Disintegrins in health and disease

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1. ABSTRACT

Few of the proteins isolated and characterized from snake venom have proven to be more chemically diverse, exquisitely specific or promiscuously active than the family known as disintegrins. These small proteins have shown structural homology with hundreds of cell surface molecules from plants and animals other than snakes, and their precise mimicry of native receptor ligands speaks to evolutionary niches related to survival and geographic locale. Over 100 disintegrins have been named and studied, with the most recent efforts into molecular techniques providing significant clues to taxonomic relationships among four different snake families. Investigators have evaluated disintegrin applications in therapies for cancer, asthma, osteopenia and inappropriate angiogenesis. Crystal and NMR studies have confirmed hypotheses regarding ligand-receptor interactions while illuminating the complexities of structure-function evidence. Disintegrin chimeras with viruses, microbubbles and fluorescent labels have become useful tools in many investigations. While many disintegrin studies still involve platelets, previously unexplored interactions with glial cancer, T lymphocytes and the bacteria Yersinia have blazed new trails for this field. This review will summarize disintegrin investigations since 2003.

2. INTRODUCTION

Disintegrins are small (4-16 kDa) molecular weight, cysteine-rich molecules which are produced by every snake studied thus far from four families: Atractaspididae, Elapidae, Viperidae and Colubridae (1). They show remarkable sequence homology owing to their conserved cysteine arrangement. The "classical" disintegrin is either monomeric or dimeric, has the ability to bind integrins in a dose-dependent manner, and possesses an R/K/M/W/VGD, MLD, MVD or K/RTS sequence within what is known as the "RGD adhesive loop", which has a critical role in the proteins' interactions with cell-surface integrin receptors (2). Viper venom disintegrins result from proteolytic processing of larger mosaic PII and PIII metalloprotease precursors (3) or are synthesized from short-coding mRNAs (4). Monomeric disintegrins are classified by size into short (41-51 residues and 4 disulfide bonds), medium (about 70 amino acids and 6 disulfide bonds) and long (~84 residues and 7 disulfide bonds). An additional class of disintegrins is dimeric, containing subunits of about 67 residues with 10 cysteines involved in 4 intrachain and 2 intrachain disulfide linkages. We previously reviewed the disintegrins described in the literature before 2003 (1). Since that time, a significant increase in interest, spurred on by advances in protein and

DNA technologies, has occurred concerning the evolutionary development of all classes of venom proteins. In addition, disintegrins continue to provide significant insights into the biology of cancer both *in vivo* and *in vitro*. The purpose of this review is to summarize the newest discoveries for the members of this naturally-occurring protein family made since 2003, highlighting their uses in health and disease.

3. TAXONOMIC CHALLENGE

Viper venoms from the Elapidae, Hydrophiidae, Atractaspididae, Viperidae and Colubridae families contain at least 25 separate classes of biologically active compounds (5). The technology of proteomics (6, 7), enabling sequence deduction from cloned cDNA (6), as well as actual recombinant expression of disintegrins even before the protein has been isolated from the crude venom (8, 9), have underscored the need for a standardized method of nomenclature for these molecules. To that end, in 2007, as a publication of the Exogenous Factors Registry of the International Society for Thrombosis and Haemostasis, we suggested a nomenclature standard for disintegrins be used. The full description of the recommendations may be found at http://www.med.unc.edu/isth/ssc/communications/exogeno us/disintegrins/disintegrins.htm. In brief, the recommendations included (1) no longer naming isolated venom proteins only by their HPLC fraction elution number; (2) naming DNA, mRNA and cDNA clones in italics to distinguish each from the protein derived from that gene, and then using that same name for all forms of the expressed protein, whether found in the venom itself or made by recombinant technology; (3) placing the letter "r-" before the name of any disintegrin isolated by recombinant DNA techniques rather than being isolated from crude venom; (4) sequentially numbering the disintegrins from the same genus/species/subspecies, so that future investigators will more easily be able to distinguish their newly isolated protein from those discovered previously. Figures 1-4 list the disintegrins and their sequences for these proteins named since 1998.

4. DISINTEGRIN STRUCTURE

4.1. Evolution

Since 2003, the number of evolutionary structural comparison studies has greatly increased in the field of disintegrins due to improved methods and protein sequence databases. Methods include genomic, transcriptomic and proteomic studies. The genomic approach utilizes the actual DNA present in the cells of snakes. In these studies, DNA is extracted from the tissue of snakes (venom glands or liver) using DNA isolation kits (10). Transcriptomic studies are based on the formation of cDNA libraries using extracted snake mRNA, usually from individual venom glands (7). Proteomic studies are based on proteins actually isolated from the venom, and therefore sequences that are transcribed and translated (11). To isolate proteins, a majority of researchers use variations of High Pressure Liquid Chromatography (HPLC) (7, 11-13). Reverse phase and normal phase HPLC have been used most frequently in disintegrin research. Electrospray-ionization mass spectroscopy is also an important tool for the structural characterization of these proteins as monomers or dimers (12).

Transcriptomic analysis of snake venom glands has been the focus of multiple investigators (7, 13-17). Sequenced cDNA are compared for clues of evolution. Soto isolated six cDNAs of three Crotalus species (*Crotalus atrox*, *Crotalus scutulatus scutulatus* and *Crotalus viridis viridis*). Three RGD disintegrins, atroxatin, mojastin and viridistatin, and three PIII-snake venom metalloproteases (SVMP), catroriarin, scutiarin and viristiarin were compared. cDNA for atroxatin and viridistatin showed 90% sequence identity, while they were 87% identical to mojastin. The cDNAs for the PIII-SVMPs from the Prairie and Mojave rattlesnakes, scutiarin and viristiarin respectively, were identical, and it was anticipated that the cDNAs for the RGD disintegrins from these snakes would also be the same. Surprisingly, mojastin and viridistatin had only 93% amino acid identity. From these comparisons, conclusions about relatedness of snakes by the shared amino acids in their proteins were proposed, and at times the phylogenetic tree findings were not congruent with taxonomic classification (16). For example, viridian and viridistatin, two RGD disintegrins from *Crotalus viridis viridis*, were grouped in two separate clades (a taxonomic group comprising a single common ancestor and all the descendants of that ancestor) with bootstrap values of 74% and 53%, respectively. In addition, transcriptomic analysis has uncovered mysteries regarding translation of DNA into protein. Juarez (18), using cDNA derived from a PII disintegrin transcript of *Bitis arietans,* discovered BA-5A which was undetected proteomically from isolated venom. It was proposed that the disintegrin may have been translated but not secreted into the venom.

Sanz (13) performed molecular cloning of disintegrins from *Cerastes vipera* and *Macrovipera lebetina transmediterranea* venom gland cDNA libraries. These authors proposed a common ancestral link between the mRNA precursors coding for short disintegrins and dimeric disintegrin subunits. This evolutionary pathway involved (1) replacement of three N-terminal cysteine residues by other amino acids; (2) the appearance of a cysteine between the ninth and tenth cysteine in the precursor; (3) proteolytic processing of the N-terminus and (4) formation of short-disintegrin-specific disulfide bonds. It is noteworthy that cDNA-deduced sequence analysis shows serine is the most common residue in the position that is usually occupied by a short-disintegrin-specific cysteine residue. Equally interesting is that the codon for cysteine (TGC or TGT) or serine (TCC or TCT) can be accomplished by a single G to C mutation. These investigators also proposed a docking model for disintegrins and integrins based on the crystal structure of the extracellular segment of alpha V beta 3 in complex with an RGD ligand (19). The peptide fits into a crevice between the alpha V propeller and the beta 3 A-domain. The R side chain is held in place by interaction with alpha V carboxylates 218 and 150, the glycine residue makes several hydrophobic interactions with alpha V, and the

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Accutin (114)                            GAQCTAGPCCWPC----EGTICRRARGDDLDDYCNGISADCPRNPYY
Echistatin alpha (A32029)                   ECESGPCCRNCKFLKEGTICKRARGDDMDDYCNGRTCDCPRNPHKGPAT
Echistatinα2(A35982)                     QCESGPCCRNCKFLKEGTICKRARGDDMDDYCNGKTCDCPRNPHKGP
Echistatin alpha(S53431)                 DCASGPCCRDCKFLKEGTICKRARGDNMDDYCNGKTCDCPRNPHKGEHDP
Eristocophin (P22826)                    ZRQEEPCATGPCCRRCKFKRAGKVCRVARGDWNNDYCTGKSCDCPRNPWNG
Eristocophin I(AAB25265)  MNSANPCCDPITCKPRKGEHCVSGPCCRNCKFLNPGTICKRRRGDNLNDYCTGVSSDCPRNP
Eristocophin II(AAB25266) ESAGPCCDPVTCKPRRGEHCVSGPCCRNCKKLNAGTVCWPAMGDWNDDYCTGISSDCPRNP
Echistatin gamma(S53432)                 DCASGPCCRDCKFLEEGTICNMARGDDMDDYCNGRTCDCPRNPHKWPAP
Eristostatin(C40003)                     QEEPCATGPCCRRCKFKRAGKVCRVARGDWNDDYCTGKSCDCPRNPWNG
Jerdostatin (AAP20878)                   PLCTTGPCCRQCKLKPAGTTCW——RTSVSSHYCTGRSCECPSYPGNG
Lebestatin (57)                          CTTGPCCRQCKLKPAGTTCW--RTSRTSHYCTGKSCDCPSYPG
Leucogastin B(63)                        DCESGPCCRDCKFLKEGTVCRIARGDNRHDYCNGRTCDCPSNPHKGKHH
Multisquamatin(63)            EGEECESGPCCRNCKFLKEGTICKRARGDDMDDYCNGKTCDCPRNPHKGPAT
Obtustatin(P83469)                       CTTGPCCRQCKLKPAGTTCW--KTSLTSHYCTGKSCDCPLYPG
Ocellatusin(115)                         DCESGPCCDNCKFLKEGTICKVARGDNMHDYCNGKTCDCPRNPYKGEHD
Pyramidin A(63)                          DCASGPCCRDCKFLKEGTICKRARGDNMDDYCNGKTCDCPRNPHKGEHDP
Viperistatin (66)                        CTTGPCCRQCKLKPAGTTCW--RTSRTSHYCTGKSCDCPVYQS
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Figure 1. Amino acid sequences of short length disintegrins. The one-letter code for amino acids is used. The stars indicate the motif commonly referred to as the "RGD adhesive loop" and any RGD-like motif is underlined. Where available, databank accession numbers are given from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed. The amino acid sequences of the following disintegrins were taken from primary references: accutin (114), leukogastin (63), multisquamatin (63), ocellatusin (115), pyrimidum A (44), viperistatin (66) and lebestatin (57). Dashed lines represent spacers providing optimal alignment due to the difference in the sequence of accutin.

