. 2. $\alpha v\beta 3$ integrin cell surface expression on untransduced, scrambled shRNA, and αv stable knocked down SK-Mel-28 cells. $p < 0.001$ (***). $\alpha v\beta 5$ integrin expression was not examined since we demonstrated that SK-Mel-28 cells do not express the $\beta 5$ subunit (Seoane et al. 2010).

Fig. 3. r-Moj-DL, r-Moj-DM, and r-Moj-DN peptides induced apoptosis of SK-Mel-28 cells by binding to the αv integrin. $p = 0.05$ (*), $p = 0.01$ (**), $p < 0.001$ (***).

Fig. 4. r-Moj-DL, r-Moj-DM, and r-Moj-DN peptides induced chromatin fragmentation of SK-Mel-28 cells. A representative cell is shown in each panel.

Fig. 5. All r-Moj-D_ mutant peptides inhibited proliferation of SK-Mel-28 cells by binding to the αv integrin. The scrambled shRNA control treated cells are not shown, since these cells grew at much slower rate than untransduced or αv knocked down cells.

1 **Fig. 6.** (A) All r-Moj-D_ mutant peptides inhibited cell migration. (B) *rac-1* mRNA
2 expression was downregulated as early as 1 h after treatment.

3

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6