UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

PROGRAMA INTERINSTITUCIONAL DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS PIPGCF UFSCar – UNESP

BLOQUEIO DA INTEGRINA $\alpha_v\beta_3$ INIBE APOPTOSE E INDUZ AUTOFAGIA EM CÉLULAS TUMORAIS DE CANCÊR DE MAMA

RAFAEL LUIS BRESSANI LINO

SÃO CARLOS

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Dissertação apresentada ao Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas Associação Ampla UFSCar/ UNESP, para obtenção do título de Mestre em Ciências, Área de Concentração Ciências Fisiológicas.

Orientação: Profa. Dra. Heloisa Sobreiro Selistre de Araújo

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Centro de Ciências Biológicas e da Saúde
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Assinaturas dos membros da comissão examinadora que avaliou e aprovou a Defesa de Dissertação de Mestrado do candidato Rafael Luis Bressani Lino, realizada em 17/12/2018:

Profa. Dra. Heloisa Sobreiro Selistre de Araujo
UFSCar

Profa. Dra. Marinilce Fagúndes dos Santos
USP

Prof. Dr. Ricardo Carneiro Borra
UFSCar

"Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes."

(Marthin Luther King)

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RESUMO

As integrinas são receptores-chave que regulam a adesão celular à matriz extracelular (ECM), além de modularem o comportamento e a migração das células. Particularmente, a integrina $\alpha_v\beta_3$, receptor de vitronectina, desempenha um papel fundamental tanto na angiogênese fisiológica quanto na progressão tumoral. Este receptor reconhece o motivo arginina-glicina-ácido aspártico (RGD) nas proteínas da MEC e pode ser antagonizado pelos peptídeos RGD, resultando na diminuição da migração e da invasão celular. Fármacos baseadas no motivo RGD mostraram resultados não promissores em ensaios clínicos, no entanto, as razões para sua falta de atividade ainda permanecem desconhecidas. Para uma melhor compreensão dos mecanismos moleculares da inibição de integrinas, testamos uma desintegrina RGD recombinante (Dis*Ba*-01) em dois tipos de linhagens celulares: 4T1BM2 de tumor de mama murino e fibroblastos L929. Apenas as células tumorais apresentaram diminuição da motilidade e da adesão celular, bem como alterações morfológicas após tratamento com Dis*Ba*-01. Estes resultados foram atribuídos aos níveis mais altos de $\alpha_v\beta_3$ nas células 4T1BM2, tornando-as mais sensíveis ao bloqueio feito pela desintegrina. A análise por citometria de fluxo mostrou que Dis*Ba*-01 induziu parada do ciclo celular na fase S em células 4T1BM2, mas não induziu apoptose, o que foi condizente com a diminuição da expressão gênica de caspase-3, 8 e 9. A presença de caspase-3 ativa não foi detectada por Western Blotting em ambas as células. Em contrapartida, foi observado que Dis*Ba*-01 aumenta a expressão proteica de LC3B, um indicador de autofagia. Em conclusão, a especificidade de Dis*Ba*-01 em relação às células 4T1BM2 quando comparada às células L929, pode ser devido ao maior nível de expressão de integrinas α_v e β_3 nas células tumorais. Estas observações sugerem novos mecanismos sobre os efeitos de inibidores baseados em RGD, considerando sua importância terapêutica para a saúde humana.

Palavras-chave: câncer de mama, integrina $\alpha_v\beta_3$, MMP-2, adesão celular, anoikis, apoptose, autofagia, desintegrinas-RGD.

ABSTRACT

Integrins are key receptors that mediate cell adhesion to the extracellular matrix (ECM) and regulate cell behavior including cell migration. Particularly, $\alpha_v\beta_3$ integrin, the vitronectin receptor, plays a key role in both physiological angiogenesis and in tumor progression. This receptor recognizes the arginine-glycine-aspartic acid (RGD) motif in ECM proteins and it can be antagonized by RGD-peptides, resulting in decreased cell migration and invasion. RGD-based drugs have shown disappointing results in clinical trials; however, the reasons for their lack of activity are still obscure. Aiming to contribute to a better understanding on the molecular consequences of integrin inhibition, we have tested a recombinant RGD-disintegrin (Dis*Ba*-01) in two types of murine cell lines, breast tumor 4T1BM2 cells and L929 fibroblasts. Only tumor cells showed decreased motility and adhesion as well as morphologic alterations upon Dis*Ba*-01 treatment. This result was attributed to the higher levels of $\alpha_v\beta_3$ in 4T1BM2 cells making them more sensitive to Dis*Ba*-01 blocking. Flow cytometry analysis showed that Dis*Ba*-01 induced cell cycle arrest at S phase in 4T1BM2 cells, but did not induce apoptosis, which was consistent with the decrease in caspase-3, 8 and 9 expression at mRNA and protein levels. It was also observed that this disintegrin increases LC3B expression in both cell lines, an indicator of autophagic induction. In conclusion, Dis*Ba*-01 specificity towards 4T1BM2 cells when compared to L929, could be due to the higher level of expression of α_v and β_3 integrins in these cells, and these observations suggest new insights on the effects of RGD-based inhibitors considering their importance on drug development for human health.

Keywords: breast cancer**,** $\alpha_v\beta_3$ integrin, MMP-2, cell adhesion, anoikis, apoptosis, autophagy, RGD-disintegrin.

LISTA DE FIGURAS

MOTIVOS ADESIVOS DAS DESINTEGRINAS

ECD - Ácido glutâmico - Cisteína - Ácido aspártico

DCD - Ácido aspártico - Cisteína - Ácido aspártico

KTS - Lisina - Treonina - Serina

KGD - Lisina - Glicina - Ácido aspártico

MGD - Metionina - Glicina - Ácido aspártico

MLD - Metionina - Leucina - Ácido aspártico

MVD - Metionina - Valina - Ácido aspártico

RGD - Arginina - Glicina - Ácido aspártico

RTS - Arginina - Treonina – Serina

VGD - Valina - Glicina - Ácido aspártico

WGD - Triptofano - Glicina - Ácido aspártico

LISTA DE ABREVIATURAS E SIGLAS

EMT – Transição epitélio-mesenquimal (*Epitelial-Mesenquimal Transition*)

FAK – Quinase de adesão focal (*Focal Adhesion Kinase*)

MMPs – Metaloproteases de Matriz (*Matrix Metalloproteases*)

MEC – Matriz Extracelular

OMS – Organização Mundial da Saúde

SVMP – Metaloprotease de veneno de serpente (*Snake venom Metalloprotease*)

VEGF – Fator de crescimento endotelial vascular (*Vascular Endothelial Growth Factor*)

VEGFR – Receptor do fator de crescimento endotelial vascular (*Vascular Endotelial Growth Factor Receptor*)

SUMÁRIO

1. INTRODUÇÃO

1.1. CÂNCER, DESENVOLVIMENTO TUMORAL E MESTÁSTASE

Segundo dados divulgados pela Organização Mundial da Saúde (OMS), em 2030 ocorrerão 27 milhões de novos casos de câncer em todo o mundo, contabilizando 17 milhões de mortes e 75 milhões de pessoas vivendo com a doença. Neste contexto, no Brasil estima-se o registro de 596 mil novos casos de câncer, sendo 300 mil em mulheres e 295 mil em homens, excluindo-se os de pele não melanoma. Em mulheres os mais prevalentes são: mama, cólon e reto, colo de útero, traqueia, brônquios e pulmão e estômago. Sendo assim, esta doença é considerada um problema de saúde pública não apenas no Brasil, mas no mundo (INCA, 2016).

O câncer é o nome dado a um conjunto de mais de 100 doenças que têm em comum o crescimento desordenado de células e a capacidade destas de invadirem tecidos e órgãos de diferentes partes do corpo humano, promovendo a formação de uma massa tumoral de crescimento contínuo (INCA, 2016). Considerada de origem multifatorial crônica, é também caracterizado por uma série de alterações genéticas e epigenéticas, que desregulam os mecanismos de controle dos processos celulares resultando na quebra da homeostase, proliferação descontrolada das células e evasão ao sistema imune, acarretando ao câncer uma resistência aos tratamentos (BAU et al., 2006; CHATTERJEE; MCCAFFREY, 2014; LIU et al., 2015; MBEUNKUI; JOHANN, 2009).

As células cancerígenas não agem de forma independente, elas são influenciadas pelo microambiente que as cerca, podendo adaptar seu fenótipo à medida que o meio externo é modificado (BISSELL; HINES, 2011; FRIEDL; ALEXANDER, 2011). No microambiente onde o tumor se desenvolve estão presentes diferentes elementos incluindo desde a matriz extracelular (MEC), vasos sanguíneos e linfáticos, assim como diversos tipos celulares e fatores de crescimento, sendo estes responsáveis por influenciar a transformação de células normais em células cancerígenas, assim como a progressão do câncer (MBEUNKUI; JOHANN, 2009; PICKUP; MOUW; WEAVER, 2014).

A metástase se dá por uma cascata de eventos celulares e moleculares interconectados e ainda não totalmente elucidados, que culminam no crescimento de um ou mais tumores secundários em localizações distintas ao tumor primário, sendo responsável por 90% das mortes entre os pacientes com câncer (ALIZADEH; SHIRI; FARSINEJAD, 2014).

De acordo com Alizadeh et al., 2014 a cascata metástatica pode ser dividida em cinco etapas: dissociação, invasão, intravasamento, extravasamento e dormência. Em todas as etapas, os processos de invasão e adesão das células tumorais são essenciais para que elas sejam capazes de se aderirem aos componentes do microambiente, de modo que possam migrar através da matriz e, consequentemente, se instalarem em locais distantes do tumor primário, conforme representado pela Figura 1 (ALIZADEH; SHIRI; FARSINEJAD, 2014; YILMAZ; CHRISTOFORI, 2010).

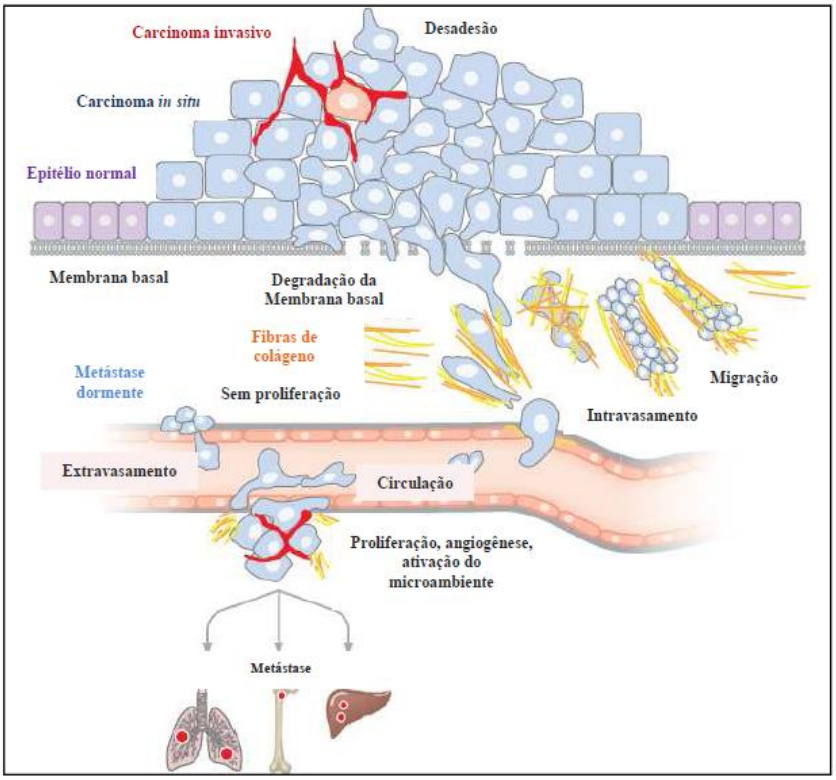

Figura 1. Principais etapas do processo metastático. O carcinoma *in situ* evolui para carcinoma invasivo, quando as células perdem suas conexões de adesão iniciando o processo de invasão celular. Desta forma, após a degradação da membrana basal, as células tumorais invadem o estroma, migram e intravasam para os vasos sanguíneos e linfáticos, sendo, portanto, transportadas até aderirem em capilares de órgãos distantes, formando assim, nos tecidos alvos, as metástases. **Fonte:** Extraído e modificado de BACAC; STAMENKOVIC, 2007.

1.2 TRANSIÇÃO EPITÉLIO-MESENQUIMAL E MIGRAÇÃO CELULAR

A metástase se inicia através de um processo denominado de Transição Epitélio-Mesenquimal (EMT: *Epithelial-Mesenquimal Transition*), realizado por células tumorais localizadas nas margens do tumor. Estas células, sofrem uma série de alterações fenotípicas epiteliais, que resultam no aumento da mobilidade celular (CATALANO et al., 2013) capacitando-as a sobreviverem em microambientes hostis (WONG et al., 2014).

A migração celular em doenças como o câncer ocorre de forma desregulada e disseminada se tornando um mecanismo chave para a progressão de alguns tipos de tumores

(CHARRAS; SAHAI, 2014; VEISEH et al., 2011). O processo tem início com a protrusão da membrana na frente de migração, causada pela polimerização dos filamentos de actina. A protrusão se conecta às fibras da MEC através de *clusters* formados por receptores de adesão (integrinas), que estão conectados ao citoesqueleto de actina na sua porção intracelular. Imediatamente à frente de migração ocorre a degradação da matriz por metaloproteases (MMPs), enquanto o citoesqueleto contrai o corpo e cauda celular causando a translocação da célula. Desta forma, as adesões presentes na cauda da célula começam a se desfazer, a cauda da célula retrai e a membrana presente na frente de migração dá início a uma nova protrusão e a reciclagem gradual das adesões ocorre (FRIEDL; ALEXANDER, 2011; PARSONS; HORWITZ; SCHWARTZ, 2010; WOLF et al., 2013).

As adesões focais começam a se formar bem próximas à membrana celular em protrusão (lamelipódia) e são denominadas de adesões nascentes que ao amadurecerem formam adesões focais. O contínuo movimento celular faz com que as adesões se localizem de forma cada vez mais posterior e sempre aumentando de tamanho, sendo denominada de adesões fibrilares e finalmente adesões traseiras, quando atingem a "cauda" da célula e se desfazem permitindo a retração da membrana (DI CIO; GAUTROT, 2016; VICENTE-MANZANARES; HORWITZ, 2011).