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Albolabrin(P17496)          EAGEDCDCGSPAN---PCCDAATCKLLPGAQCGEGLCCDQCSFMKKGTICRRARGDDLDDYCNGISAGCPRNPLHA
Applagin(P16338)            EAGEDCDCGSPAN---PCCDAATCKLLPGAQCGEGLCCDQCSFMKKGTICRRARGDDLDDYCNGISAGCPRNPLHA
Atrolysin E(P34182)         ECDCGSLEN---PCCYATTCKMRPGSQCAEGLCCDQCRFMKKGTVCRVSMVDRNDDTCTGQSADCPRNG
Barbourin(P22827)           EAGEECDCSSPEN---PCCDAATCKLRPGAQCADGLCCDQCRFMKKGTVCRVAKGDWNDDRCTGQSADCPGLYG
Basilicin(P31981)           AGEECDCGSPAN---PCCDAATCKLRPGAQCADGLCCDQCRFIKKGKICRRARGDNPDDRCTGQSADCPRNHGHA
Batroxostatin(P18618)       EAGEECDCGTPEN---PCCDAATCKLRPGAQCAEGLCCDQCRFKGAGKICRRARGDNPDDRCTGQSADCPRNRF
Brevicaudin 1a (A59411)     GEECDCGSPGN---PCCDAATCKLRQGAQCAEGLCCDQCRFMKKGTVCRIARGDIMDDYCNGISAGCPRNPFHA
Brevicaudin 1b (A59410)     EAGEECDCGSPGN---PCCDAATCKLRQGAQCAEGLCCDQCRFMKKGTVCRIARGDIMDDYCNGISAGCPRNPFHA
Brevicaudin 2b (A59409)     GEECDCGSPGN---PCCDAATCKLRQGAQCAEGLCCDQCRFMKEGTICRRARGDDLDDYCNGISAGCPRNPFHA
Cerastin(P31982)            EAGEECDCGTPEN---PCCDAATCKLRPGAQCADGLCCDQCRFMKKGTVCRVARGDQMDDTCTGQSADCPRNGLYG
Cereberin(P31985)           EAGEECDCGSPAN---PCCDAATCKLRPGAQCADGLCCDQCRFIKKGTVCRPARGDNPDDRCTGQSADCPRNRFH
Cotiarin(P31988)            EAGEECDCGAPEN---PCCDAATCKLRPGAQCAEGLCCDQCRFKGAGKICRRARGDNPDDRCTGQSADCPRNRFH
Crotatroxin(P31980)         AGEECDCGSPAN---PCCDAATCKLRPGAQCADGLCCDQCRFIKKGTVCRPARGDWNDDTCTGQSADCPRNGLYG
CTF I/II (P23323)           ELLEEGEDCYCHIPPN---PCCDPATCKLTPGSQCAEGLCCDQCRFKKKGTICRIARGDFPDDRCTGLSDDCPRWNDLI
DISBA-01 AA075107)          EAGEECDCGTPGN---PCCDAATCKLRPGAQCAEGLCCDQCRFIKRARGDIMDDYCNGISAGCPRNPFHA
Durissin(P31980)            AGEECDCGSPAN---PCCDAATCKLRPGAQCADGLCCDQCRFIKKGTVCRPARGDWNDDTCTGQSADCPRNGLYG
Elegantin(P17349)           EAGEECDCGSPEN---PCCDAATCKLRPGAQCADGLCCDQCRFKKKRTICRRARGDNPDDRCTGQSADCPRNGLYS
Eo1-1 [7]                   MNSAH---PCCDPWMCKPKRGEHCISGPCCRNCKFLSPGTICKKAWGDIMNDYCTGISSDCPRNPWKD
Eo10 [7]                    NSAH---PCCDPVTCQPKQGEHCISGPCCRNCKFLNSGTICKRARGDNLHDYCTGIPSDCPRNPYKGKYD
Flavoridin(P18619)          GEECDCGSPSN---PCCDAATCKLRPGAQCADGLCCDQCRFKKKRTICRIARGDFPDDRCTGLSNDCPRWNDL
Flavostatin(P80949)         GEECDCGSPSN---PCCDAATCKLRPGAQCAEGLCCDQCRFIKRARGDNPDDRCTGQSADCPRNS
Halysin(P21858)             EAGEECDCGSPGN---PCCDAATCKLRQGAQCAEGLCCDQCRFMKKGTVCRIARGDIMDDYCNGISAGCPRNPF
Halystatin-2(BAA06027)      EAGEDCDCGAPAN---PCCDAATCKLRPGAQCAEGLCCDQCRFMKEGTICRMARGDIMDDYCNGISAGCPRNPFHA
Horrdistatin 1 (67)         GEECDCGSPAN---PCCDAATCKLRPGAQCAEGLCCDQCRFMKKGTVCRIARGDNPDDRCTGQSADCPRNGL
Horridstatin 2 (67)         GEECDCGSPAN---PCCDAATCKLRPGAQCADGLCCDQCRFIKKGTVCRPARGDWNDDTCTGQSADCPRNGLYG
Jararin(AED34833)           EVGEDCDCGPPANCQNPCCDAATCKLTTGSQCAEGLCCDQCRFIKARQICRKGRGDNPDDRCTGGSGDCPRNS
Jararacin(P31989)           EAGEECDCGPPGN---PCCDAATCKLRPGAQCAEGLCCDQCRFKGAGKICRRARGDNPDDRCTGQSADCPRNRFHA
Jarastatin(116)             EAGEECDCGTPEN---PCCDAATCKLRPGAQCAEGLCCDQCRFMKEGTVCRRARGDIMXXYCNGISAGCPRN
Kistrin(P17494)             GKECDCGSSPEN---PCCDAATCKLRPGAQCGEGLCCEQCKFSRAGKICRIPRGDMPDDRCTGQSADCPRZH
Lachesin(P31990)            EAGEECDCGAPAN---PCCDAATCKLRPGAQCAEGLCCDQCRFIKKGKICRRARGDNPDDRCTGQSADCPRNGYYG
Lutosin(P31986)             EAGEECDCGSPAN---PCCDAATCKLRPGAQCADGLCCDQCRFIKKGTVCRVARGDQMDDTCTGQSADCPRNGLYG
MD2(AAF61189)               EAGKDYDRDSSAN---PCYDAATCKLNQGAQCTAGPCCDQGRFKEEGTICRRARGDDLDDYCNGISGDCPRNPYHA
Mojastin 1 (68)             GEECDCGSPAN---PCCDAATCKLRPGAQCADGLCCDQCRFIKKGTVCRPARGDWNDDTCTGQSADCPRNGLYG
Mojastin 2 (68)             EAGEECDCGSPAN---PCCDAATCKLRPGAQCADGLCCDQCRFIKKGTVCRPARGDWNDDTCTGQSADCPRNGLYG
Molossin(P31984)            EAGIECDCGSPEN---PCCDAATCKLRPGAQCADGLCCDQCRFIKKGKICRRARGDNPDDRCTGQSADCPRNRFHA
MT-C(AF051790)              EAGKECDCGAPAN---PCCDAETCKLRPGQQCAEGLCCDQCRFMKEGTICQEAKGDWNDDTCNGISAGCPRNGFYG
MT-d-1(AF051789)            EAGKECDCGAPAN---PCCDAATCKLRPGAQCAEGLCCDQCRFMKEGTICRMARGDIMDDYCNGISAGCPRNPFHA
Salmosin 1(AAC08997)        ELLEAGEECDCGSPGN---PCCDAATCKLRQGAQCAEGLCCDQCRFMKEGTICRRARGDDLDDYCNGISAGCPRNPFHA
Salmosin 2(AAC42597)        EAGEECDCGAPAN---PCCDAATCKLRPGEQCAEGLCCDQCRFMKEGTICQEAKGDWNDDTCTGQSADCPRNGFYG
Salmosin 3(AAC42596)        VGEDCDCGPPANCQN---PCCDAATCGLTTGSQCAEGLCCDQCRLXKAGTICRKARGDNPDDRCTGQSGVCPRNT
Saxatilin(AAG01882)         EAGEECDCGAPAN---PCCDAATCKLRPGAQCAEGLCCDQCRFMKEGTICRMARGDIMDDYCNGISAGCPRNPFHA
Tergeminin(P22828)          EAGEECDCGSSPAN---PCCDAATCKLRPGAQCADGLCCDQCRFMKKGTVCRVARGDWNDDRCTGQSADCPGLYG
Triflavin(P21859)           GEECDCGSPSN---PCCDAATCKLRPGAQCADGLCCDQCRFKKKRTICRIARGDFPDDRCTGQSADCPRWNGL
Trigramin(P15503)           EAGEDCDCGSPAN---PCCDAATCKLIPGAQCGEGLCCDQCSFIEEGTVCRIARGDDLDDYCNGRSAGCPRNPFH
Trimestatin (63)            GEECDCGSPSN---PCCDAATCKLRPGAQCADGLCCDQCRFKKKRTICRIARGDFPDDRCTGQSADCPRWNDL
Trimucrin(AAP80729)         GEECDCGSPEN---PCCDAATCKLRPGAQCAEGLCCDQCRFKKKRTICRRARGDNRDDRCTGQSADCPRNGLYS
Ussuristatin-1(A5943)       GEECDCGSPGN--PCCDAATCKLRPGAQCAEGLCCEQCRFIKAGTVCRVARGDWNDDKCTGQSADCPRNGFYG
Viridin (P31987)            AGEECDCGSPAN---PCCDAATCKLRPGAQCAEGLCCDQCRFIKKGKICRRARGDNPDDRCTGQSADCPRNPRFH
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Figure 2. Amino acid sequences of medium length disintegrins. The one-letter code for amino acids is used. The stars indicate the motif commonly referred to as the "RGD adhesive loop" and any RGD-like motif is underlined. Where available, databank accession numbers are given from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed. The amino acid sequences of the following disintegrins were taken from primary references: Eo1-1 (7), Eo10 (7), horrdistatin 1 and 2 (67), jarastatin (116), mojastin 1 and 2 (68) and trimestatin (63). Dashed lines represent spacers providing optimal alignment due to the difference in the sequence of jararin.

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Albolatin (23)           VSPPVCGNYYLEVGEDCDCGPPANCQNPCCDATTCKLTPGSQCAEGLCCAQCKFIEEGTVCRVARGDWNDDHCTGQSGDCPWIGYYG
Bilitoxin-1 (117)         SSPPVCGNDILEVGEECDCGCPRNCQDPCCNAATCKKYSWVQCESGECCDQCRFKGAGTECRRAMGDDPGGRCTGQSADCPRN
Bitistatin(P17497)       VSPPVCGNKILEQGEDCDCGSPANCQDQCCNAATCKLTPGSQCNHGECCDQCRFKKARTVCRIARGDWNDDYCTGKSSDCPWNH
Bothrostatin(AAKL5542)   VSTPVSGKNFGAGEECDCGTPGN---PCCDAVTCKLRPGAQCAEGLCCDQCRFMKEGTVCRRARGDDMDDYCNGISAGCPRNPFHA
Jararin(ABD34833)        VSPPFCGNYYPEVGEDCDCGPPANCQNPCCDAATCKLTTGSQCAEGLCCDQCKFIKARQICRRGRGDNPDDRCTGQSGDCPRNS
Jerdonitin (AY364231)    VSPSVCGNYYMEVGEDCDCGPPANCQNPCCDAATCRLTPGSQCADGLCCDQCRFMRKGTICRIARGDDLDDYCNGISAGCPRNPFHA
Salmosin3(AAC42596)      VSPPVCGNYYPEVGEDCDCGPPANCQNPCCDAATCGLTTGSQCAEGLCCDQCRLKKAGTICRRARGDNPDDRCTGQSGVCPRNT
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Figure 3. Amino acid sequences of long length disintegrins. The one-letter code for amino acids is used. The stars indicate the motif commonly referred to as the "RGD adhesive loop" and any RGD-like motif is underlined. Where available, databank accession numbers are given from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed. The amino acid sequences of albolatin (23) and bilitoxin-3 (117) were taken from their primary reference.

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1TEJ-A (9)               NSVNPCCDPVICKPRDGEHCISGPCCNNCKFLNSGTICQRARGDGNHDYCTGITTDCPRNRYNV
1TEJ-B (9)               NSVNPCCDPQTCKPIEGEHCISGPCCENCYFLRSGTICQRARGDGWNDYCTGITPDCPRNRYNV
Acostatin2α(58)          IQPKNPCCDAAPCKLTPGSQCAEGLCCDQCKFIKAGKICRRARGDNPDYRCIGQSGDCPRKHF
Acostatin2β(58)          DAPANPCCDAAPCKLTTGSQCADGLCCDQCKFMKEGTVCRRARGDDLDDYCNGISAGCPRNPF
Agkistin-s (AY071905)    GPPANCQNPCCDAATCRLTPGSQCAEGLCCEQCSFMKEGTVCRIARGDDLDDYCNGISAGCPRNPSHA
Bitisgabonin-1A (Q6T6T3)    NAHPCCDPVTCKPKRGEHCTSGPCCRNCKFLNAGTICKRGRGDSLHDYCTGVTPDCPRNPNKGESDE
Bitisgabonin-1B (Q6T271) ZNSPHPCCDPVTCKPKAWEHCISGPCCRDCKFLRPGTVCRVARGDWNDDFCTGRSSECESNPWNFWNH
Bitisgabonin-2A (Q6T6T2)   NSAHPCCDPVTCKPKRGEHCISGPCCRNCKFLNAGTICKKTMLDGLNDYCTGVTPDCPRNPNKGESDE
Bitisgabonin-2B (Q6T271) ZNSPHPCCDPVTCKPKAWEHCISGPCCRDCKFLRPGTVCRVARGDWNDDFCTGRSSECESNPWNFWNH
CC8A(P83043)             MNSAHPCCDPVTCKPKRGEHCISGPCCRNCKFLSPGTICKKARGDDMNDYCTGISSDCPRNRIKK
CC8B(P83044)             MNSAHPCCDPVTCKPKRGEHCISGPCCRNCKFLSPGTICKKARGDDMNDYCTGISSDCPRNRIKK
Contortrostatin(AAF65171) DAPANPCCDAATCKLTTGSQCADGLCCDQCKFMKEGTVCRRARGDDLDDYCNGISAGCPRNPFH
EC3A(P81630)             NSVHPCCDPVKCEPREGEHCISGPCCRNCKFLRAGTVCKRAVGDDVDDYCSGITPDCPRNRYKGKED
EC3B(P81631)             NSVHPCCDPVKCEPREGEHCISGPCCRNCKFLNAGTICKRAMLDGLNDYCTGKSSDCPRNRYKGKED
EC6A(P82465)             NSVHPCCDPVTCEPREGEHCISGPCCRNCKFLNAGTICKKAMLDGLNDYCTGISSDCPRNRYKGKEDD
EC6B(P82466)             NSVHPCCDPVTCKPKRGKHCASGPCCENCYIVGVGTVCNPARGDWNDDNCTGVSSDCPPNPWNGKPSDN
EMF10A(P81742)           MNSANPCCDPITCKPKKGEHCVSGPCCRNCKFLNPGTICKKGRGDNLNDYCTGVSSDCPRNPWKSEEED
EMF10B(P81743)           ELLQNSGNPCCDPVTCKPRRGEHCVSGPCCDNCKFLNAGTVCWPAMGDWNDDYCTGISSDCPRNPVFK
EMS11A(24)               NSAHPCCDPVKCEPREGEHCISGPCCRNCKFLNAGTICKKAMLDGLNDYCTGISSDCPRNRYKGKEDD
EO4A(24)                 NSAHPCCDPVTCQPKQGEHCISGPCCRNCKFLNSGTICKRARGDNLNDYCTGISSDCPRNPYKSEEED
EO5A(24)                 NSAHPCCDPVTCQPKKGEHCISGPCCRNCKFLNSGTICKKTMLDGLNNYCTGVTSDCPRNRYKDKED
EO5B(24)                 NSAHPCCDPVTCQPKRGEHCISGPCCRNCKFLNSGTVCKRAVGDDMDDYCTGITSCCPRNPYKD
Lebein A (P83253)        NSGNPCCDPVTCKPRRGEHCVSGPCCRNCKFLNAGTICNRARGDDMNDYCTGISSDCPRNPYRD
Lebein B (P83254)        NSGNPCCDPVTCKPRRGWHCVSNPCCDNCKFMRAGTICNRARGDDMNDYCTGISSDCPRNPYRD
Lebetase(CAA66471)       ELLQNSGNPCCDPVYCQPRRGEHCVSGKCCRNCKFLRAGTVCKRAVGDDMDDYCTGISSDCPRNPYK
Piscivostatin 2A(BACC55947) GAVQPKNPCCDAATCKLTPGSQCAEGLCCDQCKFIKAGKICPRARGDNPDYRCTGQSGDCPRKHF
Piscivostatin 2B(BAC55946)  ESDFDAPANPCCDAATCKLTPGSQCAEGLCCDQCKFMKEGTVCHRAKGDLLDDYCNGISAGCPRNPFH
VA6(24)                  NSANPCCDPVTCKPRRGEHCVSGPCCRNCKFLNAGTICRYARGDDMNDYCTGVTSDCPRNPYKS
VB7A(24)                 NSGNPCCDPVTCKPRRGEHCVSGPCCRNCKFLNAGTICKYARGDDMNDYCTGISSDCPRNPYRD
VB7B(24)                 ELLQNSGNPCCDPVTCKPREGEHCISGPCCRNCKFKRAGTVCLDARGDWMNNYCTGISSDCPRN
VLO4(24)                 MNSGNPCCDPVTCKPRRGEHCVSGPCCRNCKFLNAGTICKRARGDDMNDYCTGISPDCPRNPWRG
VLO5B(24)                MNSANPCCDPITCKPRRGEHCVSGPCCRNCKFLNPGTICKRTMLDGLNDYCTGVTSDCPRNPWKSEEKD
VLO5A(24)                NSGNPCCDPVTCQPRRGEHCVSGKCCRNCKFLRAGTVCKRAVGDDMDDYCTGISSDCPRNPYRD
Ussuristatin-2(A59412)   EAGEECDCGAPANPCCDAATCKLRPGAQCAEGDCCEQCRFVKEGTVCREARGDWNDDSCTGQSADCPRNGF
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Figure 4. Amino acid sequences of dimeric disintegrins. The one-letter code for amino acids is used. The stars indicate the motif commonly referred to as the "RGD adhesive loop" and any RGD-like motif is underlined. Where available, databank accession numbers are given from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed. The amino acid sequences of the following disintegrins were taken from primary references: 1TEJ (9), Acostatin2 (4), EMS11 (26), EO4 (26), EO5 (26), VA6 (26), VB7 (26) and VLO4 (26).

aspartate interacts primarily with the beta A residues. In disintegrins, the conserved aspartate (D) residue might be responsible for the binding of disintegrins to integrin receptors which share a beta subunit, while the other two residues of the integrin-binding motif (RG, KG, MG, WG, ML, VG) may dictate the integrin specificity through their interaction with the alpha subunit.

Qinghua (15) constructed a cDNA library from the venom gland of *Agkistrodon acutus* and expressed sequence tags (ESTs) were analyzed. Disintegrins made up 0.05% of the toxin components, with two genes, uni31322309 and uni64408, showing 85.7% and 89.7% identity to genes coding for a disintegrin from *Gloydius saxatilis* and trigramin from *Trimeresurus gramineus*,

respectively. In addition, another gene, uni27922989, had a best protein match by BLASTN for the dimeric piscivostatin's alpha chain from *Agkistrodon piscivorus piscivorus*.

Analysis of the pre-sequence of the cDNA structure of jerdostatin from *Trimeresurus jerdonii* (8) suggested that this disintegrin is not derived from proteolysis of a metalloprotease precursor as the majority of other disintegrins are, but instead is derived from a short-coding gene similar to that of acostatin-alpha, one subunit of the dimeric disintegrin acostatin from *Agkistrodon contortrix contortrix* (20). This would make these the only two disintegrins derived in this way.

A novel alpha V beta 3-blocking disintegrin, Dis*Ba*-01, was isolated from a cDNA library made from the venom gland of *Bothrops alternatus* (17). Three dimensional modeling of Dis*Ba*-01 with alpha V beta 3 predicted a large surface of contacts with the beta 3 subunit. The aspartate D58 in the RGD motif interacted with the metal ion coordinated at the beta 3 subunit MIDAS. The side chains of residues R53 and R56 (within the RGD motif and R73 all point to negative regions of the alpha V subunit, including residues D150 and D218. The C-terminus also exhibit several interactions with both the alpha V and beta 3 subunits.

Although transcriptomic studies have proven to be a valuable asset, Calvete (12) criticized this approach since transcriptomes include mRNA that is not translated. In fact, transcriptomic diversity is larger than proteomic diversity due to non-venom-expressed transcripts such as Eo-10 and Eo1-1 from *Echis ocellatus* that encode "putative dimeric disintegrin subunits" (7). Proteomic studies have proven to be a valuable tool in disintegrin structural studies. Interestingly, some findings of the proteomic approach even go undetected by transcriptomic analysis. Calvete (12) reported that eleven out of thirty-five proteins proteomically identified in *B. gabonica gabonica* were not reported transcriptomically, and thus the cDNA library was missing numerous transcripts of venom-expressed proteins. These investigators also found numerous examples of transcripts encoding putative secreted proteins which could not be found in the proteomic analysis of *B. gabonica gabonica* venom. Clearly, such instances may be a result of limits of technology as well as differences in source material for the DNA versus the protein testing.

While not strictly speaking a classical disintegrin, the discovery of jerdonitin (21), a protein from the venom of *T. jerdonii*, warrants mention. Jerdonitin's cDNA structure shows similarity to agkistin-s (22) and albolatin (23), and forms a new class of P-II SVMPs in having the disintegrin domain remain attached to the inactive metalloprotease domain via a spacer domain. This protein arrangement is made possible by the placement of an additional cysteine residue within both the spacer and disintegrin domains. Proteolytic processing of this pre-protein possibly hinders the release of the disintegrin domain (24). Only jerdonitin has actually been isolated from *T. jerdonii* venom (21), but the disintegrin domain of all three have been expressed by recombinant DNA technology (21, 23, 25), and have all been shown to have inhibitory activities as described below. Both agkistin and jerdonitin possess an RGD motif and are considered monomers, while albolatin has KGD and is thought to be a homodimer.

In addition to proteomic and transcriptomic studies, genomic analyses are a valuable asset in disintegrin evolutionary studies. Although used less often, they provide critical insight into intron sequences that suggest evolutionary pathways. Bazaa (10) performed a genomic study on *Macrovipera lebetina transmediterranea* and *Echis ocellatus* venom gland DNA which was previously analyzed transcriptomically. Interestingly, Bazaa's findings suggest that the evolutionary path of disintegrins began with cell-membrane anchored <u>A</u> <u>D</u>isintegrin <u>and</u> <u>M</u>etalloprotease (ADAM) precursors diverging into PIII SVMPs, which contain disintegrin-like and cysteine-rich domains and three introns. The conceptualized phylogenetic tree proposed that PIII genes evolved, through loss of intron sequences coding for the cysteine-rich domain, first to PII disintegrins with an extra disulfide (like BA-5A (18)) , then to long disintegrins to medium-sized disintegrins, possessing two introns. Loss of one more intron and rearrangement of cysteine linkages led to dimeric disintegrins. With the loss of the final intron arrived the short disintegrins.

As noted above, cDNA Eo1-1 of *E. ocellatus* yielded a WGD-containing polypeptide not found in proteomic studies (7). The reason behind the transcription but not translation of these sequences remains unclear, although Juarez speculated about temporal expression and activation for adaptation to prey habits and evolving ecological niches. Sequences transcribed but not expressed may very well be intermediates in an evolutionary pathway which leads to the "short disintegrin ocellatusin from a short-coding dimeric disintegrin precursor" (7). Sanz (13), using cDNAs, also found a short disintegrin (called CV-short), not found with the proteomic approach, from *C. vipera* that is identical to jerdostatin from *Trimeresurus jerdonii*. This short disintegrin displayed a high resemblance to the KTS-disintegrin obtustatin of *Macrovipera lebetina* and viperistatin of *Vipera palestinae.* One notable difference among these disintegrins is an RTS motif in CV-short and jerdostatin instead of the KTS tripeptide found in obtustatin and viperistatin. Bazaa (10) summarized that "analysis of cDNAs from *M. l. transmediterranea* and *E. ocellatus* venom gland libraries encoding disintegrins argue strongly for a common ancestry of the messengers of short disintegrins and those of precursors of dimeric disintegrin chains."

A major component in evolutionary studies involves phylogenic analysis. In conjunction with genetic and biochemical data, these analysis have led to the conclusion that disintegrin structural diversification can be linked to loss of disulfide bonds and cysteine-rich domains (18). These losses are separate incidences, such that the loss of cysteine-rich domains occurred first followed by the loss of disulfide bonds (18). This finding is a key to disintegrin evolutionary studies. Soto, in a comparison of disintegrins and SVMPs from three Crotalus species, found that the genes encoding these proteins separate into distinct clades. Further analysis suggested that rapid evolution occurred between the genes coding for the SVMPs, and this resulted in the production of the RGD disintegrin genes (16). This agrees with the model of disintegrin evolution proposed by Calvete (12, 26). All of these comparison processes for DNA, RNA and protein have been advanced by the development of data bases like UniProt (7), GenBank (10, 18), NCBI (16) and Swiss-Prot (14).

4.2. NMR and crystallization data

Nuclear Magnetic Resonance (NMR) data has played a major role in advancing knowledge of disintegrin structures. Monleon described obtustatin, a monomeric disintegrin from *Vipera lebetina obtusa* lacking an RGD sequence (27). Interestingly, NMR examination of the backbone Trp20, His27 and Tyr28 suggested a hinge mechanism, allowing increased flexibility of the integrin recognition loop. Similar to the NMR solution structures of echistatin (28), flavoridin (29), albolabrin (30) and kistrin (31), the C-terminus of obtustatin has close proximity to the "RGD loop". What is unique about obtustatin is finding the KTS motif instead of an RGD-like sequence in the RGD loop. This tripeptide is directly involved in giving obtustatin its selectivity for the alpha 1 beta 1 integrin, with Lys21 and Ser23 becoming the functional homologues of the arginine and aspartic acid, respectively, and Thr22 being important for maintaining the conformation of the loop.