Neste contexto, as integrinas são as moléculas que dão início às adesões através de *clusters*, compostos de 3 ou 4 moléculas (adesões nascentes). Moléculas adaptadoras fazem a ligação entre as integrinas e os filamentos de actina, participando das adesões desde sua formação. Imediatamente após, uma série de moléculas se reúnem à adesão reforçando a ligação aos filamentos do citoesqueleto de actina, além de proteínas sinalizadoras como a FAK (*Focal Adhesion Kinase*). Estas são fosforiladas criando sítios de ligação para outras proteínas e promovendo a polimerização da actina. Desta forma, à medida que a protrusão avança, as adesões amadurecem, o número de moléculas cresce, consequentemente, aumentando o tamanho das adesões, assim como sua força contrátil e o *turnover* (DI CIO; GAUTROT, 2016; GEIGER; SPATZ; BERSHADSKY, 2009; VICENTE-MANZANARES; HORWITZ, 2011; WEHRLE-HALLER, 2012).

1.3 APOPTOSE, ANOIKIS E AUTOFAGIA

A apoptose, conhecida como morte celular programada, desempenha um papel essencial durante diversos processos fisiológicos e patológicas. Este tipo de falência celular mantém a homeostase pela eliminação controlada das células que não são mais necessárias (HASSAN et al., 2014; SAYERS, 2011), porém uma vez perdido este controle, as células se

desregulam levando a formando de tumores. Características importantes são aparentes em células em apoptose: encolhimento celular, formação de vesículas, formação de corpos apoptóticos no citoplasma, ausência de sinais inflamatórios, degradação da membrana celular e condensação da cromatina (ELMORE, 2007).

A morte por apoptose possui duas vias principais: extrínseca e intrínseca. A via extrínseca é conhecida por ser a via ativada por receptores de morte que se associam com a pro-caspase-8. Nesse processo, ela é ativada e consequentemente, recruta e ativa as caspases efetoras 3 e 7, além de mediar a interligação das vias extrínseca e intrínseca, por induzir a ativação de Bax, membro da família Bcl-2, importante na via intrínseca (FULDA, 2014). Nesta via, também conhecida como mitocondrial, Bax induz a permeabilização da membrana da mitocôndria, em seguida, o Citocromo C ativa a pro-caspase-9 que ativa as caspases efetoras 3 e 7, ocorrendo então a apoptose (ELMORE, 2007).

Atrelado a isto, anoikis é uma morte celular programada induzida pela perda a longo prazo da adesão celular (mediada por integrina) à matriz extracelular. No contexto tumoral, comporta-se como um mecanismo crítico na prevenção do crescimento celular independente de aderência e na ligação a uma matriz inadequada, evitando assim a colonização de órgãos distantes. Porém este evento, a curto prazo, leva as células cancerígenas a inibirem a maquinaria de apoptose, desencadeando sinais pró-sobrevivência (KENIFIC; WITTMANN; DEBNATH, 2016; VLAHAKIS; DEBNATH, 2017).

Este evento protetivo celular, pode se dar por autofagia, um mecanismo que retarda a morte celular, permitindo que as células sobrevivam até encontrar um novo local para a adesão (GUADAMILLAS; CEREZO; DEL POZO, 2011; KIM et al., 2012). Este processo, refere-se ao mecanismo de degradação lisossomal, que existe em condições normais, essencial para a sobrevivência, desenvolvimento, diferenciação e homeostase celular (BADADANI, 2012; BAEHRECKE, 2005). No entanto, o papel da autofagia no câncer permanece controverso (DALBY K. et al.2010), pois foi descrito que a autofagia apoia a progressão do câncer através de sua ação pró-sobrevivência nas células (GUO JESSIE; XIA; WHITE, 2013). A autofagia é rigorosamente regulada por uma rede de cascatas de sinalização (TANIDA; UENO; KOMINAMI, 2008), sendo a proteína chave para este processo a cadeia leve 3 (LC3B), necessária para a montagem dos autofagossomos.

1.4 METALOPROTEASES

Metaloproteases de matriz (MMPs) são enzimas precursoras inativas e dependentes de zinco, caracterizadas pela presença de três histidinas e um zinco no motivo catalítico, seguidos de um resíduo metionina conservada (APTE; PARKS, 2015; KESSENBROCK; PLAKS; WERB, 2010). A formação das MMPs se dá por um peptídeo sinal, um pró-peptídeo e o domínio catalítico, além do domínio tipo-hemopexina que está presente em quase todas as MMPs. Sua ativação ocorre no momento da dissociação entre um resíduo de cisteína, presente no pró-domínio, e o zinco do domínio catalítico, dentro ou fora das células (KESSENBROCK; PLAKS; WERB, 2010).

No câncer, tanto suas funções catalíticas como não catalíticas podem promover a invasão celular e, normalmente, sua presença em alguns tipos de tumores malignos tem sido associada a um pior prognóstico. Em células de câncer de mama, foi comprovado que MMPs clivam as E-caderinas e integrinas, induzindo a EMT e aumentando a expressão de MMPs em eventos metastáticos (BROWN; MURRAY, 2015; BOURBOULIA E STETLER-STEVENSON, 2010; RADISKY E RADISKY, 2010).

Além de agir na EMT, as MMPs participam ativamente do processo de invasão celular, no qual, a integrina $\alpha_v\beta_3$ se co-localiza com a MMP-2 na frente migratória, realizando uma degradação da MEC, para a passagem celular. É proposto, que a forma ativa da MMP esteja ligada a integrinas na superfície de células endoteliais (VARTAK et al., 2010). Foi ainda demostrado, a ação das gelatinases, MMP-2 e 9, na interação com a integrina $\alpha_v\beta_3$ em melanoma *in vivo*, promovendo a invasão e em células de câncer de mama, induzindo a migração (BOURBOULIA & STETLER-STEVENSON, 2010).

1.5 MATRIZ EXTRACELULAR E INTEGRINAS

A MEC é responsável por fornecer suporte para a arquitetura celular e organização dos tecidos, através de uma estrutura composta por proteínas, glicosaminoglicanos, proteoglicanos e fatores de crescimento produzidos principalmente por fibroblastos e células endoteliais, (HARISI; JENEY, 2015; HYNES, 2011; HYNES; NABA, 2012; WORTHLEY; GIRAUD; WANG, 2010). A MEC tumoral influência o comportamento do tumor, agindo de forma a favorecer sua sobrevivência e crescimento, além de fornecer estrutura tridimensional, necessária para sustentação do tecido (NABA et al., 2014). As moléculas presentes na MEC são únicas, assim proteínas como a fibronectina, vitronectina e colágeno podem estar presentes em quantidades variáveis nos diferentes tumores (GOETZ, 2012; HARISI; JENEY, 2015; PICKUP; MOUW; WEAVER, 2014).

As integrinas são moléculas chave na adesão e migração celular. Atualmente, sabe-se da existência de 18 subunidades α e 8 subunidades β formando 24 heterodímeros diferentes (GINSBERG, 2014; HYNES, 2002; IWAMOTO; CALDERWOOD, 2015). A estrutura de cada subunidade se dá por uma porção extracelular longa (ligante de proteínas da MEC no meio extracelular), um domínio transmembrana e uma curta cauda citoplasmática, que se liga à quinases e diversas proteínas acessórias, regulando processos como migração, proliferação, sobrevivência celular e apoptose (Figura 2).

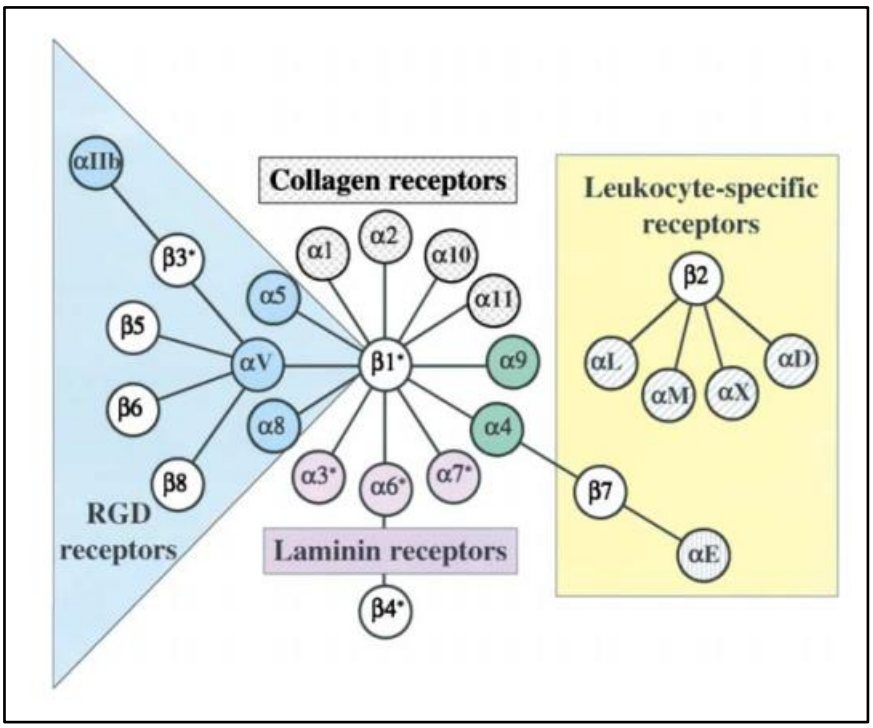

Figura 2. Representação das diversas famílias das integrinas. A associação direta entre as 8 subunidades β e as 18 subunidades α das integrinas, formam 24 heterodímeros funcionais que são divididos, de acordo com seus respectivos ligantes, em quatro grupos: receptores específicos de leucócitos, de colágeno, de laminina e de RGD. **Fonte:** HYNES, 2002.

Muito além de funções como de ancoragem, as integrinas por serem receptores, transduzem sinais para o meio intracelular ativando cascatas de sinalização, regulando a expressão de genes e mudanças no fenótipo das células (DESGROSELLIER; CHERESH, 2010; GINSBERG, 2014). Desta forma, a sinalização via integrinas pode ocorrer de forma bidirecional. Quando o heterodímero interage com uma proteína da MEC o sinal transmitido de fora para dentro é denominado de sinalização *outside-in* (de fora para dentro), enquanto que quando moléculas do meio intracelular interagem com determinada integrina, essa sinalização é denominada de *inside-out* (de dentro para fora).

Uma integrina pode ter afinidade por diferentes alvos e diferentes integrinas podem se ligar a um mesmo alvo, mas mediar funções específicas. As integrinas $\alpha_5\beta_1$ e $\alpha_v\beta_3$, neste contexto, são receptores clássicos que se ligam à sequência RGD presente na fibronectina, que estão envolvidas em funções diferentes relacionadas a motilidade celular sobre esta proteína (REEKS; FRY; ALEWOOD, 2015; DI CIO; GAUTROT, 2016; MISSIRLIS et al., 2016).

No câncer, a expressão de determinadas integrinas podem ser relacionadas a uma maior ou menor prognóstico (DESGROSELLIER; CHERESH, 2010; LAWSON; BURRIDGE, 2014). A integrina $\alpha_v\beta_3$ é mais expressa em células cancerígenas de mama do que em células normais e as subunidades α_3, α_5, α_6, α_v, β_1 e β_3 são mais expressas em células metastáticas sendo, portanto, indicadores de metástase (RATHINAM; ALAHARI, 2010).

1.6 METALOPROTEASES DE VENENO DE SERPENTES E DESINTEGRINAS

Dada a relevância das integrinas nos processos metastáticos, as desintegrinas, cujo alvo molecular são as integrinas, são inibidores nos processos de adesão, proliferação e na migração de células cancerígenas (MARCINKIEWICZ, 2013; REEKS; FRY; ALEWOOD, 2015). Estes inibidores são polipeptídios ricos em cisteínas, que contém de 40-100 aminoácidos e podem ser subdivididos de acordo com sua estrutura ou função.

Estas são derivadas de precursores proteoliticamente processados, denominados de metaloproteases de veneno de serpentes (SVMPs) (CALVETE et al., 2005), sendo tradicionalmente classificadas em três classes (P-I, P-II e P-III) de acordo com sua organização estrutural.

Os membros da classe P-I são compostos apenas pelo domínio MP. As proteínas pertencentes à classe P-II possuem o domínio MP e um domínio desintegrina adicional, e as proteínas de classe P-III possuem o domínio MP, um domínio desintegrina-símile e um domínio rico em cisteína, este último sendo substituído, em alguns casos, por um domínio lectina. Além disso, as classes P-II e P-III podem ser subdivididas de acordo com o processamento proteolítico de seus domínios e sua capacidade de formar estruturas diméricas (Figura 3) (CALVETE et al., 2005).

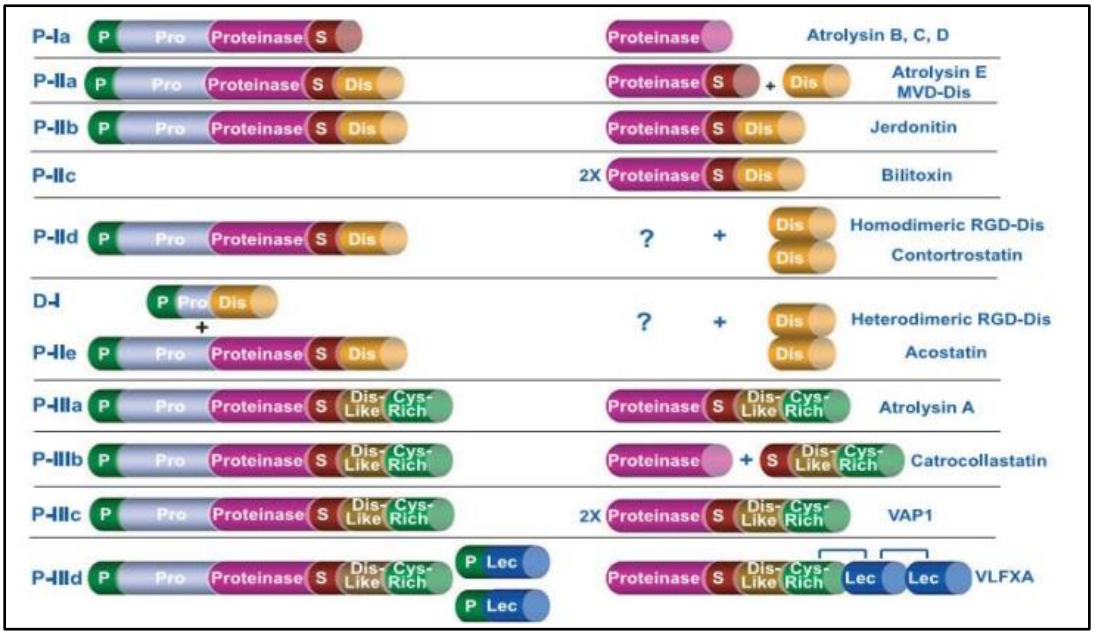

Figura 3. Classes de SVMPs. As SVMPs são sintetizadas como precursores multimodulares que sofrem diferentes estágios de processamento. P: pré-domínio, Pro: pró-domínio, Proteinase: domínio metaloproteinase, S: sequência de aminoácidos entre o domínio catalítico e desintegrina ou desintegrina-símile, Dis: desintegrina, Dis-Like: domínio desintegrina-símile, Cys-Rich: domínio rico em cisteínas, Lec: domínio ligante de lectina. **Fonte:** FOX; SERRANO, 2008.