Further research of echistatin through an improved homonuclear NMR (32) provided a more conclusive structure. The C-terminal polypeptide of this disintegrin folds parallel to the RGD loop, exposing Pro43, His44, and Lys45. In addition, both the C-terminus and the RGD loop carry a positive charge. Although no hinge effect was observed in the C-terminus, one is found in the base of the RGD loop. As expected, the cysteines arranged in disulfide linkages restrict mobility of the protein. These authors also built a model for an echistatin-alpha V beta 3 complex using HADDOCK software and based on the crystal structure of alpha V beta 3 complexed with an RGD ligand (PDB code 1L5G). The intramolecular contacts proposed by this modeling suggested that Arg26 in eh adhesive loop interacts with Ser123 of the beta 3 chain, as well as the MIDAS cation, while Arg24 associates with Asp150, Tyr178, Gln180, Asp218 and Asp219 of the alpha V subunit. This is in conflict with the crosslinking studies of Yahalom (33), who proposed the RGD motif interacts with beta 3 residues between 109 and 118. Interestingly, the flanking amino acids of echistatin, long proposed to provide disintegrin selectivity (26), interact selectively with the alpha V (Arg22) or the beta 3 (Asp27, Met28, Asp29) subunits. The C-terminus of echistatin has been associated with the affinity of the disintegrin to its integrin binding partner (34). In Monleon's studies, His44 of echistatin associates with Asp150, Ala215, Ile216 and Asp218 of the alpha V subunit, while Lys45 of the disintegrin complexes with Gln214 and Ile216 of alpha V. Note that in this model, there are no interactions between the C-terminus of echistatin and the beta 3 subunit.

A major obstacle to crystallization studies continues to be the amount of disintegrin that needs to be isolated. Fujii (35) attested that NMR and chemical methods lead to poorly defined structures due to the high mobility of the disintegrin backbone scaffold. Therefore, crystallization data is needed to provide definitive structural data. These investigators presented a crystal structure of trimestatin from the venom of *Trimeresurus flavoridis*, resolved to 1.7 angstroms. Their data suggested the RGD-containing loop protrudes at one end of a long axis of the elongated molecule. This would confirm the NMR evidence that the adhesive loop of disintegrins

extends 14-17 angstroms from the core of the molecule (29). They also proposed a docking model for trimestatin within the binding cleft of alpha V beta 3 integrin. Similar to Monleon's studies mentioned above, Arg49 of the RGD motif forms a salt bridge with alpha V residues Asp150, Asp218 or Thr212, while Asp51 binds to a manganese cation at the MIDAS site in the beta A domain. At the C-terminus of trimestatin, the following interactions were proposed by their model: (1) Arg66 forms salt bridges to Asp48 and Asp150 in the alpha V subunit; (2) Trp67 contacts Asp148 in alpha V, while Asn68 has multiple interactions with alpha V, including Thr116, Lys119, Glu121 and Asp148; (3) the only beta 3 interaction for the C-terminal residues is Trp67 of trimestatin and Tyr166 and Asp179 in beta 3. They also discussed the importance of Arg214 in the integrin's beta A domain. Patients with Glanzmann's thrombocytopenia, in which Arg214 is replaced with either Trp or Glu, show deficient binding to fibrinogen, decreasing platelet aggregation activity. The evidence from trimestatin's Pro53 in the RGD loop interacting with Arg214 suggests beta 3's Arg214 may be critical in these ligand binding interactions.

Bilgrami and colleagues have reported the crystal structures of schistatin, a homodimeric disintegrin, at 2.5 angstrom (36) and of a heterodimeric disintegrin named 1TEJ_A/B at 1.9 angstrom resolutions (9), both from the saw-scaled viper *Echis carinatus* using the sitting drop vapor diffusion method. The overall folding of the monomers of both disintegrins is similar. In addition, the arrangement of interchain disulfide bonds is the same for both disintegrins, with two N-terminal disulfide bonds between Cys7 (A chain) and Cys12 (B chain), plus Cys12 (A chain) and Cys7 (B chain). This contrasts with the arrangement found in heterodimeric disintegrin EMF10 from *Eristocophis macmahoni* (37). An analysis of the surface charges showed that they were distributed on one face of the C-terminal domain only, and the charged faces lie opposite to each other, allowing two integrins to bind to the single dimeric disintegrin. Such an arrangement may help explain the receptor clustering phenomenon described for another homodimeric disintegrin, contortrostatin (38).

Fernandez chose to use the NMR structure of salmosin (39) as a template to predict the three-dimensional structure of bothrostatin, a medium-sized disintegrin which was not isolated from the venom of *Bothrops jararaca* but expressed as a recombinant protein only (40). Molecular modeling suggested that the RGD motif is exposed at the tip of a loop and is very close to the C-terminus. In addition, residues Met55, Pro67 and Phe71 form a small hydrophobic core that supports the stability of the loop structure.

1. *IN VIVO* STUDIES

5.1. Tumor inhibition
Due to the well-known anti-integrin activity of disintegrins, and the importance of integrins in all cell function, including cancer cells, numerous investigators have envisioned the use of injected disintegrins as an inhibitory treatment for cancer (41-43). Earlier attempts at such interventions with the anti-platelet properties of

echistatin (44) and kistrin (45, 46), however, clearly demonstrated the long-term immunogenicity of these purified venom proteins. One of the newest ways to surmount the problems with immune recognition while maintaining biological activity of disintegrins *in vivo* is a liposomal formulation. Kang (47) previously described the anti-tumor effect of the disintegrin salmosin from *Gloydius saxatilis* (48). Kim and colleagues developed a unique lipoplex method for delivering salmosin DNA in experimental and spontaneous models of metastasis. For experimental metastasis, B16BL6 mouse melanoma cells were administered via the tail vein into C57BL/6 female mice. DNA (pAAV-CMV-Sal, pCMV-FLAG-Sal, or empty vector) was complexed with cationic liposomes, and the resulting lipoplexes injected subcutaneously once daily near the dorsal midline 1 day before tumor inoculation and then on every fifth day until day 25. Reduction of pulmonary metastases by treatment with pAAV-CMV-Sal and pCMV-FLAG-Sal was 89 and 84%, respectively. For spontaneous metastasis, B16BL6 cells were administered subcutaneously into the foot pad of C57BL/6 female mice. The primary tumors were surgically removed by amputating below the knee when they reached a volume of 400–600 cubic millimeters. The lipoplex solutions were injected subcutaneously once into the dorsal midline 1 day before tumor removal and then on every fourth day until day 25. The number of large colonies in the lungs of mice treated with pAAV-CMV-Sal or pCMV-FLAG-Sal was reduced by 92 and 88%, respectively. It was suggested that this anti-tumor effect is based on the anti-angiogenic property of salmosin. This was indirectly supported by inhibition of bovine capillary endothelial (BCE) cell growth by the salmosin peptides released from transfected human kidney 293 cells. The salmosin added to the culture media inhibited BCE cells in a dose-dependent manner and also induced a change in the morphology of the cells into a rounded shape.

Swenson and colleagues (41) created liposomes composed of disteroylphosphatidylchlorine, cholesterol, and polyethylene glycol-derived lipid and containing the dimeric disintegrin contortrostatin. While a daily intratumor injection of the disintegrin itself showed anti-tumor efficacy in an orthotopic xenograft model (49), such a mode of delivery was not clinically translatable. However, intravenous administration of a liposome-encased contortrostatin at varying doses to immunodeficient mice showed significant improvement in a number of parameters. Liposomal contortrostatin lasted markedly longer in the circulatory system, was passively accumulated in the tumor, did not react with platelets, and did not enlist an immune system response. The liposomal contortrostatin did not inhibit platelet aggregation until its lipid casing was disrupted. Incubation from 1-2 hours was not long enough to accomplish this disruption; however, a 20 hour incubation resulted in complete release of contortrostatin. Once this occurred, its 50% inhibitory concentration (IC50) was measured as 60 nM, which is comparable to its *in vitro* inhibition of ADP-induced platelet aggregation (50). Liposomal contortrostatin also showed potent anti-angiogenic activity. Thus it is clear that a liposomal formulation solves many of the problems in

biological activity retention. The administration of the liposomal contortrostatin resulted in a reduction in tumor growth, as well as a reduction in microvascular density of tumor cell area in the MDA-MB-435 breast cancer tumor line. The liposomes were much more effective in reducing tumor growth volume than either the control or the non-liposomal contortrostatin.

In subsequent research, Minea (51) used the same liposomal encapsulation procedure but instead encased a recombinant monomeric contortrostatin. This study showed "native" or non-liposome-encased contortrostatin inhibited tumor growth less effectively than the newly designed liposome-encased recombinant contortrostatin. Swenson also reported in 2005 that contortrostatin inhibited angiogenesis and tumor progression *in vivo* using the A2780SEAP human ovarian cancer cell line (49). A liposomal formulation similar to Swenson's previous research was used.

Dis*Ba*-01 from *Bothrops alternatus* (17) prevented lung colonization by a B16F10 melanoma sub-clone (B16F10-2B8) transfected with the luciferase gene, allowing non-invasive bioluminescent imaging. This activity was both time- and dose-dependent. Excised lungs at day 14 were treated with a bolus of 2 mg/kg of Dis*Ba*-01, which resulted in a 72.8% reduction of the surface covered by pigment melanoma foci. Histological analyses of these lungs confirmed the decreased number of micrometastases.

Another unique *in vivo* use of disintegrins was done when Hallak (52) combined the disintegrin echistatin with a measles virus. The measles virus echistatin vector (MV-ERV) caused regression of multiple myeloma tumors in a xenograft model of Chinese hamster ovary cells. The tumors were either completely eradicated or growth was severely retarded. The MV-ERV destroyed newly formed capillaries *in vivo* using the chorioallantoic membrane (CAM) angiogenesis assay. On the other hand, the Edmonton parental strain of measles virus (MV-Edm) had no effect on capillaries. The vector reproduced normally with the echistatin. The echistatin was critical for the virus to recognize the alpha V beta 3 receptor, through which the measles virus could gain entrance into the cells through either this integrin or the virus's natural CD46 receptor. In addition, an *in vitro* angiogenesis assay showed human umbilical endothelial cells (HUVECs) infectable by both MV-Edm and MV-ERV, but only MV-ERV could infect HUVEC capillary-like tubules formed on matrigel.

McQuade investigated the use of bitistatin (a disintegrin targeting alpha V beta 3 and alpha IIb beta 3 integrins) to determine whether it would target tumors in mice (53). Radiolabeled bitistatin was injected into mice carrying EMT-6 mouse mammary carcinoma tumor cells. The bitistatin was radiolabeled with (125)I or a beta-emitting radionuclide, (64)Cu, attached to bitistatin via 1,4,7,10-tetraazacyclododecane-N,N',N' ',N' "-tetraacetic acid (Cu64-DOTA). Copper was used because it is highly effective in positron emission tomography (PET) studies as well as being a radiotherapy agent. After it was confirmed,

by *in vitro* ADP-induced platelet aggregation, that no functional change in the disintegrin function was induced by the placement of the radiolabel, micro PET images where done on all mice at 4, 6, and 15 hours after lateral tail vein intravenous injection with Cu64-DOTA-bitistatin. The mice receiving the Cu64-DOTA-bitistatin had a significantly higher tumor uptake than the mice receiving the Cu64-DOTA and unlabeled bitistatin at 6 and 15 hours, suggesting receptor-mediated uptake. There was no difference at 4 hours time. The investigators noted that tumor uptake of Cu64-DOTA-bitistatin was 87% lower than with the I125 analogue. One possibility was a labeling phenomenon involving a lysine close to the integrin binding site of bitistatin. To test this, the authors replaced DOTA with bromoacetamidobenzyl-1,4,7,10-tetraazacyclododecane-N,N',N ',N' "-tetraacetic acid (Cu64-BAD), which was hypothesized to lessen any steric interactions, and repeated the tumor uptake assay. As with the DOTA conjugate, maximum tumor uptake did not occur before 6 hours post injection. However, tumor levels were 3-4 times higher at these time points compared with the DOTA conjugate. The mice where then euthanized and a biodistribution study was done to determine the percent dose per gram of radioactive material. The peak uptakes where 11.7 %ID/g for I125-bitistatin at 2 hours, 1.55%ID/g for Cu64-DOTA-bitistatin at 6 hours, and 5.18 %ID/g for Cu64-BAD-bitistatin, also at 6 hours. These results are similar to those for much smaller RGD-containing peptides. It is important to note that the radiolabeled bitistatin accumulates in tumors that do not themselves express the alpha V beta 3 integrin. From biodistribution studies it can be concluded that the I125 bitistatin accumulates much better in the tumor then the Cu64-DOTA-bitisatin.   The   Cu64-BAD-bitistatin accumulates well in the tumor, but also accumulates in other organs as well, making it potentially dangerous.

Yang (43) showed that the disintegrin trigramin, a medium-length disintegrin from the venom of *Trimeresurus gramineus*, inhibited growth of breast cell cancer MDA-MB-231 when both were locally injected into the bone marrow cavity of tibia of nude mice. Quantitative assessment of osteolytic lesions and tumor size showed that trigramin treatment (100 μg) inhibited each by 71.5% and 72.7%, respectively.

5.2. Angiogenesis
Kim *et al* (54) studied the effects of combinational transfer of genes from angiostatin K1-3 (a protein with a sequence identical to the first three triple-loop structures of plasminogen), endostatin (a carboxy-terminal fragment of collagen XVIII) and the 73-residue disintegrin saxatilin (from *Gloydius saxatilis*) on angiogenesis and tumor progression in a mouse model using a matrigel-impregnated B16Bl6 melanoma cell line implanted into the abdomens of C57BL/6 mice. They reported that the most effective combination of genes was the pFlag-angioK1/3, pFlag-Endo, and Pflag-Sax compared with any other combinations of these genes. Using hydrodynamic cotransfection, the three genes reduced tumor growth by 89 % and pulmonary metastasis

by 90% compared to the control group. The authors indicated that a combination of disintegrin genes may be the best way to develop an effective anti-angiogenic cancer therapy.

Obtustatin is a unique disintegrin in that it is the smallest (41 amino acids), possesses a KTS motif within a shortened adhesive loop, making it situated to the side of that loop rather than at the tip, as found in the majority of other disintegrins, and is selective for the alpha 1 beta 1 integrin (55). Marcinkiewicz and colleagues (56) showed that obtustatin potently inhibited new vessel development in the chicken chorioallantoic membrane model of angiogenesis by ~80%, compared to another structurally related disintegrin, eristostatin, which was not active in this system. These experiments strongly pointed to the importance of the alpha 1 beta 1 integrin in angiogenesis. Moreover, obtustatin significantly inhibited the development of Lewis lung carcinomas in a syngeneic mouse model, reducing tumor sizes more than 50% after 1 week of treatment of established tumors.

The inhibition by lebestatin of HMEC adhesion to type IV collagen led Olfa and colleagues to investigate whether this disintegrin could affect angiogenesis in a chick CAM assay (57). Topical application of lebestatin (0.1 microgram per embryo) inhibited spontaneous angiogenesis in a dose-dependent manner. While platelet-derived growth factor (PDGF) and VEGF both had a pronounced positive effect on angiogenesis, lebestatin, at 0.5 micrograms per embryo, caused abrupt arrest of capillary development. The authors noted comparably less activity of another KTS disintegrin, obtustatin, reported in a similar assay (56), and that this correlates with the difference in inhibitory potencies of these two disintegrins in adhesion assays.

The disintegrin Dis*Ba*-01 from *Bothrops alternatus* (17) suppressed *in vivo* angiogenesis induced by basic fibroblast growth factor in a matrigel plug injected subcutaneously in athymic nude mice. Endothelial cell number was significantly and dose-dependently reduced, at 23% with 20 nM and 61% with 1000 nM disintegrin. New vessel formation was also markedly decreased to 7.7% of control at 1000 nM Dis*Ba*-01 (IC50 = 83 nM).

Agkistin-s (25) was used in a 10-day-old chick CAM assay and showed effective anti-angiogenic effect. Spontaneous angiogenesis was markedly reduced without affecting preexisting blood vessels. Tube formation with bovine aortic endothelial cells cultured in sandwich collagen gels was strongly prevented by agkistin-s in a dose-dependent manner. In contrast, Tian investigated the ability of the short monomeric disintegrin eristostatin to block angiogenesis (58). Compared to echistatin, which effectively inhibited angiogenesis in an 8-day old quail chick CAM model, eristostatin had no such effect. The authors hypothesized that the difference is based on eristostatin's lack of activity with the alpha V beta 3 integrin.

Table 1. Comparison of disintegrin inhibitory effects in cell adhesion assays[1]

Cells	Integrin	Ligand	IC50 (nM)							
			Obtustatin	Viperistatin	Echistatin	Lebestatin	VLO5	VLO4	EC3	EO5
alpha 1K562	Alpha 1 beta 1	Coll I	0.5	0.02	>10,000					
alpha 1K562	Alpha 1 beta 1	Coll IV	2	0.08	>10,000		>10,000		>10,000	>10,000
PC12	Alpha 1 beta 1	Coll I				0.2				
PC12	Alpha 1 beta 1	Coll IV				0.4				
alpha 1CHO	Alpha 1 beta 1	Coll I				0.2				
alpha 1CHO	Alpha 1 beta 1	Coll IV				0.4				
eosinophils	Alpha 1 beta 1	Coll IV	1150	11						
alpha 2K562	Alpha 2 beta 1	Coll I	>10,000	>10,000	>10,000		>10,000		>10,000	>10,000
alpha 2K562	Alpha 2 beta 1	Coll IV	>10,000	>10,000	>10,000					
Jurkat	Alpha 4 beta 1	VCAM-1	>10,000	>10,000	>10,000		5.5		16	9.5
Ramos	Alpha 4 beta 1	VCAM-1					1.2		22	5.3
RPMI8866	Alpha 4 beta 7	MadCAM-1					185		17	120
RPMI8866	Alpha 4 beta 7	MadCAM-1					185		17	120
K562	Alpha 5 beta 1	FN	>5000	>10,000	50		3,400		150	690
alpha 6K562	Alpha 6 beta 1	LM	>10,000	>10,000	>10,000		>10,000		>10,000	>10,000
HT29-D4	Alpha 6 beta 4	LM				>10,000				
SW480-alpha9	Alpha 9 beta 1	VCAM-1	>10,000	>10,000	>10,000		2.6		35	7.2
SW480-alpha9	Alpha 9 beta 1	OP					34		>10,000	
SW480-alpha9	Alpha 9 beta 1	TSP, NoC1					100% blocked[2]	25% blocked[2]		
SW480-alpha9	Alpha 9 beta 1	TN-C					29		>10,000	
CHO-A5	Alpha iib beta 3	FG	>10,000	>10,000,	50		760		500	980
JY	Alpha v beta 3	VN	>10,000	>10000	0.01		>10,000		>10,000	>10,000
IGROV1	Alpha v beta 3	FG				>10,000				
HT29-D4	Alpha v beta 5	VN				>10,000				
HT29-D4	Alpha v beta 6	FN				>10,000				

[1]Data summarizes the findings from Kisiel (66), Olfa (57), Staniszewska (59),Bazan-Socha (60), Bazan-Socha, (95), Coll, collagen; LM, laminin; VCAM-1, vascular cell adhesion molecule-1; FG, fibrinogen; FN, fibronectin; VN, vitronectin; TSP, thrombospondin; NoC1, C-terminal domain of TSP; OP, osteopontin; Tn-C, tenascin-C. K562, SW480 and CHO cells were transfected with the indicated integrin. For other cells, the indicated integrin is native to the cell line. Blank cells =no activity reported. [2]IC50s were not calculated; data is expressed as adhesion blockage (inhibition) percent compared with control

The disintegrin VLO5, containing the MLD sequence in its binding loop, blocks proliferation and angiogenesis of dermal human microvascular endothelial cells (dHMVEC) expressing alpha 9 beta 1 integrin, as well as similar cells from brain (bHMVEC) which do not express this integrin (59). The phenomenon was partially explained by the known interaction of VLO5 with alpha 4 beta 1 integrin, which is present on all endothelial cells (60), albeit in low amounts. The inhibition of angiogenesis was also seen in experiments using matrigel plugs implanted into mice. The positive effect in the number of vessels in the presence of thrombospondin C-terminal fragment, NoC1, was abolished in the presence of VLO5.

6. *IN VITRO* STUDIES

6.1. Platelet aggregation

Disintegrins were first discovered as non-enzymatic venom components which facilitated hemorrhage in an envenomed subject (61). One of the hallmarks of disintegrin function is their ability to interact with, and many times prevent the activity of, cell surface receptors, most notably integrins. Many types of cells have been used in studies involving adhesion and integrin function (endothelium, bone, platelets, cancer), with platelet aggregation (PA) being by far the most frequently reported. We have previously compared monomeric and dimeric disintegrin inhibitory profiles with integrin alpha IIb beta 3 for the 71 disintegrins described in the literature from 1998 – 2004 (62). This comparison showed a wide variety of potencies: 50% inhibitory concentrations (IC50) less than 400nM to over 1000 nM in collagen-induced PA; less than 100 nM to over 1000 nM for ADP-induced PA; 28 – 1200 nM in thrombin-induced PA; 7-165 nM with tumor-induced PA. It was also evident that the tripeptide sequence in the RGD loop was critical in determining the potency when comparing monomeric to dimeric disintegrins. When the triad was "RGD", IC50s were always less than 200 nM, indicating significant potency, regardless of being monomeric or dimeric. As the tripeptide substituted arginine and/or glycine with residues like methionine, valine or leucine (MGD, MLD, VGD), the inhibitory activity against alpha IIb beta 3 would decrease to IC50 values between 300 to 1800 nM. Surprisingly, dimeric disintegrins with at least one KGD (piscivostatin (63), ussuristatin 2 (64), VB7 (26)) also showed decreased

ability to inhibit this receptor. This is surprising because monomeric disintegrins containing KGD are not only potent but also highly selective for alpha IIb beta 3 (65). Finally, obtustatin (56) and viperistatin (66), the shortest disintegrins, both with KTS rather than RGD in the adhesive loop, show no potency at all with the integrin alpha IIb beta 3, with IC50s over 10,000nM.

Disintegrins described since 2004 show a similar variability in their inhibition of agonist-induced platelet aggregation (Table 1). Four disintegrins have been expressed as recombinant proteins and have not actually been isolated from the snake venom, itself: bothrostatin, albolatin, agkistin-s and jerdostatin. r-Bothrostatin, a medium-sized disintegrin with 73 amino acids, was derived from a cDNA library of the venom gland of *Bothrops jararaca* (40). It shows identity with the disintegrins jarastatin (86%) and jararacin (81%), both isolated from this same snake. In collagen-induced platelet aggregation, r-bothrostatin inhibited with an IC50 of 12 nM. A portion of the disintegrin domain of albolatin from *Trimeresurus albolabris*, homologous to medium-sized disintegrins containing 13 cysteines and forming a homodimer, was produced in yeast and inhibited collagen-induced platelet aggregation with an IC50 of 976 nM (23). Both full-length agkistin and the disintegrin domain, agkistin-s, from *Agkistrodon halys*, were expressed in a baculovirus cell culture system. Both showed inhibition in ADP-induced platelet aggregation, but the authors did not calculate IC50 values. The light transmittance graphs suggested an IC50 of ~200 nM for agkistin and ~100 nM for agkistin-s. Recombinant jerdostatin from *T. jerdonii* was cloned and produced from cDNA amplified from venom glands of *Trimeresurus jerdonii* collected from Yiliang, Yunnan, China (8). After in-gel tryptic digestion, mass fingerprinting and collision-induced dissociation by tandem mass spectrometry, two recombinant isoforms were isolated, with both having the wild-type arginine residue at position 21. Neither isoform inhibited interaction between integrin alpha IIb beta 3 and its natural ligand, fibrinogen. There were, however, significant differences in the two isoforms' inhibition with other integrins, discussed below. Another protein found in this snake with disintegrin similarities, jerdonitin, inhibited ADP-induced platelet aggregation with an IC50 of 120 nM. This is noteworthy since jerdonitin does not fall into the "classical" disintegrin category, but belongs to a new class of P-II SVMPs possessing metalloprotease-spacer-disintegrin domains. The authors did not describe the metalloprotease activity of this protein, but this should be an interesting future investigation.

Galán purified two disintegrins from the venom of a *Crotalus horridus* specimen collected in Pleasanton, Tennessee by the staff of the Natural Toxins Research Center, Kingsville TX (67). Horrdistatin 1 and horrdistatin 2 are nearly identical in sequence, differing by only 220 daltons due to the C-terminal Tyr-Gly found in horrdistatin 1 (Figure 3). They also show similar inhibitory potency in ADP-induced platelet aggregation using a whole blood aggregometer: IC50 of 12.5nM for horrdistatin 1 and 16.2 nM for horrdistatin 2.