As desintegrinas podem exercer sua ação assumindo estruturas monoméricas ou diméricas (homodiméricas ou heterodiméricas) dependendo da classe e do tipo de processamento das SVMPs. Sua função específica se relaciona à sequência tripeptídica responsável pelas interações no sítio de ligação das integrinas. Estas sequências tripeptídicas estão presentes na forma de um *loop*. De acordo com a sequência a desintegrina possui uma classificação, sendo: desintegrina RGD (–Arg-Gly-Asp-), KTS (-Lys-Thr-Ser-) ou MLD (–Met-Leu-Asp-) (CALVETE et al., 2005; MARCINKIEWICZ, 2013).

Além da sequência adesiva, a configuração correta das pontes dissulfeto também são fatores críticos para a atividade biológica das desintegrinas, visto que são responsáveis pela estrutura tridimensional assumida pela molécula (MCLANE et al., 1998; REEKS; FRY; ALEWOOD, 2015). O resíduo de aspartato (R) conservado em diversos motivos (RGD, KGD, MGD, VGD, WGD e MLD) pode ser o responsável pela interação da desintegrina com a subunidade β da integrina, enquanto o resíduo presente no N-terminal do motivo seria o responsável por influenciar a especificidade da ligação com a subunidade α da integrina (CALVETE, 2013; REEKS; FRY; ALEWOOD, 2015).

O motivo RGD foi o primeiro a ser descoberto e está presente na maior parte das desintegrinas monoméricas (CALVETE et al., 2003). Esse tripeptídeo possui diferentes afinidades e especificidades pelas integrinas $\alpha_v\beta_3$, $\alpha_5\beta_1$, $\alpha IIb\beta_3$, $\alpha_8\beta_1$ e $\alpha_v\beta_1$, mas

principalmente pelas integrinas $\alpha_v\beta_3$, $\alpha_5\beta_1$ e $\alpha IIb\beta_3$ que são os receptores de vitronectina, fibronectina e fibrinogênio, respectivamente. A afinidade destas moléculas às integrinas, é elevada, sendo necessárias baixas concentrações, na escala nanomolar, para a visualização dos efeitos biológicos *in vitro* e micromolar para testes *in vivo* (SELISTRE-DE-ARAUJO et al., 2010).

Recentemente, uma molécula sintetizada na década de 90, a partir do motivo RGD das desintegrinas de venenos de serpentes, chegou à fase III de testes clínicos para o tratamento de glioblastoma. O cilengitide é um pentapeptídeo cíclico, que com seu motivo RGD, possui atividade antiangiogênica e o primeiro medicamento tendo como alvos as integrinas $\alpha_v\beta_3$, $\alpha_5\beta_1$ e $\alpha_v\beta_5$. Ele vem sendo testado em conjunto com radioterapia, porém os resultados não foram melhores do que os obtidos com drogas normalmente utilizadas. (MAS-MORUNO et al., 2010; GOODMAN; PICARD, 2012; WELLER et al., 2016).

1.7 DIS*Ba*-01 (Desintegrina de *Bothrops alternatus*)

A Dis*Ba*-01, é uma desintegrina recombinante RGD, monomérica, média, sendo composta por 78 aminoácidos e 6 ligações dissulfeto (12 cisteínas). Ela foi produzida a partir de uma biblioteca de cDNA construída com o RNAm extraído da glândula venenífera de uma única serpente do gênero *Bothrops alternatus*. Sua massa molecular é de 11,780 Da, entretanto, nele está inclusa a cauda de histidina, composta por 6 histidinas, adicionada à sequência com o objetivo de facilitar o processo de purificação da proteína.

Ramos e colaboradores, em 2008, por ressonância plasmônica, demostraram uma possível afinidade desta desintegrina pela subunidade β_3 (K_d: $1{,}6.10^{-7}$ M) das integrinas. Estes resultados, foram confirmados recentemente, onde foi demostrado, que a Dis*Ba*- 01 possui alta afinidade pela integrina $\alpha_v\beta_3$ (K_d: $4{,}63.10^{-7}$ M) e menos especificidade pela integrina $\alpha_5\beta_1$ (K_d: $7{,}62.10^{-5}$ M), assim sugerindo que a Dis*Ba*-01 é 100 vezes mais específica pela integrina $\alpha_V\beta_3$ do que $\alpha_5\beta_1$ (MONTENEGRO et al., 2017).

Em 2008, Kauskot e colaboradores, mostraram que além de inibir fortemente a agregação plaquetária e prolongar o tempo de sangramento na cauda de camundongos, a Dis*Ba*-01, também inibiu a fosforilação da FAK em plaquetas humanas (KAUSKOT et al., 2008). Além disto, a Dis*Ba*-01 foi capaz de inibir fortemente a adesão e a proliferação de células de melanoma murino B16F10 e HMEC-1 (IC_{50} = 225 e 555 nM, respectivamente) plaqueadas sobre vitronectina. Experimentos *in vivo* realizados em camundongos demonstraram uma diminuição significativa da formação de novos vasos induzida por bFGF (*basic Fibroblast Growth Factor*). Além disso, camundongos tratados com a desintegrina

tiveram uma redução de 69,8 ± 11,7 % na colonização dos pulmões por células de melanoma injetadas (RAMOS et al., 2008).

A Dis*Ba*-01 já demostrou ser eficaz em diminuir a expressão do VEGF (*Vascular Endothelial Growth Factor*) e seus receptores VEGFR1 e VEGFR2 (*Vascular Endotelial Growth Factor Receptor*) em células HMEC-1 e a expressão do VEGF em fibroblastos e MDA-MB-231 nos tempos de 24 e 48 horas, respectivamente. Além disto, aumentou a atividade enzimática de MMP-9 na MDA-MB-231 e diminuiu MMP-2 em fibroblastos (MONTENEGRO et al., 2012), bem como inibiu a migração em células de câncer de mama, próstata e fibroblastos, em ensaios de migração empregando o sistema de *transwell* (SELISTRE-DE-ARAUJO et al., 2010).

Como resultado recente de um trabalho de doutorado do nosso grupo de pesquisa, foi demostrado que a Dis*Ba*-01, a partir de ensaio *time-lapse* em células de carcinoma oral, promoveu diminuição da direcionalidade e migração celular, além de ser capaz de aumentar a maturação das adesões focais (paxicilina), da área destas adesões e da protrusão celular. Estes efeitos foram correlacionados com a integrina β_3, pois quando havia a superexpressão desta integrina, os resultados supracitados eram suprimidos (MONTENEGRO et al., 2017).

2. JUSTIFICATIVA

O presente estudo se torna relevante, devido ao impacto social, econômico e político que o câncer acarreta à sociedade contemporânea, já que é a segunda causa de óbitos na população brasileira, apesar dos avanços no diagnóstico, técnicas cirúrgicas e tratamentos (INCA, 2016).

A integrina $\alpha_v\beta_3$, neste contexto, passou a ser amplamente estudada devido a sua participação em eventos cruciais no avanço do estudo contra o câncer, sendo considerada um alvo terapêutico interessante (BELVISI et al., 2005; SCARINGI et al., 2013). Além disto, os processos de adesão e migração celular que são etapas essenciais no câncer, se tornam também alvos atraentes para estudos, já que bloqueadores destas funções limitariam a progressão tumoral (WELLS et al., 2014).

Além disto, estudos recentes mostraram que a perda a longo prazo da adesão celular mediada por integrina leva a Anoikis, um tipo específico de morte celular apoptótica. No entanto, por curto período, as células podem ser protegidas contra a apoptose promovendo autofagia, como um mecanismo para retardar a morte celular, permitindo que as células sobrevivam até encontrar um novo local para a adesão (KENIFIC; WITTMANN; DEBNATH, 2016; VLAHAKIS; DEBNATH, 2017). Este mecanismo se torna importante para as células tumorais que precisam sobreviver por períodos mais longos em comparação com as células normais e, assim, promoverem metástases (GUADAMILLAS; CEREZO; DEL POZO, 2011; KIM et al., 2012).

Portanto, tendo em vista este contexto e o potencial terapêutico de bloqueadores de receptores de adesão tais como as desintegrinas, faz-se necessário aprofundar o conhecimento sobre as consequências do bloqueio da integrina $\alpha_v\beta_3$ a nível molecular.

3. HIPÓTESE

O bloqueio da integrina $\alpha_v\beta_3$ promovido pela desintegrina Dis*Ba*-01, inibe a invasão e a adesão celular, além de diminuir a proliferação de células tumorais de mama *in vitro*. Ademais, as células frente à vigência da morte programada ocasionada por este bloqueio, promovem autofagia como mecanismo compensatório e protetivo contra a apoptose.

4. OBJETIVOS

Determinar os efeitos do bloqueio da integrina $\alpha_v\beta_3$ pela Dis*Ba*-01 sobre células tumorais de mama, investigando a influência deste bloqueio nas etapas principais da cascata metastática *in vitro*, assim como na resistência das células tumorais à morte celular programada.

Objetivos Específicos

- Avaliar os efeitos *in vitro* do bloqueio da integrina $\alpha_v\beta_3$ pela Dis*Ba*-01 na proliferação, invasão e adesão de células normais e tumorais de mama sobre componentes da MEC: fibronectina e vitronectina;

- Avaliar a atividade de metaloproteases (MMP-2 e MMP-9) de células normais e tumorais de mama durante o processo de migração celular;

- Verificar a morfologia das células normais e tumorais tratadas com Dis*Ba*-01 através de técnicas de microscopia de fluorescência e eletrônica;

- Avaliar a influência do bloqueio da integrina $\alpha_v\beta_3$ pela Dis*Ba*-01, no ciclo celular e na indução da apoptose por Anoikis;

- Verificar os mecanismos autofágicos envolvidos na proteção celular frente a morte programada em células tumorais de mama.

Os resultados e a discussão deste trabalho serão apresentados na forma de artigo científico.

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6. MANUSCRITO

BLOCKING $\alpha_v\beta_3$ INHIBITS APOPTOSIS BY INDUCING AUTOPHAGY IN MURINE BREAST TUMOR CELLS

Rafael Luis Bressani Lino[a]; Patty Karina dos Santos[a]; Graziéle Fernanda Deriggi Pisani[a]; Wanessa Fernanda Altei[a]; Normand Pouliot[b]; Marcia Regina Cominetti[c]; Heloisa Sobreiro Selistre-de-Araújo[a‡].

[a]Department of Physiological Sciences, Center of Biological and Health Science, Federal University of São Carlos, Rod. Washington Luis, Km 235-SP-310-São Carlos, São Paulo, Brazil, CEP 13.565-905.

[b]Matrix Microenvironment and Metastasis Laboratory, Olivia Newton-John Cancer Research Institute, School of Cancer Medicine, La Trobe University, Melbourne, Australia.

[c]Department of Gerontology, Center of Biological and Health Science, Federal University of São Carlos, Rod. Washington Luis, Km 235-SP-310-São Carlos, São Paulo, Brazil, CEP 13.565-905.

‡Corresponding author: Heloísa Sobreiro Selistre-de-Araújo
E-mail: hsaraujo@ufscar.br. orcid.org/0000-0002-2372-7814.

ABSTRACT

Integrins are key receptors that mediate cell adhesion to the extracellular matrix (ECM) and regulate cell behavior including cell migration. Particularly, $\alpha_v\beta_3$ integrin, the vitronectin receptor, plays a key role in both physiological angiogenesis and in tumor progression. This receptor recognizes the arginine-glycine-aspartic acid (RGD) motif in ECM proteins and it can be antagonized by RGD-peptides, resulting in decreased cell migration and invasion. RGD-based drugs have shown disappointing results in clinical trials; however, the reasons for their lack of activity are still obscure. Aiming to contribute to a better understanding on the molecular consequences of integrin inhibition, we have tested a recombinant RGD-disintegrin (Dis*Ba*-01) in two types of murine cell lines, breast tumor 4T1BM2 cells and L929 fibroblasts. Only tumor cells showed decreased motility and adhesion as well as morphologic alterations upon Dis*Ba*-01 treatment. This result was attributed to the higher levels of $\alpha_v\beta_3$ in 4T1BM2 cells making them more sensitive to Dis*Ba*-01 blocking. Flow cytometry analysis showed that Dis*Ba*-01 induced cell cycle arrest at S phase in 4T1BM2 cells, but did not induce apoptosis, which was consistent with the decrease in caspase-3, 8 and 9 expression at mRNA and protein levels. It was also observed that this disintegrin increases LC3B expression in both cell lines, an indicator of autophagic induction. In conclusion, Dis*Ba*-01 specificity towards 4T1BM2 cells when compared to L929, could be due to the higher level of expression of α_v and β_3 integrins in these cells, and these observations suggest new insights on the effects of RGD-based inhibitors considering their importance on drug development for human health.

Keywords: breast cancer**,** $\alpha_v\beta_3$ integrin, MMP-2, cell adhesion, anoikis, apoptosis, autophagy, RGD-disintegrin.

INTRODUCTION

Cell adhesion and migration are key processes for cancer progression and metastasis development [1]. During tumor progression, cancer cells must detach from primary tumor and partially degrade the extracellular matrix (ECM) to allow migration towards the blood and lymphatic vessels. After extravasation, tumor cells must adhere to the ECM at the new site before starting proliferation. Throughout the steps for tumor progression, cells are constantly interacting with the ECM through surface receptors including the integrins [1].

The integrin family comprises 19 α subunits and 8 β subunits, which associate in 24 different combinations to form distinct ECM heterodimeric transmembrane receptors [2–4]. Integrins mediate cell adhesion by connecting the cell cytoskeleton to a variety of extracellular matrix proteins including fibronectin (FN), collagen and vitronectin (VN). Beyond their role as adhesion receptors, integrins regulate a great diversity of cellular properties including cell migration, survival, and proliferation by triggering a set of intracellular signaling pathways [5].

Integrin receptor clustering at cell surface is crucial for focal adhesion formation, with association of a number of signaling accessory proteins that mediate ECM connections with the cytoskeleton [3]. Conversely, long-term loss of integrin mediated cell adhesion leads to anoikis, a specific type of apoptotic cell death induced by cell detachment. However, in a short-term period, cells can be protected from apoptosis by promoting autophagy, as a mechanism to delay cell death, allowing cells to survive until they find a new site for adhesion [6, 7]. This is important for tumor cells that need to survive for longer periods compared to normal cells and thus promote metastasis. Therefore, anoikis resistance has been suggested as a key process for metastasis development [8, 9].