Sanchez and colleagues isolated and characterized two monomeric disintegrins from the venom of the Mojave rattlesnake, *Crotalus scutulatus scutulatus* (68). Mojastin 1 and 2 contained 71 and 73 amino acids, respectively, with masses of 7.436 and 7.636 kDa, and had 97% sequence identity to each other (Figure 3). Mojastin 1 was actually identical in sequence to horrdistatin 2, isolated from *Crotalus horridus* (67). In whole blood platelet aggregation, using ADP as agonist, the IC50 of the two disintegrins was also identical, at 13.8 nM.

In developing an anti-thrombotic molecule based on disintegrins, it is critical to have the molecule not be immunogenic. Jing and colleagues used an octapeptide sequence (CAKGDWNC), derived from the integrin barbourin (69), and produced a chimera by introducing it into an inactive human proinsulin molecule (70). Expressed as a recombinant protein in *E. coli*, the chimera inhibited ADP-induced platelet aggregation (IC50 = 830 nM) while not binding to the insulin receptor. The investigators hypothesized that the chimera would not be immunogenic since its CD spectrum suggests it has very close secondary structural properties compared with native insulin. The chimera showed specificity for the alpha IIb beta 3 integrin by inhibiting the attachment of K2 melanoma cells to fibrinogen-coated wells but not to fibronectin-coated wells.

In a comparison of echistatin to the anti-platelet orally active drugs orbofiban, roxifiban, eptifibatide and tirofiban, Hantgan and colleagues (71) reported that all of the drugs bound to platelets, inhibited platelet aggregation and adhesion to fibrin better than echistatin. In addition, analytical centrifugation revealed that the drugs perturbed the resting alpha IIb beta 3 receptor less than echistatin did. The authors proposed that the same mechanism used for the binding of the drugs or disintegrin with the receptors is responsible for interfering with the integrin's resting conformation. The authors further proposed that their findings raised some questions as to whether or not integrin antagonists will be effective drugs, or even safe ones.

The RGD sequence of disintegrins has long been recognized as the most active site on the disintegrin. Kim (72) designed RGD peptides based on the sequence of the disintegrins salmosin (39) and saxatilin (54), which bind strongly to the integrin alpha V beta 3. Linear RGD-5 and circular RGD-6 were compared in integrin binding and platelet aggregation assays. As predicted, the circular RGD-6 (Ace-CARGDDC-NH2) was more active in both assays than linear RGD-5 (Ace-ARGDD-NH2). RGD5 (linear) showed an IC50 of 91 micromolar, while RGD6 (cyclic) and saxitilin showed IC50s of 3.6 and 0.074 micromolar, respectively. Kim also looked at the effect of electrostatic potential distribution on the RGD sequence. The more electropositive RGD sequences had higher (and hence, weaker) IC50s. Kim concluded by suggesting that these structural/functional differences could be helpful insights for the development of drugs.

Reiss and colleagues compared RGD disintegrins to KGD and KTS disintegrins in their 2006 study (73). Reiss introduced small peptides containing RGD or KGD

sequences into scaffolds of a variant of obtustatin (55) or the following cysteine knot microproteins: trypsin inhibitor EEET-II (74) and the melanocortin receptor binding domain of the human agouti-related protein AGRP (75). The scaffolds receiving the RGD sequence showed a significantly greater inhibition of fibrinogen and platelet aggregation then the original RGD counterpart. However, the scaffolds derived from the disintegrin barbourin, receiving the KGD sequence, were poor platelet aggregator inhibitors. This is unexpected since the sequence KGD has been shown, at least in barbourin, to bestow specificity for the alpha IIb beta 3 integrin (69). Noteworthy is the increased aggregation inhibitory potency of the obtustatin variant when a KGD motif is added, since obtustatin is selective for alpha 1 beta 1 integrin, and is a poor inhibitor of platelet aggregation. The authors concluded that novel functionality can result when RGD/KGD motifs are grafted onto recipient proteins.

6.2. Cell adhesion

Neither mojastin 1 or mojastin 2 were effective in the inhibition of T24 human urinary bladder carcinoma cells to fibronectin compared with the disintegrin echistatin, which inhibited this interaction with an IC50 of 256nM (68). This was surprising since crotatroxin, a closely related disintegrin also containing the sequence RGDW, could inhibit M21 melanoma cell adhesion to fibronectin (76). The authors hypothesized that T24 cells bind fibronectin via alpha V beta 3 or alpha V beta 5 rather than alpha 5 beta 1, and that neither mojastin disintegrin must interact with the alpha V receptors. Data from Scarborough had suggested that RGDW disintegrins, like mojastin 1, 2 and crotatroxin, would be inhibitory against alpha 5 beta 1integrin. In contrast, the work of Wierzbicka (34) and Juliano (77), using the RGDW disintegrin eristostatin, both showed no inhibitory activity of this disintegrin against alpha 5 beta 1.

Glioblastoma multiforme is a particularly malignant cancer which is resistant to surgery, radiation and chemotherapy due to tumor cell invasion and angiogenesis. Schmitmeier and colleagues asked (1) does soluble and/or immobilized contortrostatin (CN) bind to integrins in a manner similar to fibronectin (FN) and (2) is contortrostatin capable of interrupting cell signaling in these cells (78). Treatment of suspended A-172 and U87 glioma cells with either soluble FN or CN resulted in tyrosine phosphorylation of FAK, paxillin and p130Cas. It was noted, however, that much higher concentrations of FN were needed than CN to get the same effect. Similar results were found when the ligands were immobilized onto plates. Addition of soluble CN to FN-adherent glioma cells caused a significant reduction in the cellular phosphorylation levels, and ultimately caused the cells to round up and partially detach. Glioma cells also migrated more slowly on CN than FN. Interestingly, these effects of CN were common for both glioma cells lines despite their having differences in alpha V beta 3 expression. Therapeutic implications of these findings are promising.

Kisel compared the activities of obtustatin, viperistatin and echistatin on various integrins in a cell adhesion assay (66). Table 1 summarizes the data and illustrates the selectivity of the KTS disintegrins for alpha 1 beta 1 integrin. Additionally, viperistatin was significantly more potent in its inhibition. Only three amino acids are different in the two disintegrins: L24/R, L38/V and P40/Q in the binding loop, hydrophobic core and C-terminus, respectively. The authors used linear peptides, created from the binding loops of the two disintegrins, in a series of adhesion and ELISA assays. They found that replacement of a leucine (as in obtustatin) with an arginine (as in viperistatin) increased by 6- to 10-fold the inhibitory activity towards alpha 1 beta 1. This once again highlights the critical role of the residues adjoining the "RGD" motif for enhancing the inhibitory activity of these proteins.

Elegantin is a specific antagonist of the alpha 5 beta 1 integrin (79). Sumathipala investigated potential alpha 5 beta 1 binding domains within this disintegrin (80) by creating seven chimeric disintegrins from sequences of elegantin and kistrin (from *Calloselasma rhodostoma*), specifically changing the integrin binding loop, the C-terminus and the "linker domain" just N-terminal to the binding loop. In studies assessing inhibition of binding of K562 cells, expressing alpha 5 beta 1 integrin, to immobilized fibronectin, chimeras with the wild type ARGDN were strong inhibitors regardless of the sequences at the linker or C-terminus. Ala50 was critical to this inhibition. Those chimeras without elegantin's linker domain, XRXKKKRTXX, were weak inhibitors of the adhesion of Chinese hamster ovary (CHO) cells, transfected with alpha 5 subunit, to fibronectin. The precise interaction site for elegantin's linker on integrin alpha 5 beta 1 was not identified in this study, but data suggested it is located within residues 10232 on the alpha 5 subunit.

For the first time, disintegrins were used in studies of adhesion of alpha 9 beta 1 integrin to nerve growth factor (NGF) (81). The heterodimeric disintegrin VLO5, selective for alpha 4 and alpha 9 integrins, inhibited adhesion of alpha 9-transfected SW480 cells to NGF while the RGD-containing dimeric disintegrin VLO4, selective for alpha 5 beta 1, did not. VLO5 also inhibited intracellular signaling, migration and proliferation of these cells, as described below.

The monomeric disintegrin echistatin is undoubtedly the most commonly used venom protein in cellular functional studies. Alimenti (82) reported that echistatin detached mouse GD25 cells, which do not express beta 1 integrins, from immobilized fibronectin. This phenomenon was specific, non-toxic, dose- and time-dependent. In an interesting aspect of the study, echistatin was more potent in promoting cell detachment of wild type GD25 cells from vitronectin than GD25 cells transfected with beta 1 subunit. The opposite was true with the transfected cells regarding detachment from fibronectin.

Echistatin also increased the adhesion of quail epiphyseal chondrocytes (in suspension to collagen II by 40 percent while kistrin and flavoridin were less effective (83). Echistatin's activity was proposed to be through a receptor-mediated mechanism. The binding in echistatin's presence was a function of collagen density. With fibronectin as the

Table 2. Comparison of disintegrin inhibitory effects in cell adhesion assays[1]

Cells	Integrin	Ligand	IC50 (nM)		
			Bitisgabonin-1	**Bitisgabonin-2**	**DisBa-01**
Jurkat	alpha 4 beta 1	VCAM-1	312	32.6	
K562	alpha 5 beta 1	FN	12.4	167	
SW480-alpha9	alpha 9 beta 1	VCAM-1	528	78	
HMEC-1	alpha v beta 3	VN			555
B16F10	alpha v beta 3	VN			225

[1]Data summarizes the findings from Calvete (12), Ramos (17). VCAM-1, vascular cell adhesion molecule-1; FN, fibronectin; VN, vitronectin. SW480 cells were transfected with the indicated integrin. For other cells, the indicated integrin is native to the cell line. Blank cells =no activity reported.

immobilized ligand, all three disintegrins inhibited adhesion in a dose-dependent manner. Investigations into the integrin partner bound by echistatin suggested alpha V beta 3 rather than alpha 5 beta 1. In addition, there was evidence that engagement of alpha V beta 3 may down-regulate beta 1 affinity in the chondrocytes. The authors linked this affinity change to the chondrocytes' ability to transition rapidly from a suspension to an adherent state.

In a 2004 study by Li (84), echistatin decreased attachment of XR1 retinal glial cells (from Xenopus frogs) to fibronectin coated on coverslips. Its potency was 700 fold greater than the linear peptide GRGDSP, and trypan blue exclusion analysis revealed no viability difference between untreated and echistatin-treated cells. Cells exposed to the disintegrin showed disruption of retinal lamination, particularly in the outer retina, and the formation of ectopic photoreceptor rosettes was reduced. Signaling experiments, described below, pointed to an integrin involvement in retinal morphogenesis.

The alpha V beta 3-blocking disintegrin, DisBa-01, from *Bothrops alternatus* (17) possesses the RGD motif and inhibited adhesion of human microvascular endothelial cell line-1 (HMEC-1) and murine melanoma cell line B16F10 to vitronectin with IC50s of 555 and 225 nM, respectively (Table 2).

Olfa (57) studied the KTS disintegrin lebestatin in cell adhesion and found that it inhibited alpha 1 beta 1 integrin-mediated cell adhesion on collagen types I and IV (Table 1) but not adhesion involving alpha 2 beta 1, alpha V (beta 3, 5 or 6), alpha 5 beta 1 or alpha 6 beta 4. The authors noted the similarity between the viperistatin and lebestatin amino acid sequence compared with obtustatin (Figure 1). Lebestatin showed a 10-fold increased in adhesion inhibitory potency against alpha 1 beta 1 when compared with obtustatin. This change was attributed to the presence of Arg24 in place of Leu24 in the adhesive loop adjacent to the KTS motif. Kisiel observed this change with viperistatin (66). In addition, lebestatin partially inhibited adhesion of human microvascular endothelial cells (HMEC-1) to type IV collagen.

Yang (43) showed that the disintegrin rhodostomin, a medium-length disintegrin from the venom of *Calloselasma rhodostoma*, inhibited breast (MCF-7 and MDA-MB-231) and prostate cancer cell (PC-3) adhesion to unmineralized and mineralized bone in a dose-dependent manner, and without affecting tumor cell viability. It accomplishes this without apoptosis. The action mechanism of rhodostomin in this adhesive inhibition was mediated predominately by blocking alpha V beta 3 integrin.