The $\alpha_v\beta_3$ integrin, first described as the vitronectin receptor, has been strongly related to tumor development and progression in many types of human cancers. Additionally, $\alpha_v\beta_3$ has a key role in physiological and tumor angiogenesis, an important contributor to tumor progression [10, 11]. Activation of this integrin upon ligand binding triggers signaling pathways that cross-talk with those activated by the vascular endothelial growth factor/vascular endothelial growth factor receptor (VEGF/VEGFR) axis, resulting in angiogenesis promotion [12–14].

Therefore, $\alpha_v\beta_3$ integrin became an important target for pharmacological inhibition of tumor angiogenesis. In addition, this integrin regulates the activity of matrix metalloprotease-2 (MMP-2) by association to membrane type 1-MMP (MT1-MMP). MMP-2 has an important role in tumor progression due to its ability to digest type IV collagen in the basement

membranes thus facilitating endothelial cell migration [15]. Furthermore, enhanced MMP-2 was also correlated to tumor metastasis [16, 17].

Once $\alpha_v\beta_3$ integrin recognizes the tripeptide sequence arginine-glycine-aspartic acid (RGD) in several ECM proteins including VN and FN. This property was the basis for the design of RGD-peptides and peptidomimetics for specific inhibition of this integrin [18]. Cilengitide is a cyclic RGD pentapeptide currently in a number of clinical trials against some types of tumors. Despite of the high levels of $\alpha_v\beta_3$ integrin expression in glioblastoma, cilengitide has failed to increase survival in a phase III study in patients newly diagnosed with this type of tumor. The reasons for the lack of cilengitide effect are still unknown, but unfavorable pharmacokinetics, heterogeneous responses and insufficient knowledge on the biological effects of the drug on tumor vasculature were suggested [19].

Snake venoms are rich sources of Cys-rich, integrin-binding proteins named disintegrins. A great number of disintegrins has the RGD motif, being effective inhibitors of $\alpha_v\beta_3$ integrin at nanomolar range. There is a large structural and functional diversity among snake venom disintegrins, which are products from proteolysis of larger precursor proteins having a metalloprotease domain [20]. Post-translational modifications also contribute to this structural diversity generating monomeric, homodimeric or heterodimeric disintegrins with natural variations of the adhesive RGD motifs such as WGD, KTS and MLD [21]. This diversity gave rise to molecules targeting different integrins such as R/KTS- disintegrins that bind to $\alpha_1\beta_1$ integrin leading to inhibition of cell adhesion to collagen I and IV [22, 23]. As a result, many researchers have used disintegrins as tools in pre-clinical studies in order to understand the role of specific integrins in the pathogenesis of several diseases including cancer [24].

We have previously demonstrated that Dis*Ba*-01, a recombinant RGD-disintegrin from *Bothrops alternatus* venom, binds to and blocks $\alpha_v\beta_3$ integrin inhibiting cell migration, MMP-2 activity, angiogenesis and experimental metastasis [25–28]. Dis*Ba*-01 also inhibited the expression of VEGFR in endothelial cells [25]. In addition to inhibition of cell migration, oral carcinoma cells treated with Dis*Ba*-01 have lost cell directionality [26]. In this study, we show that this disintegrin does not induce cell death by interference with anchorage (anoikis). Instead, cells were protected from death by entering a program of autophagy, which is crucial for cell survival in a short-term period. These observations suggest new insights on the effects of RGD-based inhibitors considering their importance on drug development for human health.

MATERIALS AND METHODS

Dis*Ba*-01 expression, purification and characterization

Recombinant disintegrin Dis*Ba*-01, a His-tag protein (GenBank, Accession: AY259516) was obtained from a cDNA venom gland library of *Bothrops alternatus*, accordingly to a previously described protocol [27] and slightly modified. After transformation of the *Escherichia coli* BL21 (DE3) strains using the pDis*Ba*-01 (pET28-a) plasmid, expression assays were performed. Briefly, cell lysate was purified in a three-step process, until a highly purified preparation was achieved. Affinity chromatography (HIS-Select® Nickel Affinity Gel, *Sigma-Aldrich*®) was performed, followed by size-exclusion chromatography (Superdex 75 10/300 GL, *GE Healthcare*) on a FPLC (Fast performance liquid chromatography) system (ÄKTA Start, *GE Healthcare*). Remaining contaminated Dis*Ba*-01 samples were purified using an anion exchange column (Mono-Q 5/50 GL, *GE Healthcare*) on a FPLC chromatography (ÄKTA Start, *GE Healthcare*). Total protein was determined by colorimetric detection of bicinchoninic acid assay (Pierce™ BCA Protein Assay, *Thermo Scientific*, Catalog Number: 23225).

Cell Culture

4T1BM2 cells line is a triple negative murine breast cancer lineage, derived from parental 4T1 cells, with high metastatic activity for bone tissue (provided by Matrix Microenvironment & Metastasis Laboratory, Dr. Normand Pouliot / Olivia Newton - John Cancer Research Institute, Melbourne, Australia) [29]. L929 lineage (ATCC® - CCL-1™), a murine fibroblast cell, was used as control of all experiments. 4T1BM2 cells were cultured in α-MEM medium, pH 7.4 containing 5 % of fetal bovine serum (FBS), penicillin (100 U / mL), streptomycin (100 µg / mL), sodium bicarbonate and sodium chloride. L929 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), pH 7.4 containing 10 % of fetal bovine serum (FBS), L-glutamine, penicillin (100 U / mL), streptomycin (100 µg / mL) and antifungal (amphotericin B). Cell cultures were kept in an incubator with 5 % CO_2 at 37 °C.

Flow Cytometry Analysis

To verify the presence of α_v and β_3 integrin subunits in 4T1BM2 and L929 cells by flow cytometry, 1 x 10^6 cells were incubated with the following primary antibodies: anti-integrin β_3 – 1:200 (*Abcam*, Ab7167) and anti-integrin α_v – 1 µg/1x10^6 cells (*Santa Cruz* - SC, 9969), for 1h at 4 °C. After washing by PBS (Phosphate Buffered Saline) (1x), cells were centrifuged at 4 °C for 10 minutes at 150 g. Goat anti-mouse FITC IgG - 1:1000 (*Abcam*, 6785) was used as a secondary antibody and incubated in the dark at 4 °C for 45 minutes.

Once again, cells were washed with PBS (1x) and centrifuged. Subsequently, both cell lines were analysed in a flow cytometer (BD Accuri™ C6, BD *Biosciences*).

Cell Adhesion Assay

To investigate the inhibition of fibronectin and vitronectin adhesion [25], well bottoms from a black plate (96 wells) (*Greiner bio-one*, Catalog number: 655090) were individually covered with 2 μg/cm^2 of fibronectin or 0,5 μg/cm^2 of vitronectin solution (*Sigma-Aldrich*®, Catalog number: F4759 and V8379, respectively). Bovine serum albumin (BSA, in a final concentration of 2 %) was added in the negative control well. Coating was incubated overnight at 4 ºC. After this period, wells were blocked with 1% BSA for 1h in an incubator with 5% CO_2, followed by washing with PBS (1x). Concomitantly, 1 x 10^5 of L929 and 4T1BM2 cells were treated with Dis*Ba*-01 (1, 10, 100 and 1000 nM). Immediately after the treatment, cells were seeded in their respective wells and incubated at 37 °C for 30 min. Thereafter, the supernatant was removed and the wells were washed with PBS (1x). Sample fixation was performed with 4% of paraformaldehyde solution (pH 7.5) for 10 min at room temperature and excess was removed by washing with PBS (1x). Cells were stained with DAPI (0.7 ng / μL) following the manufacturer's instructions and counted on an automatic fluorescence microscope system (ImageXpress® Micro, *Molecular Devices*).

Cell Invasion Assay

Establishing how the recombinant protein Dis*Ba*-01 influences the ability of 4T1BM2 and L929 cells to invade matrigel was the goal of this assay [18]. In this procedure was used a 24-well plate (*Corning*® Biocoat™ Matrigel® Invasion Chamber, Catalog number: 354480). DMEM or α-MEM medium supplemented with FBS was pipetted into the wells, except on the negative control where serum-free medium was pipetted. 4T1BM2 and L929 (1 x 10^5 cells) were treated with 1, 10, 100 and 1000 nM of Dis*Ba*-01 on serum-free medium for 30 minutes. After that, the membrane-containing chamber was added to the well whilst treated cells were being pipetted. The invasion assay occurred for 6 hours at 37 ºC. The chamber membrane was fixed in 4% paraformaldehyde and cell nucleus was coloured using DAPI. Membranes were assembled in slides using Vectashield® mounting media (*Vector Laboratories*, Catalog Number: 101098-042) and cell counting was performed on an automated fluorescence microscope system (ImageXpress® Micro, *Molecular Devices*).

Gelatin Zymography

MMP activity was analyzed by gelatin zymography on a Wound Healing assay [25]. L929 and 4T1BM2 ($5x10^4$ cells) were seeded in 12 well plates, grown at 37 °C until confluency and scratched with a plastic tip. Confluent monolayers were washed with the appropriate medium to remove cell debris, and Dis*Ba*-01 (100 nM and 1000 nM) in serum-free medium was added to the wells. After 24 hours, culture medium was collected, centrifuged at 10,000 g for 5 min at 4 °C and incubated with sample buffer without β-mercaptoethanol. The samples were resolved at 4 °C in a 10 % polyacrylamide gel containing 0.1% gelatin. The gel was washed (2x) with 2.5% Triton X-100 and incubated at 37 ºC during 18h in 50 mM Tris buffer pH 8.0, containing 5 mM $CaCl_2$, 0.02 % NaN_3 and 10 mM $ZnCl_2$. After staining with Coomassie Blue R-250 and destaining with acetic acid:methanol:water (1:4:5), the clear bands were visualized on a molecular imager (ChemiDoc™ XRS, *Bio-Rad*) and quantified by densitometry using the Image J 1.48v program.

Immunostaining

L929 and 4T1BM2 (1×10^4 cells) were plated in a black plate (96 wells) (*Greiner bio-one*, Catalog number: 655090) and incubated overnight at 37 °C, 5 % CO_2. After this period, cells were treated with Dis*Ba*-01 (100 and 1000 nM), Cytochalasin D (1000 nM) (potent inhibitor of actin polymerization) for 6h [30] or Rapamycin (500 nM) (autophagic promoter) for 24h [31]. Then, cells were fixed in 3.7% paraformaldehyde for 30 min, followed by permeabilization using 0.1 % Triton X-100 for 5 min. Glycine (100 nM) was added to each well in order to block free aldehydes. Wells were washed with PBS (3 times, 5 min each), succeeded by 30 min of incubation in 2 % PBS-BSA to block unspecific sites. Cells were stained with Alexa Fluor® 488 Phalloidin, a high-affinity filamentous actin (F-actin) dye (*Thermo Fisher Scientific*, Catalog Number: 12379) or Targeted anti-LC3B primary antibody - 1:2000 (*Abcam*, Ab51520) was centrifuged at 10.000 g for 1 h at 4 °C and pipetted into the wells. After this period, secondary antibody goat anti-Rabbit APC IgG - 1:1000 (*Abcam*, 130805) were mixed in 2 % PBS-BSA and centrifuged at 10.000 g, 1 h at 4 °C before be applied on the wells. After incubation, wells were cleansed and stained with 0.7 µg/mL of DAPI for 10 min. After washing, observed on an automated fluorescence microscope system with a 60x objective (ImageXpress® Micro, *Molecular Devices*).

Morphological analysis by Scanning Electron Microscopy

L929 and 4T1BM2 ($1x10^4$) cells were plated in Lab-TeK® chamber slide™ system (LOBOV, Catalog Number: 177402) overnight at 37 ºC for adhesion. Cells were treated with

1000 nM Dis*Ba*-01 for 6 hours. After that, cells were washed with PBS at 37 ºC and fixed in 4 % glutaraldehyde (*Sigma-Aldrich*®, Catalog number: 7651) for 1h at 37 ºC. Cells dehydration was performed by incubation in increasing concentrations of ethanol (50, 60, 70, 80, 90 and 100 %, 10 min for each step) before drying with hexamethyldisilazane (*Sigma-Aldrich*®, Catalog number: 440191) [32]. Cellular morphology was characterized using a Scanning Electron Microscopy (Inspect F50 - FEI®).

Anoikis-Resistance assay

4T1BM2 cells were incubated with different concentrations of Dis*Ba*-01 (100 nM and 1000 nM) and maintained under continuous agitation for 0, 1, 3, 6, 12 and 24h. In these specific times, cells in low density (50 cells) were plated in 6 cm dishes and incubated overnight at 37 °C. Colonies (> 50 cells) formed after 7 days at 37 ºC were fixed with methanol, stained with a solution of crystal violet for 15 min, photographed and counted (number and size) using ImageJ 1.52a program.

Proliferation assay

Dis*Ba*-01 effect on proliferation was determined using the Alamar Blue assay. 96 well-plates (*Greiner bio-one*, Catalog number: 655180) were individually covered with 2 µg/cm^2 of fibronectin or 0,5 µg/cm^2 of vitronectin solution (*Sigma-Aldrich*®, Catalog number: F4759 and V8379, respectively) and incubated overnight at 4 ºC. After this period, 4T1BM2 and L929 cells (5 x 10^5) were seeded in the wells coated with vitronectin or fibronectin and allowed to adhere for 8 hours at 37 °C. Then, cells were treated with Dis*Ba*-01 (1 nM, 10 nM, 100 nM and 1000 nM) plus AlamarBlue™ Cell Viability Reagent (*Invitrogen*, Catalog number: DAL1100) during 5 days and fluorescence was measured in a spectrophotometry equipment every 24 hours (555 nm excitation and 590 nm emission) (SpectraMax i3x, *Molecular Devices*).

Cell Cycle Analysis

L929 and 4T1BM2 (1x10^6 cells) were seeded in 6 cm Petri dishes and incubated for 24 h at 37 ºC, 5 % CO_2 [33]. Cells were treated with 100 and 1000 nM of Dis*Ba*-01 following incubation for 24 h. Then, cells were detached and pelleted by centrifugation, washed with cold PBS, fixed in 70% of cold ethanol and stored for 24 h at -20 °C. Fixed cells were incubated with RNase A (0.2 mg/mL) (*Sigma-Aldrich*®, Catalog Number: 6513) at 37 °C for 30 min and stained with propidium iodide (PI) (1 µg/mL) (*Sigma-Aldrich*®, Catalog Number:

P4864). DNA content of cells (15.000 events) was analyzed by flow cytometry (BD Accuri™ C6, BD *Biosciences*).

Apoptosis Assay

The apoptotic activity of Dis*Ba*-01 on L929 and 4T1BM2 cells was analyzed by flow cytometry with the PE-Annexin V Apoptosis Detection Kit (BD *Biosciences*, Catalog Number: 559763) [33]. Cells ($1x10^5$) were seeded in 24 well plates with DMEM or α-MEM medium and incubated for 24 h. After this period, the medium was removed and cells were incubated or not with different concentrations of Dis*Ba*-01 for 24 h at 37 ºC and 5 % CO_2. Cells were harvested, centrifuged, washed twice with cold PBS and resuspended in binding buffer. Then, cells were incubated with PE-Annexin V and 7ADD for 15 min in the dark at room temperature, followed by addition of binding buffer. Analyses occurred in a flow cytometer (BD Accuri™ C6, BD *Biosciences*).