Bazan-Socha demonstrated the biological activities of the MLD-containing dimeric disintegrins, EC3, VLO5 and EO5. All three are potent inhibitors of alpha 4 beta 1, alpha 4 beta 7 and alpha 9 beta 1 integrins while inactive against numerous others (Table 1). EC3 was more active, by over one order of magnitude, than VLO5 in inhibiting alpha 5 beta 1 adhesion to fibronectin, while EC3 surpassed VLO5 and EO5 in activity against alpha 4 beta 7 adhesion of MadCAM-1. Confirming platelet aggregation studies, the three disintegrins all showed IC50s above 500 nM in adhesion assays using fibrinogen and alpha IIb beta 3. With Jurkat and Ramos cell suspensions, both of which possess alpha 4 beta 1 integrin, EC3, VLO5 and EO5 had adhesion IC50 values of 16.5, 5.5 and 9.5 nM, respectively. Using peptides derived from the adhesive loop of the 3 disintegrins, the investigators showed that CKRTMLDGLNDYC (shared by EO5 and VLO5) was more active in inhibition of alpha 4 beta 1 and alpha 9 beta 1 than CKRAMLDGNDYC (found in EC3). This could explain the selectivity of the first 2 disintegrins for alpha 4 and alpha 9 integrins versus EC3's selectivity against the RGD-dependent integrins, alpha 5 beta 1 and alpha IIb beta 3. An interesting observation was made regarding species selectivity of these heterodimeric disintegrins. Mouse melanoma cell line B16 adhered more potently to EC3 than to either VLO5 or EO5. In contrast, human melanoma cell line HS.939T adhered much better to VLO5 and EO5. This appeared to be related to cell expression of alpha 4 beta 1. One added technical note from this work was the description of a new method for purification of alpha 4 beta 1 and alpha 9 beta integrins (from Ramos cells or alpha 9-transfected SW480 cells, respectively) by using a resin column coupled with the disintegrin VLO5.

Calvete analyzed two dimeric disintegrins isolated from the venom of the snake *Bitis gabonica gabonica*: bitisgabonin-1 (containing RGD/RGD subunits) and bitisgabonin-2 (containing MLD/RGD subunits) (12). Against the alpha 5 beta 1-expressing K562 cell line, bitisgabonin-1 and 2 showed an adhesion IC50 of 12.4 and 167 nM respectively. When tested on the Jurkat cell line (alpha 4 beta 1), the IC50s were 312 and 32.6 nM respectively, while the alpha9-Sw480 cell line (alpha 9 beta 1) showed IC50s of 528 and 78 nM respectively (Table 2).

6.3. Cell signaling and motility

Tian and colleagues performed *in vitro* studies with the disintegrin eristostatin to determine its effect on the migration of six human melanoma cell lines: radial growth phase SBCl2, vertical growth phase WM164, and metastatic MV3, 1205LU, C8161 and M24met (58). Eristostatin interfered with migration using transwell migration and *in vitro* wound closure assays. Eristostatin inhibited cell migration on fibronectin in a dose-dependent manner but had no effect on collagen IV or laminin. However, no effect was detected on cell proliferation or angiogenesis. The authors suggested that eristostatin interacts with the melanoma cells through fibronectin-binding integrins. Eristostatin has been described as being selective for the alpha IIb beta 3 integrin (2, 34). Since none of the melanoma cells expressed this integrin, these investigators suggested that a different mechanism and/or integrin is involved in eristostatin's inhibitory action then traditionally thought. It is unlikely that the integrin involved would be alpha 5 beta 1 since several investigators (34, 77) have shown that this disintegrin does not interact with the alpha 5 integrin.

Ren and colleagues in 2006 provided evidence that agkistin-s, a recombinant disintegrin from the domain of the SVMP agkistin, inhibited bovine aortic endothelial cell (BAEC) migration on gelatin in a dose-dependent manner (25). Agkistin-s also inhibited proliferation and migration with *in vitro* tests.

Skeletal unloading is a phenomenon experienced by astronauts in space flight and patients on bed rest. It is associated with a loss of weight bearing in these individuals, and causes osteopenia (85). Sakata used the small monomeric disintegrin, echistatin, to investigate whether integrins are involved in IGF-I resistance induced by skeletal unloading (86). Echistatin mimicked results induced by unloading on bone marrow osteoprogenitor cells: inhibition of IGF-I-stimulated phosphorylation of the IGF-I receptor and reduced mRNA levels both of alpha V and beta 3 integrin subunits in the cells. Interestingly, this inhibition was not associated with a concurrent alteration of IGF-I binding to its receptor. The authors concluded that resistance to IGF-I- induced by unloading can be attributed to decreased integrin alpha V beta 3/IGF-I receptor interaction.

Li used echistatin with retinal glial cells to assess the influence of integrins on retinal development (84). Cells exposed to this disintegrin rarely formed focal adhesions, and the F-actin cytoskeleton was severely disorganized. Many cells showed round or spindle-shaped morphology at echistatin exposure levels of 2.5 micrograms per milliliter. At higher doses, the cells retracted and detached. This effect was not due to cytotoxicity. The authors hypothesized that echistatin disrupted integrin-mediated XR1 cell interactions with extracellular matrix, and this blocked cell spreading, focal adhesion assembly and formation of actin stress fibers.

Kim demonstrated that saxatilin inhibited the proliferation of human ovarian cancer cell line (MDAH

2774) by inhibiting TNF-alpha-induced proliferation (87). When cells were treated with/without saxatilin, there was minimal effect on cell proliferation. When control cells were treated with TNF-alpha, the cell proliferation percentage nearly doubled, thus showing TNF-alpha has a great effect on ovarian cancer cell proliferation. However, cells treated with saxatilin and TNF-alpha, resulted in only a minimal amount of cell proliferation. This strongly suggested that saxatilin almost completely negated the effects of the chemokine. In addition, saxatilin inhibited IL-8 production, which is normally induced by TNF-alpha. These results suggested a possible mechanism of this disintegrin's antiangiogenic properties.

Surazynski (88) compared the effects of echistatin to thrombin on collagen production in confluent human dermal fibroblast cultures. Echistatin ($\geq$ 100 nM) significantly decreased the collagen biosynthesis by 30% of control, while thrombin ($\geq$ 0.1 IU) caused an increase by 25%. These effects seemed connected to a concurrent change in prolidase activity, which plays an important role in collagen biosynthesis. In addition, similar directional changes in FAK and SOS-protein expression, and phosphorylation of the MAP-kinases, ERK1 and ERK2, were found with echistatin and thrombin. The authors concluded that regulation of collagen biosynthesis may involve signaling molecules induced by beta 1-integrin receptor.

Alimenti (82) also found a decrease in FAK, but not Shc, phosphorylation in pre-apoptotic signals induced by echistatin in GD25 cells (lacking beta 1 integrins), and the process involved was termed "integrin-mediated death" (89). This form of apoptosis is defined as occurring in adherent cells induced by the recruitment of caspase-8 to the beta subunit tails of unligated integrins. Echistatin may be causing this form of apoptosis on GD25 and beta 1-transfected GD25 cells adhering to vitronectin and fibronectin by binding alpha V beta 3 and alpha 5 beta 1 integrins.

Olfa's experiments with alpha 1 beta 1-specific lebestatin demonstrated an inhibition of CHO-alpha1, PC12 and HMEC-1cell migration toward types I and IV collagens in a modified Boyden chamber assay (57). This inhibition was dose-dependent, with a half-maximal inhibition at 0.1 nM.

Yang (43) showed that the disintegrin rhodostomin, a medium-length disintegrin from the venom of *Calloselasma rhodostoma*, inhibited breast (MDA-MB-231, MCF-7) and prostate (PC-3) cancer cell migration in a modified Boyden chamber assay, using fetal bovine serum as the chemoattractant. Tumor cells were also stimulated to penetrate uniform layer of matrigel (collagen type IV, heparin sulfate proteoglycan, entactin, laminin), but were inhibited by rhodostomin (1-10 micrograms per milliliter), trigramin and triflavin (both at 10 micrograms per milliliter).

In another experiment dealing with rhodostomin, Tang and colleagues (90) studied the mechanism associated

with ultrasound stimulation of accelerated fracture healing in animal models. Rhodostomin reduced ultrasound-induced COX-2 expression. In addition, pretreatment of osteoblasts for 30 minutes with 280 nM rhodostomin markedly attenuated the ultrasound-induced phosphorylation of the p85 subunit of phosphatidyl inositol-3-kinase (PI3K). In addition, this pretreatment also reduced Akt phosphorylation. Ultimately, kistrin helped to establish that NF-kappa B is the downstream effector for integrin/FAK/PI3K/Akt and ERK pathways in ultrasound-induced COX-2 expression.

Staniszewska studied the interaction between thrombospindin-1, its C-terminal fragment NoC1 and the alpha 9 or alpha 4 beta 1 integrins using two disintegrins from *Vipera lebetina obtusa* venom, VLO4 and VLO5, in a series of solid phase adhesion assays (59). Table 1 shows that VLO5 completed inhibited the adhesion of dHMVEC to immobilized NoC1, while VLO4 inhibited adhesion by only 25%. VLO4's inhibition was most likely through alpha 5 beta 1 integrin since it is not active against alpha 9 beta 1. In addition, the investigators demonstrated that adhesion of alpha9-transfected SW480 cells to immobilized NoC1 increased phosphorylation of ERK1/2, and this activation was inhibited by blockers of alpha 9 beta 1, like the disintegrin VLO5. In 2008, Stanszewska also demonstrated a VLO5-associated decrease in the phosphorylation of ERK1/2 and paxillin in alpha 9-transfected SW480 cells adherent to neural growth factor (NGF) (81). VLO5 also inhibited NGF-induced proliferation of these SW480 cells. Interestingly, neither VLO5 nor eristostatin inhibited the migration of these alpha 9-expressing cells to mouse NGF, but an alpha 9 beta 1-specific antibody, Y9A2, did. The authors hypothesized that paxillin signaling is linked with migration.

In an interesting study on "platelet-derived supernatant" (PDS), Jang tested the effects of saxatilin from the Korean viper, *Gloydius saxatilis*, on collagen-induced platelet activation and the angiogenic properties of PDS (91). PDS promotes angiogenic responses in HUVECs. One growth factor in PDS that was responsible for mediating this property was platelet-derived growth factor-AB (PDGF-AB). Saxatilin inhibited the release of PDGF-AB from collagen-activated platelets and therefore decreased the ability of PDS to promote HUVEC migration and invasion. This is noteworthy since saxatilin does not interact with collagen integrin receptors alpha 1 beta 1 or alpha 2 beta 1. The authors hypothesized that saxatilin's inhibition is actually through interaction with alpha IIb beta 3: engagement of alpha IIb beta 3 by this disintegrin blocks collagen-induced Syk activation, leading to reduced platelet granular secretion and reduced phosphorylation of MAP-Kp42/44 In separate studies using the lung tumor cell line NCI-H460, Jang and colleagues demonstrated a 50 percent loss in induction of HUVEC migration and tube formation after exposing the HUVEC cells to supernatant from tumor cells previously treated with 100 nM saxatilin (92). A critical observation showed that saxatilin, itself, in the media could not inhibit either migration or tube formation. This strongly suggested the disintegrin was affecting the NCI-H460 cells, themselves. While saxatilin

did not affect NCI-H460 cell proliferation, it down-regulated, at the transcriptional level, VEGF expression in these cells in a dose- and time-dependent manner. Their data further suggested that this repression was through the ILK/Akt pathway.

Scmittmeir had shown that the homodimeric disintegrin contortrostatin could better stimulate glioma cell intracellular signaling than the ECM molecule fibronectin (FN). To follow up those studies, they questioned whether contortrostatin could impinge on intracellular signaling already fully activated through prior adhesion to immobilized FN. Human glioma cell liens U87 and A-172 were adhered to FN for 3 hours and then treated with 100 nM contortrostatin for 4 hours. There was a significant reduction in phosphorylation levels of FAK, paxillin and Src. Surprisingly, not even soluble FN could achieve this competition (78). They hypothesized that this phenomenon was due to the higher binding affinity of CN for the integrin receptors than FN itself.

6.4. Interactions with leukocytes

In another study using rhodostomin, Tseng incubated the disintegrin with whole blood, and, by flow cytometric analysis, found that it interacted with neutrophils as well as with platelets (93). Rhodostomin attenuated super-oxide production and blocked adhesion of neutrophils to fibrinogen, leading the authors to suggest that rhodostomin can be used for its anti-inflammatory properties.

Coelho (94) compared the RGD disintegrin jarastatin to a heterodimeric MLD disintegrin EC3 as chemotactic agents with neutrophils. Both disintegrins inhibited the chemotaxis of neutrophils induced by fMLP and were, themselves, strong chemotactic agents. Interestingly, jarastatin was able to inhibit EC3-induced chemotaxis, while other RGD disintegrins (kistrin, flavoridin, echistatin) were unable to do so. Also, EC3 had no inhibitory effect on jarastatin-induced chemotaxis. Jarastatin interacted with the alpha M beta 2 integrin while EC3 interacted with the alpha 9 beta 1 integrin. Jarastatin, after 5 minutes incubation with neutrophils, promoted accumulation of F-actin at the cells' margins while EC3-incubated neutrophils showed F-actin distribution near the nucleus. Actin polymerization induced by both disintegrins was abrogated by the tyrosine kinase inhibitor, genistein, suggesting the importance of tyrosine kinase activation is a critical step for cell chemotaxis.

Eosinophils have never before been shown to interact with disintegrins. Bazan-Socha (95) demonstrated the increased expression of alpha 1 beta 1 and alpha 2 beta 1 integrins on peripheral blood eosinophils of asthmatic patients. The alpha 1 beta 1-selective disintegrins viperistatin and obtustatin (KTS motif) inhibited eosinophil adhesion to collagen type IV with IC50 values of 11 nM and 1150 nM, respectively. Neither monomeric echistatin (RGDD) nor heterodimeric VLO5 (MLD/VGD) inhibited this adhesion. The authors suggested these KTS-disintegrins or their structural analogs could hold promise for bronchial asthma therapy.

Neto and colleagues (96) demonstrated for the first time in 2007 that disintegrins can interact with lymphocytes. Medium length flavoridin and kistrin, as well as short length echistatin (each at 1 micromolar), modulated activation of human T lymphocytes as evidenced by induction of T cell proliferation (1.7- to 2.9-fold) and CD69 expression. This activation paralleled actin cytoskeleton reorganization and tyrosine phosphorylation of FAK as well as PI3K activation. A ninety minute incubation with flavoridin showed a significant increase in nuclear translocation of transcription factor NK-kappaB, while kistrin and echistatin showed much less activity. The disintegrins had variable cooperating impact on the mitogenic effect of concanavalin A (ConA): Flavoridin + ConA showed 23% increased effect, while echistatin pretreatment inhibited ConA-induced proliferation by 19%. Kistrin had no effect with ConA. Finally, proto-oncogene c-Fos expression was increased following stimulation by all three disintegrins, suggesting integrins (perhaps alpha V beta 3 and alpha 5 beta 1) activate multiple downstream signaling events that regulate T-cell proliferation.

The disintegrin effect on calcium signaling was the focus of experiments by Sun and colleagues (97). Immobilized recombinant rhodostomin enhanced platelet spreading within minutes of their adherence. In addition, calcium oscillation continued for hours after adherence, and the source of calcium was identified as coming from internal stores. There was also a rearrangement of cytoskeletal proteins in the platelets which was attributed to the PI3K pathway. The authors described a synergistic regulation of PI3K and calcium signaling via alpha IIb beta 3 activation.

7. UNIQUE USES OF DISINTEGRINS

One of the more unique uses of disintegrin function in the literature is the inhibition of adipogenesis. Lin and colleagues (98) found that rhodostomin (kistrin) inhibited adipogenesis in a concentration-dependent manner. Rhodostomin acted by inducing detachment and apoptosis of the preadipocyte, preventing it from developing into a more mature adipocyte. This activity involves the alpha V beta 3 integrin and is RGD-dependent. Rhodostomin also caused changes in pp125FAK phosphorylation, which plays an important role in the intracellular arrangement of vinculin and the actin cytoskeleton, a key process of adipogenesis. The authors further suggested that this could eventually be developed into a possible obesity treatment.

In an interesting use of disintegrins, Leong-Poi (99) tested molecular imaging with contrast-enhanced ultrasound and microbubbles targeted to endothelial integrins (alpha V beta 3) for noninvasive assays to investigate if imaging of integrin expression could be used to assess peripheral arterial disease. The researchers used an *in vivo* rat hindlimb ischemia model. They prepared a microbubble attached to echistatin and performed ultraharmonic imaging on the hind tails of the experimental rats. The experimenters reported that this method of imaging may, in fact, be useful in evaluating proangiogenic

therapies, as well as be an assessment tool for therapeutic treatments of arteriogenesis.

Hantgan and colleagues studied the effects of a recombinant form of echistatin on various integrin interactions (100). Two recombinants were used: rEch (1-40), truncated at the C-terminus, and full-length rEch (1-49). ADP-induced platelet aggregation showed that rEch (1-49) had an IC_{50} of 126 nM while rEch (1-40) did not have any inhibitory effect on platelet aggregation. rEch (1-49) also inhibited platelet adhesion to clotted fibrinogen with an IC_{50} of 124 nM. Transmission electron microscopy of rotary shadowed alpha IIb beta 3 integrin in the presence or absence of echistatin revealed a variety of integrin conformers, including many large ones. This indicated that the recombinant echistatin is able to promote integrin oligomerization. Further tests indicated that rEch causes a conformational change of the alpha IIb beta 3receptor. Truncated echistatin was also able to perturb alpha IIb beta 3's solution conformation, suggesting the C-terminus of this disintegrin is not required. In addition, using a dynamic light scattering technique, rEch's binding to the alpha IIb beta 3 integrin produced a more thermally stable conformation of the integrin. The author concluded that echistatin may serve as a model for understanding the binding prerequisites for multidomain adhesive large molecules. Subsequent research on the interactions between integrin alpha IIb beta 3 and echistatin yielded a conclusion that echistatin binding is an entropy-driven phenomenon (101). The integrin-disintegrin complex formed is critically dependent upon electrostatic interactions, primarily ion pairing of the Asp27 in echistatin's adhesive loop with a divalent cation in the MIDAS site of the integrin receptor. The thermodynamic stability of the complex is comparable to many receptor: drug interactions.

Flavoridin inhibits *Yersinia enterocolitica* uptake into fibronectin-adherent HeLa cells (102), but echistatin and kistrin exhibit less potency. The addition of 50 micrograms per milliliter of echistatin, kistrin or flavoridin caused HeLa cell detachment from fibronectin-coated 96-well dishes, 44%, 10% and 85%, respectively. To assess the disintegrins' activity on *Y. enterocolitica* uptake by FN-adherent HeLa cells, the non-cytotoxic dose of 1 microgram per milliliter was used. At that dose, flavoridin almost blocked uptake by 85%, followed by echistatin (57%) and flavoridin (7%). Whereas neither echistatin nor kistrin had any effect on HeLa cell intracellular signaling, 15 minutes of incubation with flavoridin caused a reduction in the disruption of FAK-Cas association caused by *Y. enterocolitica* infection. None of the disintegrins affected the FAK-paxillin association. The authors conclude that flavoridin's binding to alpha 5 beta 1 integrin on HeLa cells may block signaling pathways leading to the disassembly of focal adhesions needed for *Y. enterocolitica* entry into the cells.

Disintegrins can affect the activity of insulin growth factor (IGF). In a 2004 study, Sakata (86) showed that echistatin blocked IGF-1-induced osteoblast phosphorylation and osteoblast proliferation in dwarf rats, *in vivo*. This effect was similar to "skeletal unloading", a phenomenon experienced in space flight and bed rest. This

was attributed to echistatin's binding to alpha V beta 3, and supports the possibility that this integrin has a significant role in enhancing activation of IGF-1 signaling pathways. In separate studies, Antonia Bayes-Genis (103) found that the disintegrin kistrin completely halted IGF-1-stimulated migration of human coronary artery smooth muscle cells, cultured in serum-containing medium until confluent, then in serum-free conditions for 48 hrs on tissue culture plates, and attributed this to inhibition of binding via the alpha V beta 3 integrin. Kapur tested whether integrin activation is involved in the synergy between IGF-I and sheer stress in TE85 osteosarcoma cells using echistatin in cell proliferation and IGF-IR phosphorylation (104). Echistatin decreased proliferation by ~60% and the sheer stress-induced mitogenic response by ~20%, while completing abolishing the mitogenic effect of IGF-I and that of IGF-I/sheer stress combined. The authors hypothesized from this that the combination of IGF-I and sheer stress on proliferation may involve integrin beta 3 activation. Similarly and in confirmation, Sekimoto (105), using 3T3-L1murine preadipocytes, found that echistatin decreased phosphorylation of Src and MAPK when the cells were exposed to IGF-I, and this inhibition was through the alpha V beta 3 integrin.

Fox (106)reported that disintegrins can affect megakaryopoiesis. Specifically, they used kistrin as a "pan-integrin blockage" since it is reported to block beta 1, beta 2, beta 3 and beta 5 integrins (107). Although there was megakaryocyte (MK) development without thrombopoeitin (TPO) stimulation, the addition of TPO significantly accelerated that development. This effect was nearly obliterated in the presence of 10 nanograms per milliliter of kistrin. Interestingly, no inhibition of MK development occurred with the incubation with the alpha 5 beta 1-specific dimeric disintegrin EMF-10 from *Eristicophis macmahoni*. Following the use of specific monoclonal antibodies to alpha 4 beta 1, it was concluded that this integrin plays a critical role in TPO-induced MK growth.

Disintegrin fusion proteins have been developed in order to obtain the platelet aggregation inhibition of the disintegrin while decreasing the immunogenicity of disintegrin infusion. Sheffield examined BLAH6, a recombinant protein combining barbourin with hexahistidine-tagged rabbit serum albumin, produced in *Pichia pastoris* yeast (108). A single bolus of BLAH6 was infused into New Zealand white rabbits, and within 4 weeks, the animals' plasma contained detectable antibodies against barbourin. These antibodies did not affect, however, the anti-platelet ability of the fusion or cause development of thrombocytopenia. The authors sought to create additional fusion proteins with minimal sequences from barbourin which would still inhibit platelet aggregation. Of the sequences chosen, VCKGDWPC and VCRGDWPC inhibited human or rabbit platelet aggregation by less than 10 micromolar, which is significantly more potent than linear RGD or KGD peptides (109). The authors had not yet tested the immunogenicity of these smaller fusion proteins.

Frausin used echistatin as a tool to study imidazolium *trans*-imidazole dimethyl sulfoxide tetrachlororuthenate (NAMI-A), a ruthenium compound with *in vitro* proadhesive effects, and *in vivo* with antimetastatic properties (110). By using echistatin as an integrin antagonist, Frausin observed that the disintegrin (note: concentration used was not given) had an inhibitory effect on the adhesion of a human epidermal carcinoma cell line, KB to immobilized fibronectin after the cells were exposed to a low dose of NMAI-1. There was only partial inhibition with higher NAMI-1 doses. The latter observation suggested that other cell surface targets relevant for cell adhesion may be involved at those doses.

Butera and colleagues created two biomarkers from a fusion of eristostatin and either alkaline phosphatase (Er/APv) (111) or enhanced green fluorescent protein (EGFP-Er) (112). Er/APv had selectivity toward platelets similar to native eristostatin is evidenced by a dot blot assay. This was confirmed using CHO cells transfected with the integrin alpha IIb beta 3 (K1) or alpha V beta 3 (VNRC3). Er/APv bound to K1 cells with intensity similar to that with platelets, and significantly less than with VNRC3 cells. This confirms the findings of differential selectivity of this disintegrin described by Wierzbicka (34)and Juliano (77). Er/APv also retained full enzymatic activity similar to free APv. Specificity to platelets was also observed with the EGFP-Er fusion protein. EGFP-Er bound to washed platelets and to platelets on whole blood smears and was easily visible by confocal microscopy. The binding of EGFP-Er to both single platelets and platelets in clumps confirmed that eristostatin binds to both resting and activated platelets.

8. PERSPECTIVE

When disintegrins were first discovered in 1983 (113) and named in 1990 (61), it was immediately appreciated that these small naturally-occurring proteins had significant promise as tools in the analysis of cell biochemistry and function. In more recent times, these highly conserved proteins have been on the cutting edge of evolutionary hypotheses, and remain viable candidates for pharmaceutical studies. Since fewer than 10% of venoms from all snakes from the Atractaspididae, Elapidae. Viperidae and Colubridae families have been analyzed for the presence of disintegrin genes, mRNAs or proteins, it is certain that these molecules will remain fertile areas of study in the future.

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