Gene Expression Analysis by qRT-PCR

For RNA extraction were used $1x10^6$ cells seeded in a 6-well plate containing DMEM or α-MEM medium supplemented with 10 % FBS for 24 h. L929 and 4T1BM2 cells were treated or not with Dis*Ba*-01 following incubation during 24 h. After this period, RNA extraction was performed using TRIZOL reagent (*Invitrogen Corporation*, Catalog Number: 15596026) according to the manufacturer's protocol. The RNA pellet was resuspended in nuclease-free water and stored at -80 ºC. The concentration and purity (260/280 nm and 260/230 nm ratios) of RNA were measured by Nanodrop 2000 (*Thermo Scientific*). Subsequently, 1 µg of total RNA from each sample was treated with deoxyribonuclease I (DNAse I; *Invitrogen Corporation*, Catalog Number: 18047019) according to the manufacturer's instructions in order to remove genomic DNA. Treated RNA was reverse-transcribed into cDNA with iScript™ cDNA Synthesis Kit (*BioRad Laboratories*, Catalog Number: 1708890) according to manufacturer's instructions. The CFX 96 real-time PCR detection system (*Bio-Rad*) was used for qPCR reaction. For each reaction, 10 ng of cDNA, 200 nM of each primer and 5 µL of SsoFast™ Evagreen Supermix (*Bio-Rad*, Catalog Number: 172-5200) were used in a total volume of 10 µL per reaction. Gene specific primers purchased from *Sigma-Aldrich*® are listed in Table 1. Thermal cycling program was performed for 10 min at 95 ºC, followed by 40 cycles of 15 s at 95 ºC, 30 s at 60 ºC and 30 s at 72 ºC. The melting curve was analyzed to confirm the specificity of the amplification products. The relative expression of the qRT-PCR products was determined through the ΔΔCt

method and calculated using the following equation: fold induction = $2^{-\Delta\Delta Ct}$ [34]. Ribosomal protein S27a (RPS27A) was used as a housekeeping gene.

Table 1. List of primers used for qRT-PCR.

Genes	Sense primer (5'-3')	Antisense primer (3'-5')	Source
Caspase-3	CATAAGAGCACTGGAATGTC	GCTCCTTTTGCTATGATCTTC	Sigma Aldrich
Caspase-8	CAAGAGAACAAGACAGTGAG	ACTCAGAGCCTCTTTATCAC	Sigma Aldrich
Caspase-9	GACCCTTACGGGGAAAACCAT	AGACAAAGTCCGGCCATCTTC	Sigma Aldrich
MMP-2	GAGATCTTCTTCTTCAAGGAC	AATAGACCCAGTACTCATTCC	Sigma Aldrich
MMP-9	CTTCCAGTACCAAGACAAAG	ACCTTGTTCACCTCATTTTG	Sigma Aldrich
RPS27A	GACCCTTACGGGGAAAACCAT	AGACAAAGTCCGGCCATCTTC	Sigma Aldrich

Western Blotting

For western blotting analysis, 4T1BM2 and L929 cells were lysed using 100 µL of lysis buffer (50 mM Tris-HCl pH 7.4, 150 nM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1% Tween 20, 0.25% sodium deoxycholate, 0.1 mM phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin and 1 µg/mL leupeptin). Cell lysates were centrifuged at 14000 g for 20 min at 4 °C. Supernatant protein content was determined by a Pierce™ BCA Protein Assay Kit (*Thermo Fisher Scientific*, Catalog Number: 23225). Cellular proteins (30 µg) were separated on a 20% SDS-PAGE, transferred to 0.45 µm nitrocellulose membranes (*Bio-Rad*, Catalog Number: 1939) and blocked with Tween-TBS buffer (140 mM NaCl, 2.6 mM KCl, 25 mM Tris, pH 7.4, 0.05% Tween 20) plus 5% powdered milk. Western Blotting was performed using the following antibodies: anti-Integrin β_3 - 1:200 (*Abcam*, Ab7167), anti-Integrin α_v – 1:1000 (*Santa Cruz* - SC, 9969), anti-Caspase 3 – 1:1000 (*Abcam*, 179517) and anti-LC3B - 1:3000 (*Abcam*, Ab51520). Anti-GAPDH – 1:10000 (*Abcam*, Ab181602) or anti-β-actin – 1:1000 (*Abcam*, Ab8227) were used as a loading control and Goat anti-mouse IgG HRP - 1:5000 (*Abcam*, Ab97040) or Goat anti-rabbit IgG HRP - 1:5000 (*Abcam*, Ab97051) were used as secondary antibodies. Bands were visualized on a molecular imager (ChemiDoc™ XRS, *Bio-Rad*) after development of chemiluminescence using Clarity™ Western ECL Substrate (*Bio-Rad*, Catalog Number: 1705061).

Statistical analysis

Data were analyzed using GraphPad Prism 5.01 for Windows. The results were compared statistically using One or Two-way Analysis of Variance (ANOVA) and Bonferroni's statistical test was used when p level were $*p < 0.05$.

RESULTS

4T1BM2 and L929 cell lines have distinct integrin profiles

In order to compare the disintegrin effects in 4T1BM2 and L929 cells, we analyzed the profile of α_v and β_3 integrin subunits in both cell lines using flow cytometry and western blotting. The results indicated similar amounts of β_3 subunits in the two cell lines, while α_v was observed only in 4T1BM2 cells at a moderate level (Figure 1A and 1B). Since Dis*Ba*-01 binds to $\alpha_v\beta_3$ integrin, these results have demonstrated that these cell lines comprise a valuable model for comparison of disintegrin effects.

Dis*Ba*-01 inhibits adhesion and invasion of 4T1BM2 cells but not of L929 cells

RGD disintegrins are known to inhibit cell adhesion and invasion due to integrin blocking [20-24]. All tested concentrations of Dis*Ba*-01 (1-1000 nM) inhibited matrigel invasion of 4T1BM2 cells without affecting L929 (Figure 1C). In addition, Dis*Ba*-01 (1-1000 nM) strongly inhibited the adhesion of 4T1BM2 cells to both vitronectin and fibronectin (Figure 1D). L929 cells adhesion was affected only when vitronectin was used as substrate.

Dis*Ba*-01 inhibits MMP-2 synthesis but increases MMP-9 activity in tumor cells

To achieve a better understanding on the effects of integrin blockage during migration, we analyzed, by Gelatin Zymography, the activities of MMP-2 and MMP-9 in the conditioned medium of cells obtained from wound healing assays. Dis*Ba*-01 (100 and 1000 nM) inhibited MMP-2 synthesis in 4T1BM2 cells as demonstrated by the lower levels of proMMP-2 in zymographic gels and by qRT-PCR (Figure 2Aa, 2Bb and 2Cb). No effects were observed on the amount of intermediate or active forms of MMP-2 (Figure 2Ab-c) in this cell line. The inhibitory effect on MMP-2 expression was also evident in L929 cells but only with 1000 nM of Dis*Ba*-01 (Figure 2Af, 2Ba and 2Ca). MMP-9 activity levels in tumor cells increased with both concentrations of Dis*Ba*-01 as evidenced by the presence of pro and active MMP-9 bands in the zymographic gels (Fig. 2Ad-e, 2Bb and 2Cc). L929 cells have not expressed MMP-9 in our assay conditions.

Dis*Ba*-01 shorten actin filaments and changes morphology of 4T1BM2 tumor cells

RGD disintegrins were suggested to inhibit cell migration by inducing cell detachment. To address this possibility, we studied the morphology of cells after Dis*Ba*-01 treatment using phalloidin staining. Interestingly, 4T1BM2 cells were highly sensitive to Dis*Ba*-01, either in 100 or 1000 nM concentrations (Figure 3A and 3B). Stress fibers were strongly affected and actin filaments apparently were shortened (Figure 3B). It is clear that the rounded-shape of cells seen here was induced by cell detachment processes (Figure 3C and 3D). To confirm this hypothesis, we used the actin filament inhibitor Cytochalasin D, which induced very distinct morphologically effects in tumor cells and in fibroblasts (Figure 3A). Contrastingly, the morphology of L929 cells was not changed by Dis*Ba*-01 treatment, even at the highest concentration. Cell spreading was similar to the control, with visible actin filaments (Figure 3A, 3C and 3D).

Dis*Ba*-01 inhibit cell growth and does not induce Anoikis-Resistance in breast tumor cells

Considering that Dis*Ba*-01 impairs 4T1BM2 adhesion properties, we tested if this disintegrin would affect cell proliferation and Anoikis-Resistance. In fact, Dis*Ba*-01 inhibited colony formation in a concentration-dependent way, decreasing the size of colonies (Figure 4A and 4b). Thus, our results showed that Dis*Ba*-01 does not induce Anoikis-Resistance in breast tumor cells. In addition, 4T1BM2 and L929 cells were seeded in wells coated with vitronectin or fibronectin for cell viability. Dis*Ba*-01 promotes a low cell growth rate in both cells, an effect that was evident much earlier and more prominently in vitronectin (Figure 4B).

Dis*Ba*-01 does not induce caspase-mediated apoptosis but changes cell cycle progression of 4T1BM2 cells

To further explore Dis*Ba*-01 effects on tumor cells, we analyzed the cell cycle of treated cells by cytometry. The exposure of 4T1BM2 cells to Dis*Ba*-01 increased its Sub-G1 phase and decreased its S phase (Figure 5A). Considering Sub-G1 phase as an indicator of DNA fragmentation, the results suggest that 4T1BM2 cells are undergoing apoptosis upon Dis*Ba*-01 treatment. Interestingly, Dis*Ba*-01 did not affect the cell cycle of L929 cells (Figure 5A). However, we could not detect apoptotic cells by annexin assay in either cell lines (Figure 5B) as the percentage of viable cells remained similar to the controls. In an attempt to assess whether Dis*Ba*-01 effects were related to the caspase-mediated apoptosis pathway, the expression of some apoptosis hallmarks was analyzed by qPCR. In 4T1BM2 cells, Dis*Ba*-01 downregulated the expression of caspase-3, 8 and 9, mostly in 1000 nM concentration (Figure

5C). The same pattern of downregulation was observed for L929 cells, with the exception for caspase-3 which expression was not changed by Dis*Ba*-01 (Figure 5C). In addition, caspase-mediated cell death was not observed on Western Blotting assay since there was no detection of cleaved caspase-3 on both 4T1BM2 and L929 cells (Figure 5D).

Dis*Ba*-01 increases autophagic flux and promotes autophagy in breast tumor cells

As shown, Dis*Ba*-01 does not induce death by lacking of anchorage. Instead, cells are probably protected from death by entering a program of autophagy. Thus, we tested the induction of LC3B, a structural protein of autophagosomal membranes and a widely used autophagy biomarker. Increased expression of LC3B, in 4T1BM2 cells after treatment with Dis*Ba*-01 disintegrin was observed by immunofluorescence (Figure 6A) as well as in the positive control treated with Rapamycin an authophagy inducer [6, 7]. These results show that cells are induced to autophagy as a way to protect themselves from cell death in a short-term period. Lysosomal degradation of autophagosomes leads to a decrease in LC3B-II levels during autophagy. Our data showed that Dis*Ba*-01 and the blockage of autophagosomal degradation induced by chloroquine (CQ), an indirect autophagy inhibitor, caused time-dependent LC3B-II accumulation, indicating a decrease in the autophagic flux (Figure 6B).

DISCUSSION

We have previously demonstrated that Dis*Ba*-01 inhibits migration of some human tumor cell lines such as MDA-MB-231 breast tumor cells, prostate DU-145 cells [18], oral squamous carcinoma cells (OSCC) [26] and murine 4T1BM2 cells [29] at similar concentrations to those used in the present study. Here we showed that Dis*Ba*-01 also inhibits the ability of 4T1BM2 cells, but not of L929 cells, to invade matrigel layers. Cells from 4T1BM2 cell line express higher amounts of α_v integrin subunit when compared to L929 cells. This subunit can combine with the β_3 integrin subunit forming the $\alpha_v\beta_3$ dimer, which presence explains the higher sensibility of 4T1BM2 cells to Dis*Ba*-01. The ability of snake venom disintegrins to inhibit cell migration and invasion is well documented [35], however, the mechanisms by which these proteins impair cell mobility are not fully understood. Recently, we have shown that Dis*Ba*-01 decreases the migration speed and directionality of OSCC cells while increases the adhesion sizes and the number of maturing focal adhesions [26].

Furthermore, β_3 overexpression restored cell migration velocity and the number of maturing adhesions that were modified by Dis*Ba*-01 [26]. These results suggest that the disintegrin preferentially targets $\alpha_v\beta_3$ integrin, although binding to $\alpha_5\beta_1$ integrin can not be

excluded; however, with lower affinity. RGD-disintegrins are believed to be nonspecific and able to bind to both integrins, however, we have demonstrated that Dis*Ba*-01 affinity to $\alpha_v\beta_3$ integrin is about 100 times higher than to $\alpha_5\beta_1$ integrin [26]. In addition, previous reports indicated distinct roles for the two integrins during the migration process with the demonstration that $\alpha_5\beta_1$ integrins are responsible for supporting adhesion strength whereas $\alpha_v\beta_3$ integrins start mechanotransduction [36–38].

Additionally, selective blocking of $\alpha_v\beta_3$ with integrin antagonists provided evidence for cooperation between these receptors in maintaining cell directionality [32]. These observations could explain the results of Dis*Ba*-01 in L929 cell invasion. Since these cells have a low content of $\alpha_v\beta_3$ integrin, they must be less sensitive to the disintegrin, as was observed by the lack of adhesion inhibition of L929 cell to FN. However, Dis*Ba*-01 inhibited the adhesion of L929 cells to VN, similarly to 4T1BM2 cells. These results suggest that L929 cells must rely on $\alpha_v\beta_3$ integrin for VN binding even in lower amounts and in this case, these cells become responsive to Dis*Ba*-01 inhibitory effect.

Extracellular matrix degradation and remodeling are critical steps in cancer progression and frequently depend on the activity of matrix metalloproteases (MMPs). Under normal physiological conditions, MMP activity is precisely regulated at several levels such as transcription, activation and by the presence of tissue inhibitors of MMPs (TIMPs) [39, 40]. Abnormal MMP activity is frequently observed in tumors and increased MMP-2 activity is correlated to increase in cell mobility and metastasis [41]. MMP-2 expression and activity are closely regulated by $\alpha_v\beta_3$ integrin but some authors have reported an increase in MMP-2 activity [42] while others suggested that overexpression of $\alpha_v\beta_3$ integrin decreased MMP-2 activation [40]. Additionally, it has been shown that MMP-2 and MMP-9 are induced by $\alpha_v\beta_3$ integrin activation and extracellular signal-related kinase (ERK) signaling pathways, processes that are reduced by $\alpha_v\beta_3$ integrin inhibitors [43].

In this study, we confirmed that Dis*Ba*-01, an $\alpha_v\beta_3$ integrin inhibitor, negatively regulated MMP-2 but not MMP-9 expression in 4T1BM2 cells. Instead, MMP-9 activity was strongly activated upon $\alpha_v\beta_3$ integrin inhibition. This data suggests that MMP-9 is not essential for 4T1BM2 cell migration since increased MMP-9 activity did not neutralize Dis*Ba*-01 effects on cell invasion. These results are in agreement with our previous data on human fibroblasts and MDA-MB-231 breast tumor cells where Dis*Ba*-01 decreased MMP-2 content and activity and increased MMP-9 activity [25]. Augmented expression of MMP-9 was associated to a favorable prognosis in patients with node-negative breast cancer [44], an effect probably due to MMP-9 ability to generate angiogenesis inhibitors such as angiostatin

and tumstatin [45–47]. In agreement with these observations, we have previously reported the antiangiogenic effect of Dis*Ba*-01 in mice although we have not tested MMP-9 expression in this model to confirm this possibility [28].

Integrin inhibition by RGD-peptides is believed to induce apoptotic cell death [48, 49]. However, despite the cytoskeleton disorganization induced by Dis*Ba*-01, our results showed that cells do not undergoing apoptosis. Cell cycle progression and annexin V analyses showed that integrin inhibition leads to S phase reduction of treated cells without further progression into apoptotic death. Indeed, mRNA expression of caspases 3, 8 and 9 were downregulated as well the protein levels of caspase-3. These results were obtained in both 4T1BM2 and L929 cell lines and suggest that cells might be undergoing to a process of autophagy, in an attempt to survive without being fully attached to the ECM. Autophagy is an anti-apoptotic process induced upon loss of integrin-mediated cell adhesion to the ECM [6, 7].

Thus, we tested the induction of LC3B, a structural protein of autophagosomal membranes, widely used as a biomarker of autophagy, in 4T1BM2 cells after treatment with Dis*Ba*-01 [6, 7, 31]. It was observed an increase in the expression of LC3B, similar to the positive control treated with Rapamycin [6, 7, 31]. Western Blotting analysis showed a higher expression of LC3B on 4T1BM2 cells and the blockage of autophagosomal degradation induced by chloroquine (CQ), an indirect autophagic inhibitor, caused time-dependent LC3B-II accumulation, indicating a decrease in the autophagic flux. This process protects cells from cell death over short-term periods and seems to be critical for tumor cell survival in order to produce metastasis [50, 51]. Furthermore, anoikis resistance has been suggested as an important factor contributing for metastasis development [6, 7]. Interestingly, Dis*Ba*-01 promotes low cell growth rate and it does not induce Anoikis-Resistance in breast tumor cells.

In conclusion, our results demonstrated that the RGD-disintegrin, Dis*Ba*-01, blocks preferentially $\alpha_v\beta_3$ integrin but does not induce anoikis, at least in a short-term period. Instead, autophagy is induced and cells lose their ability to invade tissues, an effect also supported by decreased MMP-2 activity. These actions are more evident in cells expressing high levels of α_v and β_3 integrin subunits such as many tumor cells. To our knowledge, this is the first study describing these effects for a RGD-disintegrin and may be helpful to achieve a better understanding on the mechanism of action of new integrin-targeted pharmaceutical compounds.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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FIGURES DESCRIPTION

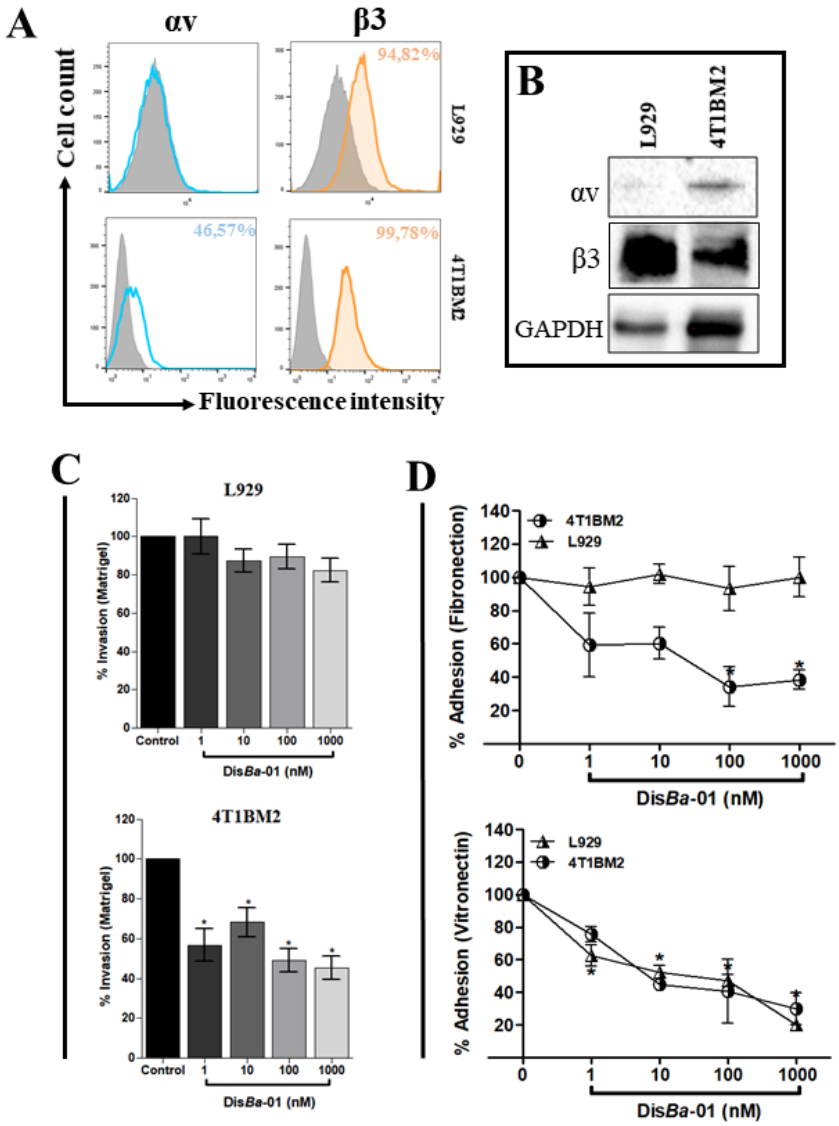


Figure 1. Dis*Ba*-01 inhibits adhesion and invasion of 4T1BM2 cells to components of ECM, but not of L929 cells. (A) Expression of α_v and β_3 integrin subunits in L929 and 4T1BM2 cells was analyzed in a flow cytometer. The presence of integrin receptors on the cell surface was determined with FITC dye and specific antibodies. Data was analysed using FlowJo v10 software. The grey curve represents the experiment control. **(B)** Cells lysates of L929 and 4T1BM2 were submitted to Western Blotting for analysis of integrin subunits. The anti-GAPDH antibody was used as loading control. **(C)** Quimiotatic invasion towards matrigel was measured in transwell chambers in the absence of serum. Dis*Ba*-01 was able to decrease cell motility in all tested concentrations in 4T1BM2. There was not inhibition of cell invasion in matrigel in these concentrations in L929. Cells were counted using a microscope (five random fields per experiment) (Carl Zeiss, Axio Vision - Objective Lens 20x) coupled with a camera (Axio Vision CAM) and invasion was expressed as a percentage of the control (100%). Assays were repeated three times in triplicate and data are represented as mean ± SD (One-Way ANOVA and Bonferroni's comparison test, *p<0.05 compared to control). **(D)** 96 wells plates were coated with fibronectin and vitronectin. After blocking, 4T1BM2 and L929 ($1x10^5$ cells/well) were incubated with different concentrations of Dis*Ba*-01 (1 nM, 10 nM, 100 nM and 1000 nM). BSA was used as negative control. Cells were then fixed, stained with crystal violet and the absorbance were read at 540 nm (SpectraMax i3, *Molecular Devices*). Data are expressed as percentage of control (considered as 100% of attachment) of three independent experiments as mean ± SD (One-Way ANOVA and Bonferroni's comparison test, *p<0.05 compared to control).

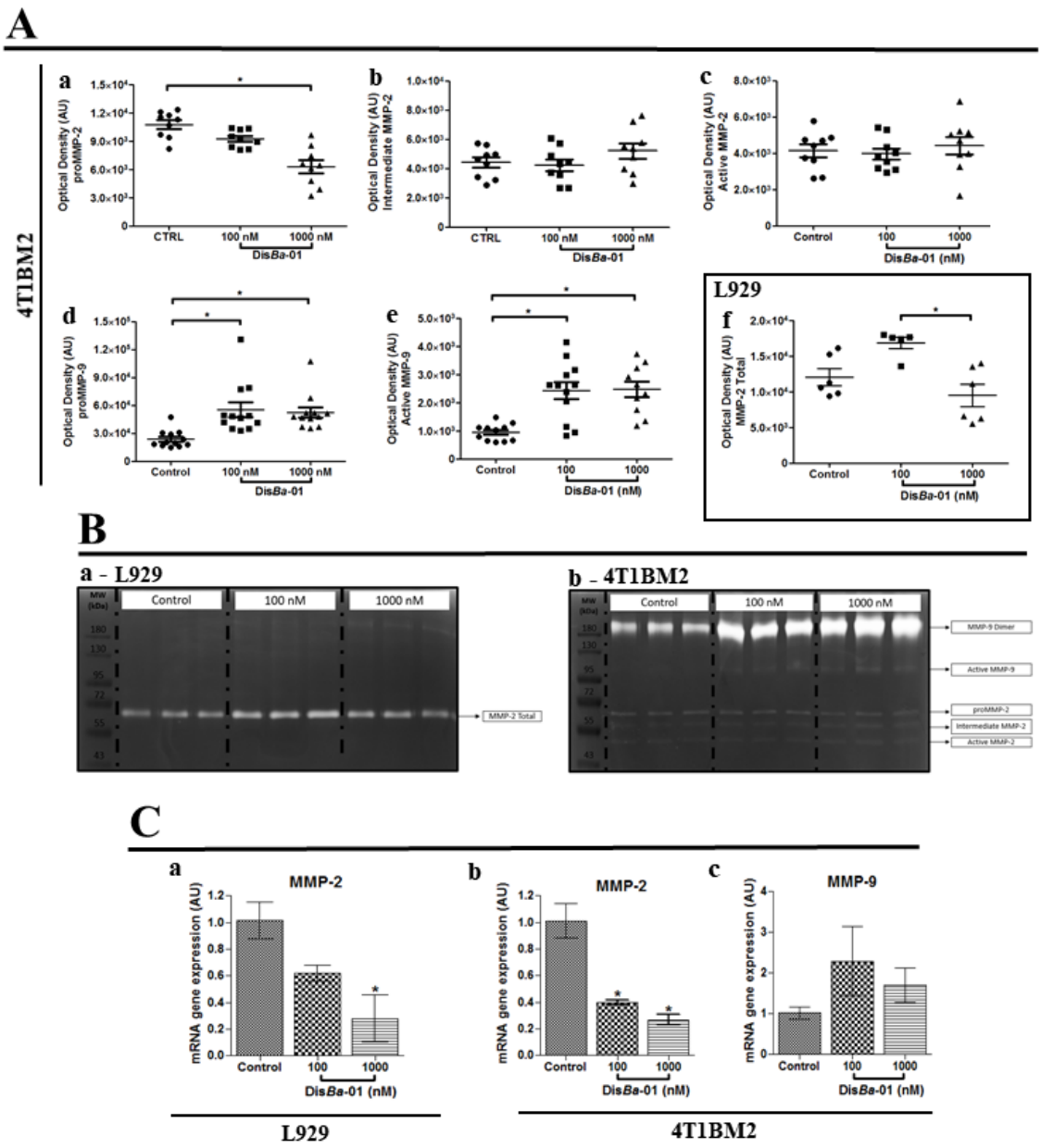


Figure 2. Dis*Ba*-01 inhibits MMP-2 synthesis but increases MMP-9 activity in tumor cells. L929 and 4T1BM2 cells were treated with the indicated concentrations of Dis*Ba*-01 for 24 hours in a wound healing assay, and the supernatants were subjected to Gelatin Zymography. **(A)** Densitometry of proMMP-2 **(Aa)**, intermediate MMP-2 **(Ab)**, active MMP-2 **(Ac)**, proMMP-9 **(Ad)**, active MMP-9 **(Ae)** in 4T1BM2 and total MMP-2 in L929 **(Af)**. **(B)** Bands representing active MMP-2 **(Ba and Bb)**, proMMP-2 **(Bb)** intermediate MMP-2 **(Bb)**, proMMP-9 **(Bb)** and active MMP-9 **(Bb)** in the gel containing gelatin. **(C)** Gene expression of MMP-2 **(Cb)** and MMP-9 **(Cc)** in 4T1BM2 and L929 cells **(Ca)** were analysed through qRT-PCR. Ribosomal protein S27a (RPS27a) was used as a housekeeping gene. Assays were repeated three times in triplicate and data are represented as mean ± SD (One-Way ANOVA and Bonferroni's comparison test, *$p<0.05$ compared to control).

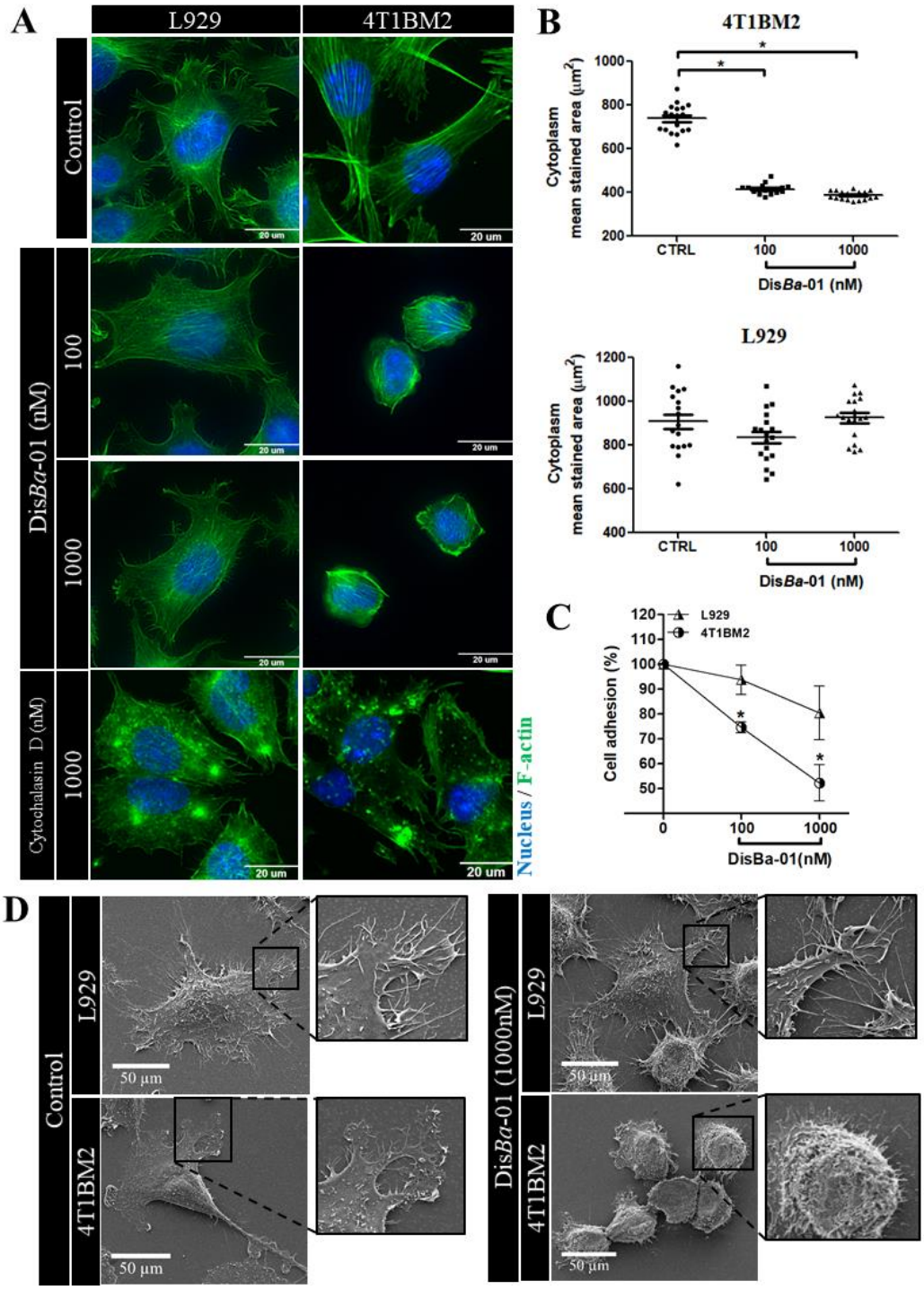


Figure 3. Dis*Ba*-01 shorten actin filaments of 4T1BM2 tumor cells changing its morphology. (A) 4T1BM2 and L929 cells were plated in 96 wells culture plates and incubated with different concentrations of Dis*Ba*-01 (100 nM and 1000 nM). Cytochalasin D (1000 nM) was used as a potent inhibitor of actin polymerization. Cells were stained with Alexa Fluor® 488 Phalloidin and images were obtained at ImageXpress® Micro equipment in a 60x increase. Scale bar = 20 µm. **(B and C)** For quantification, cytoplasm mean stain area (µm^2) was obtained using a high content screeening (ImageXpress® Micro equipment). Data were analysed using GraphPad Prism 5.01. **(D)** L929 and 4T1BM2 cells were plated in a Lab-TeK® chamber slideTM system overnight for adhesion. After this time, cells were incubated with Dis*Ba*-01 (1000 nM), fixed, washed with PBS and then dehydrated in a series of increasing ethanol concentrations. The images were obtained at a Scanning Electron Microscopy (Inspect F50 - FEI®). Scale bar = 50 µm. Assays were repeated three times in triplicate and data are represented as mean ± SD (One-Way ANOVA and Bonferroni's comparison test, *p<0.05 compared to control).

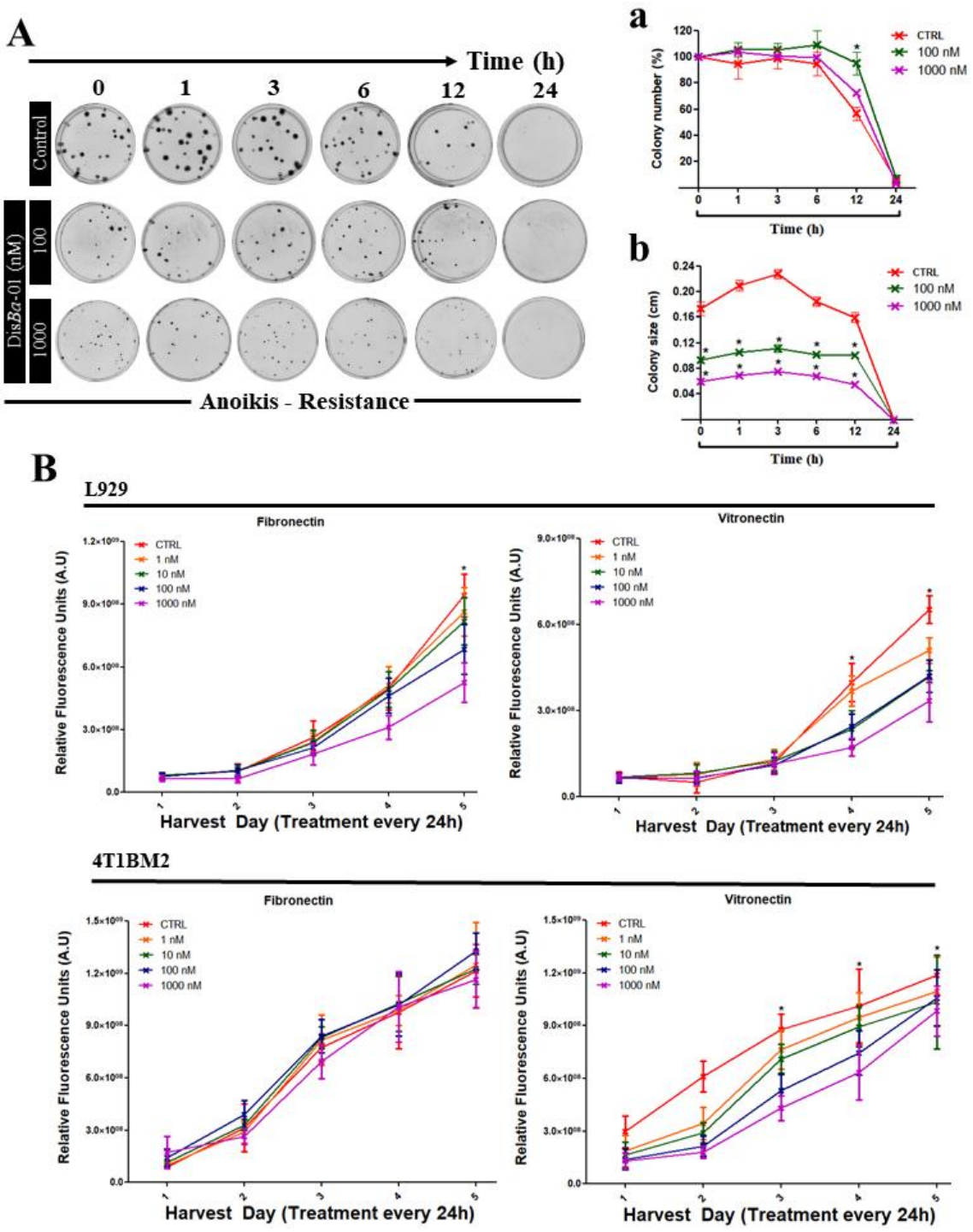


Figure 4. Dis*Ba*-01 promotes a low cell growth rate and it does not induce Anoikis-Resistance in breast tumor cells. (A) Representative image of colonies formation after incubation with different concentrations of Dis*Ba*-01 (100 nM and 1000 nM) for 0, 1, 3, 6, 12 and 24h of shaking. Colonies formed after 7 days were stained with crystal violet, photographed and counted using Image J 1.48v program. **(Aa and Ab)** Analysis of the numbers and sizes of the colonies. Assays were repeated two times in triplicate and data are represented as mean ± SD (Two-Way ANOVA and Bonferroni's comparison test, *p<0.001 compared to control). **(B)** 4T1BM2 and L929 cells (5 x 10^5) were seeded in wells coated with vitronectin or fibronectin and allowed to adhere for 8h at 37 °C. Cells were then treated with the stated doses of Dis*Ba*-01 every 24 hours for 5 days. Assays were repeated two times. Cell proliferation was expressed as fluorescence at 550 nM (SpectraMax i3x, *Molecular Devices*) and each point represents the mean ± SD of six replicate wells per dose per day (Two-Way ANOVA and Bonferroni's comparison test, *p<0.001 compared to control).

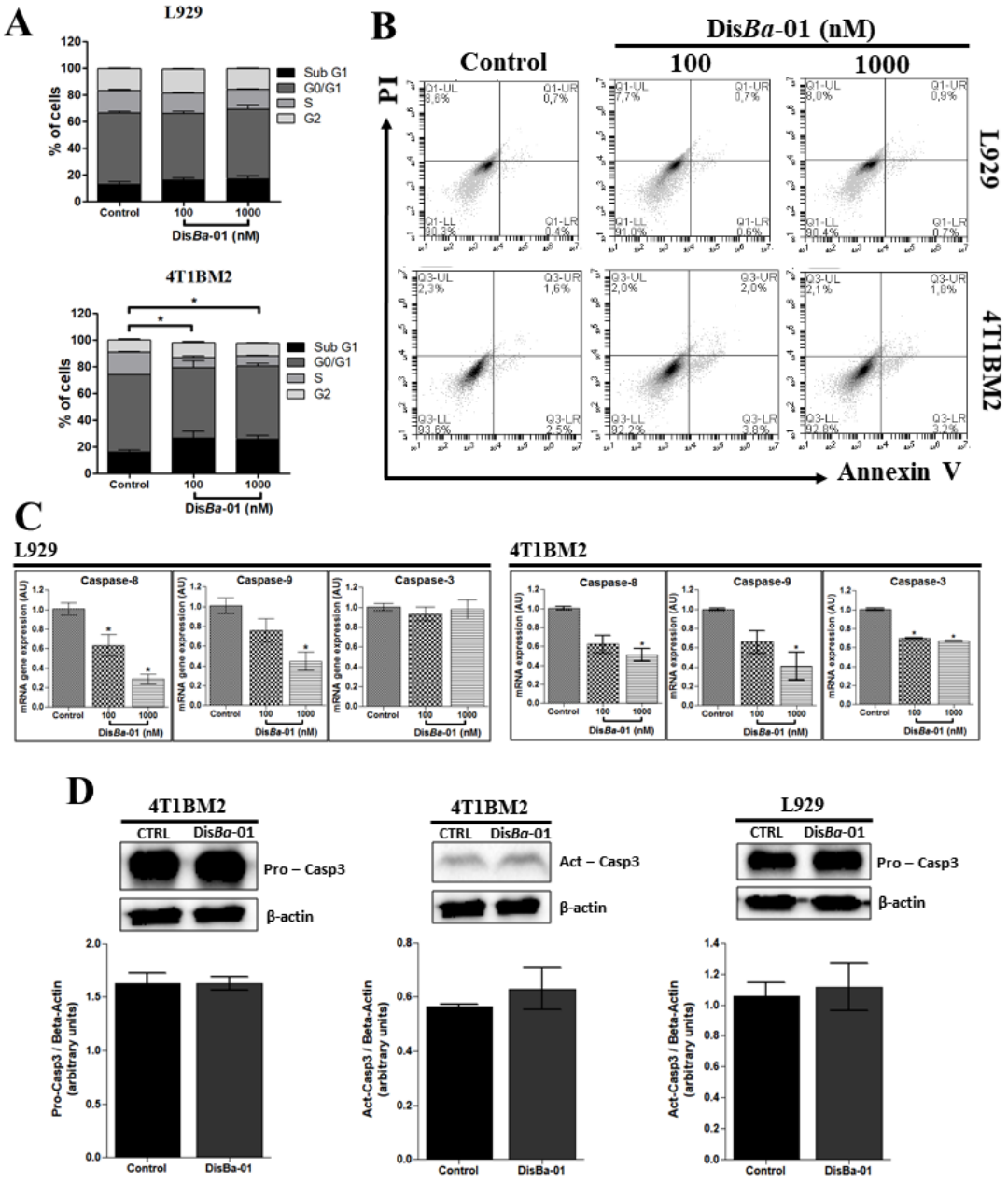


Figure 5. Dis*Ba*-01 changes cell cycle progression but does not induce apoptosis in 4T1BM2 breast tumor cells. (A) 4T1BM2 and L929 cells were treated with Dis*Ba*-01 for 24 hours at indicated concentrations and then fixed, stained with PI and analyzed by flow cytometry for distribution of cells in each cycle phase. Results indicated a decrease of cells in S phase after 24 hours of treatment with 100 and 1000 nM of Dis*Ba*-01 in 4T1BM2, without effects on L929 cells. Data are reported as means ± SD (One-Way ANOVA and Bonferroni's comparison test, *p<0.05 compared to control). **(B)** After treatment with different concentrations of Dis*Ba*-01 (100 nM and 1000 nM) for 24 hours, cells were incubated with PE annexin V and 7-AAD in the dark and analyzed by flow cytometry. **(C)** Gene expression involved in pro and anti-apoptotic effects in 4T1BM2 and L929 cells, after treatment with Dis*Ba*-01 (100 nM and 1000 nM) for 24h, were analyzed through qRT-PCR. Ribosomal protein S27a (RPS27a) was used as a housekeeping gene. Data are reported as means ± SD (One-Way ANOVA and Bonferroni's comparison test, *p<0.05 compared to control). **(D)** Cells were plated in 6 wells culture plates and incubated with Dis*Ba*-01 (1000 nM) for 24 hours. After incubation, cells were lysed and submitted to Western Blotting for analysis of Pro-Caspase 3 and Act-Caspase 3. Anti-Beta Actin antibody was used as loading control. Bands corresponding to all proteins were quantified by densitometry using the Image J 1.52a program. Assays were repeated three times and data are represented as mean ± SD (One-Way ANOVA and Bonferroni's comparison test, *p<0.05 compared to control).

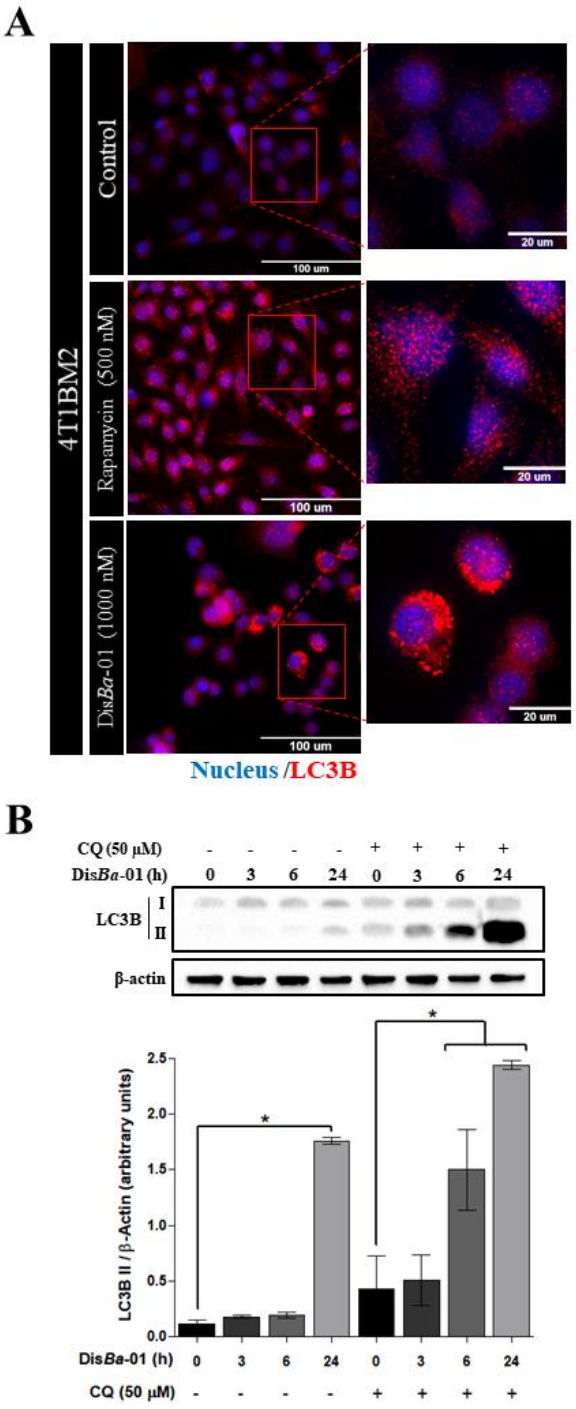


Figure 6. Dis*Ba*-01 promotes autophagy in tumor cells and increases the autophagic flux. (A) LC3B was visualized in cells by immunofluorescence. 4T1BM2 cells were plated in 96 wells culture plates and incubated with Dis*Ba*-01 (1000 nM) for 24 hours. Rapamycin (500 nM) was used as an autophagic promoter in both experiments. After incubation, cells were fixed and stained with anti-LC3B and APC secondary antibody (red-Cy5) for immunostaining procedures. Nuclei were stained with DAPI (blue). Images were obtained at ImageXpress® Micro equipment in a 60x increase. Scale bar = 100 and 20 µm. **(B)** Cells were treated with Dis*Ba*-01 (1000 nM) for the indicated time points with or without CQ (50µM). After incubation, cells were lysed and submitted to Western Blotting for analysis of LC3B. The β-actin antibody was used as loading control. Bands corresponding to all proteins were quantified by densitometry using the Image J 1.48v program. Assays were repeated three times and data are represented as mean ± SD (One-Way ANOVA and Bonferroni's comparison test, *p<0.05 compared to control).

7. CONCLUSÃO

Neste trabalho, verificamos que:

- O bloqueio da integrina $\alpha_v\beta_3$ pela Dis*Ba*-01 inibiu invasão, adesão e proliferação de células normais e tumorais de mama, sobre componentes da MEC: fibronectina e vitronectina;

- Dis*Ba*-01 inibiu a síntese de MMP-2, mas aumentou atividade de MMP-9 nas células tumorais;

- Morfologicamente as células tumorais foram evidentemente afetadas pela Dis*Ba*-01;

- A inibição da integrina $\alpha_v\beta_3$ pela Dis*Ba*-01, induziu parada do ciclo celular na fase S em células 4T1BM2, mas não promoveu apoptose, o que foi condizente com a diminuição da expressão gênica de caspase-3, 8 e 9. A clivagem de caspase-3 por Western Blotting não foi detectada em ambas as células;

- A desintegrina levou ao aumento da expressão proteica de LC3B, um indicador de autofagia, mecanismo este protetivo da célula contra a morte celular programada por Anoikis.

Em conclusão, a especificidade da Dis*Ba*-01 pela 4T1BM2 quando comparada a L929, pode ser devido ao maior nível de expressão de integrinas α_v e β_3 nestas células. Estas observações sugerem novos mecanismos sobre os efeitos de inibidores baseados em RGD, considerando sua importância terapêutica para saúde humana. O resumo desses efeitos será apresentado na figura 4 a seguir:

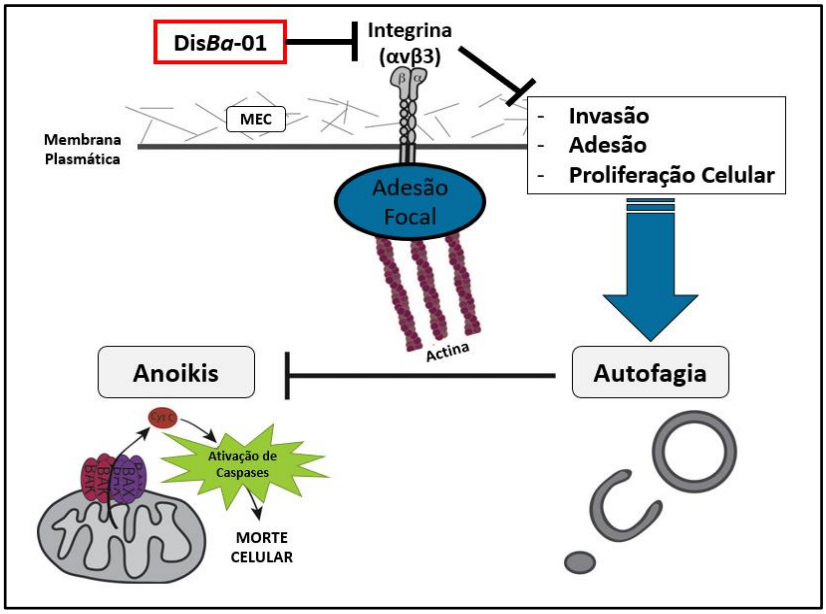
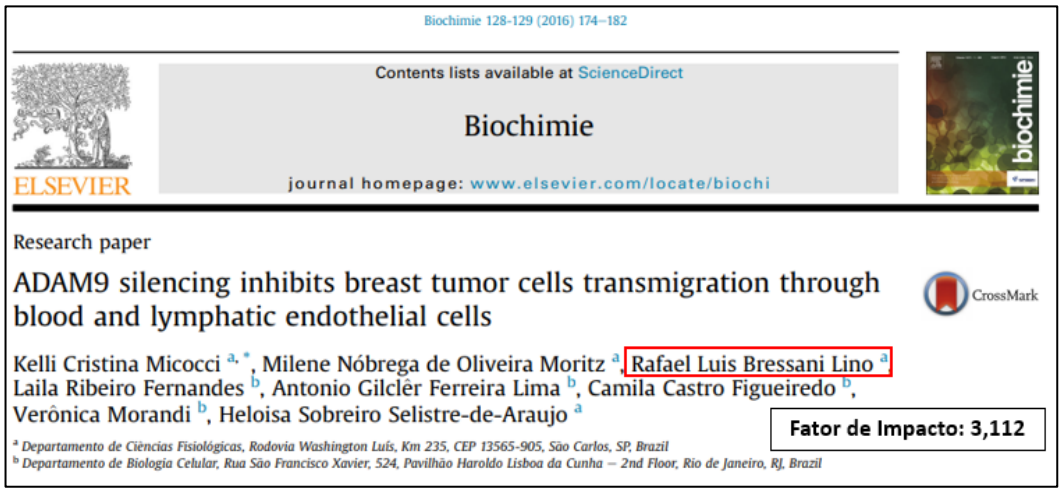


Figura 4. Resumo do bloqueio da integrina $\alpha_v\beta_3$ pela Dis*Ba*-01. O bloqueio da integrina $\alpha_v\beta_3$ promovida pela Dis*Ba*-01, inibiu invasão e adesão celular, além de ter diminuído a proliferação de células tumorais de mama *in vitro*. Ademais, as células frente a vigência da morte programa ocasionada por este bloqueio, promoveu autofagia, como mecanismo compensatório e protetivo da apoptose. **Fonte:** Adaptado de VLAHAKIS et a.l, 2017.

8. ANEXO

ARTIGOS PUBLICADOS EM COLABORAÇÃO



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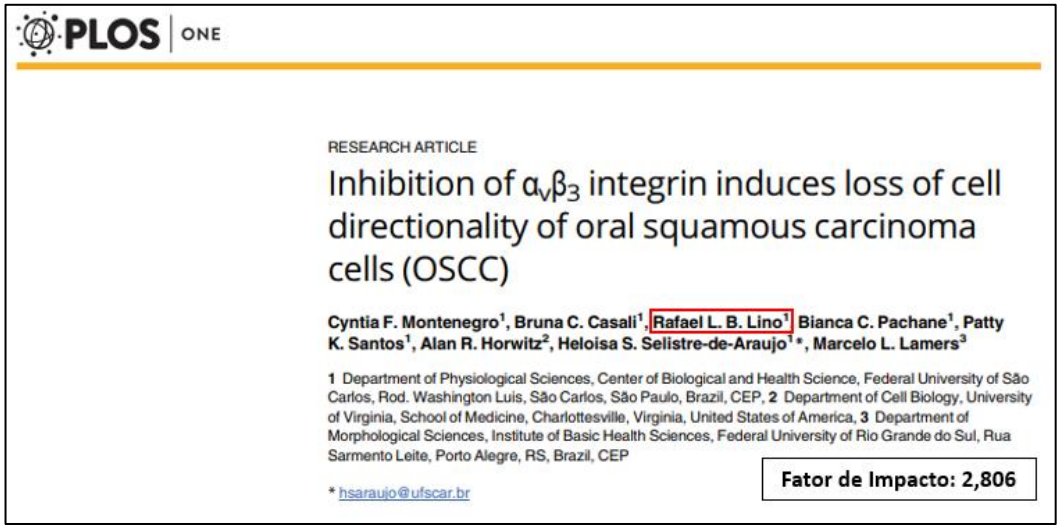
Research paper

ADAM9 silencing inhibits breast tumor cells transmigration through blood and lymphatic endothelial cells

Kelli Cristina Micocci [a, *], Milene Nóbrega de Oliveira Moritz [a], Rafael Luis Bressani Lino [a], Laila Ribeiro Fernandes [b], Antonio Gilclêr Ferreira Lima [b], Camila Castro Figueiredo [b], Verônica Morandi [b], Heloisa Sobreiro Selistre-de-Araujo [a]

[a] Departamento de Ciências Fisiológicas, Rodovia Washington Luís, Km 235, CEP 13565-905, São Carlos, SP, Brazil
[b] Departamento de Biologia Celular, Rua São Francisco Xavier, 524, Pavilhão Haroldo Lisboa da Cunha – 2nd Floor, Rio de Janeiro, RJ, Brazil

Fator de Impacto: 3,112



PLOS ONE

RESEARCH ARTICLE

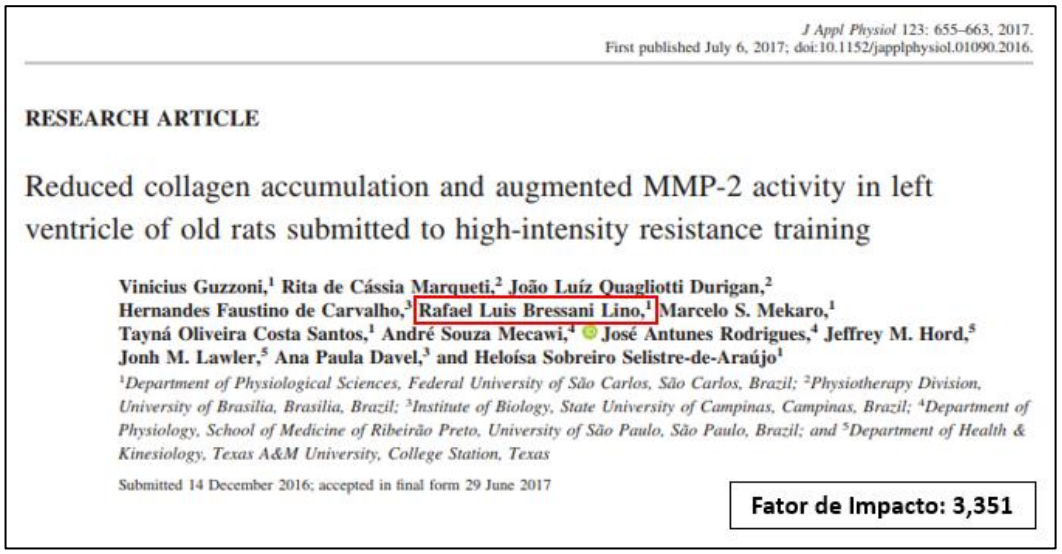
Inhibition of $\alpha_v\beta_3$ integrin induces loss of cell directionality of oral squamous carcinoma cells (OSCC)

Cyntia F. Montenegro[1], Bruna C. Casali[1], Rafael L. B. Lino[1], Bianca C. Pachane[1], Patty K. Santos[1], Alan R. Horwitz[2], Heloisa S. Selistre-de-Araujo[1]*, Marcelo L. Lamers[3]

1 Department of Physiological Sciences, Center of Biological and Health Science, Federal University of São Carlos, Rod. Washington Luis, São Carlos, São Paulo, Brazil, CEP, 2 Department of Cell Biology, University of Virginia, School of Medicine, Charlottesville, Virginia, United States of America, 3 Department of Morphological Sciences, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Rua Sarmento Leite, Porto Alegre, RS, Brazil, CEP

* hsaraujo@ufscar.br

Fator de Impacto: 2,806



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RESEARCH ARTICLE

Reduced collagen accumulation and augmented MMP-2 activity in left ventricle of old rats submitted to high-intensity resistance training

Vinicius Guzzoni,[1] Rita de Cássia Marqueti,[2] João Luíz Quagliotti Durigan,[2] Hernandes Faustino de Carvalho,[3] Rafael Luis Bressani Lino,[1] Marcelo S. Mekaro,[1] Tayná Oliveira Costa Santos,[1] André Souza Mecawi,[4] José Antunes Rodrigues,[4] Jeffrey M. Hord,[5] Jonh M. Lawler,[5] Ana Paula Davel,[3] and Heloísa Sobreiro Selistre-de-Araújo[1]

[1]Department of Physiological Sciences, Federal University of São Carlos, São Carlos, Brazil; [2]Physiotherapy Division, University of Brasilia, Brasilia, Brazil; [3]Institute of Biology, State University of Campinas, Campinas, Brazil; [4]Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, São Paulo, Brazil; and [5]Department of Health & Kinesiology, Texas A&M University, College Station, Texas

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