Vimocin and Vidapin, Cyclic KTS Peptides, Are Dual Antagonists of α₁β₁/α₂β₁ Integrins with Antiangiogenic Activity

Tatjana Momic, Jehoshua Katzeheendler, Ofra Benny, Adi Lahiani, Gadi Cohen, Efrat Noy, Hanoch Senderowitz, Johannes A. Eble, Cezary Marcinkiewicz, and Philip Lazarovici

School of Pharmacy, Institute for Drug Research, Faculty of Medicine, Hebrew University of Jerusalem, Jerusalem, Israel (T.M., J.K., O.B., A.L., G.C., P.L.); Department of Bioengineering, College of Engineering, Temple University, Philadelphia, Pennsylvania (C.M.); Department of Chemistry, Bar Ilan University, Ramat-Gan, Israel (E.N., H.S.); Center for Molecular Medicine, Department of Vascular Matrix Biology, Frankfurt University Hospital, Excellence Cluster Cardio-Pulmonary System, Frankfurt, Germany (J.A.E.); and Institute for Physiological Chemistry and Pathobiotechnology, University of Münster, Münster, Germany (J.A.E.)

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ABSTRACT

Obtustatin and viperistatin, members of the disintegrin protein family, served as lead compounds for the synthesis of linear and cyclic peptides containing the KTS binding motif. The most active linear peptide, a viperistatin analog, indicated the importance of Cys19 and Cys29, as well as the presence of Arg at position 24 for their biologic activity, and was used as the basic sequence for the synthesis of cyclic peptides. Vimocin (compound 6) and vidapin (compound 10) showed a high potency (IC₅₀ = 0.17 nM) and intermediate efficacy (20% and 40%) in inhibition of adhesion of α₁/α₂ integrin overexpresser cells to responsive collagen. Vimocin was more active in inhibition of the wound healing (53%) and corneal micropocket (17%) vascularization, whereas vidapin was more potent in inhibition of migration in the Matrigel tube formation assay (90%). Both compounds similarly inhibited proliferation (50–90%) of endothelial cells, and angiogenesis induced by vascular endothelial growth factor (80%) and glioma (55%) in the chorioallantoic membrane assay. These peptides were not toxic to endothelial cell cultures and caused no acute toxicity upon intravenous injection in mice, and were stable for 10–30 hours in human serum. The in vitro and in vivo potency of the peptides are consistent with conformational ensembles and "bioactive" space shared by obtustatin and viperistatin. These findings suggest that vimocin and vidapin can serve as dual α₁β₁/α₂β₁ integrin antagonists in antiangiogenesis and cancer therapy.

Introduction

Integrins are a family of cell surface receptors that play essential roles in eukaryotic cell adhesion and migration. These heterodimeric receptors are composed of two non-covalently linked distinct subunits, α and β, each containing a large extracellular binding domain, a transmembrane region, and a short cytoplasmic domain. Upon binding to ligand, the integrins cluster and recruit via their cytoplasmic domain cytoskeletal, adaptor, and signaling proteins, eventually forming focal adhesions. These not only anchor the cell to the subjacent extracellular matrix protein ligands, but also convey signals into the cell. The two major collagen-binding integrins, α₁β₁ and α₂β₁, preferentially bind to collagen type IV and I, respectively (Hynes, 1992). In contrast with other β₁-containing integrins, α subunits of collagen-binding integrins contain a unique 200 amino acid–long A domain that is involved in collagen binding (Leitinger and Hogg, 1999). Studies with α₁/α₂ integrin A domain chimeras confirmed a preference for different collagen types (Abair et al., 2008). As such, integrin receptors are central to the etiology and pathology of many diseases, such as cardiovascular (Clemetson and Clemetson, 1998), inflammatory (Apostolaki et al., 2008) and neurodegenerative disorders (Engelhardt and Kappos, 2008). They therefore have been actively targeted for drug discovery. Many approaches have been developed, entailing the use of classic small molecules, cyclic peptides and engineered antibodies (Goodman and Picard, 2012). To date, major drug discovery attempts have been focused on several integrins, such as glycoprotein IIbIIIa, α₄β₁, α₅β₃, and lymphocyte

ABBREVIATIONS: Acm, acetamidomethyl; bFGF, basic fibroblast growth factor; BrdU, bromodeoxyuridine; BSA, bovine serum albumin; CAM, chorioallantoic membrane; CMFDA, 5-chloromethylfluorescein diacetate; DIMEA, N,N-dimethylpropylamine; DMT, dimethoxytrityl; FMoc, fluorenlymethylxycarbonyl; GST, glutathione S-transferase; HAEC, human aortic endothelial cell; HBSS, Hanks’ balanced salt solution; HBU, 2-1[1H]-benzotriazole-1-y1-1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzo-triazole; HPLC, high-performance liquid chromatography; HUVEC, human umbilical vein endothelial cell; LDH, lactate dehydrogenase; MD, molecular dynamics; RMSD, root-mean-square deviation; RP, reverse-phase; TBS, Tris-buffered saline; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; Trt, trityl; VEGF, vascular endothelial growth factor.

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function–associated antigen 1 (α1β1) and have produced several registered drugs, directly targeting glycoprotein IbαIIa (abiximab, tirofiban, and intrifiban) and αβ1 (natalizumab) (Millard et al., 2011; Goodman and Picard, 2012).

In the past decade, the importance of collagen-binding integrins αβ1 (Pozzi et al., 2000) and αβ1 (Zhang et al., 2008) in tumor angiogenesis was demonstrated using knockout animals. Furthermore, important contributions of αβ1 (Chen et al., 2005) and αβ1 (Yoshimura et al., 2009; Ibaragi et al., 2011) in cancer and metastatic processes were revealed. These findings emphasize the role of the αβ1 and αβ1 integrins in angiogenesis and cancer and suggest their importance as targets in drug discovery and translational medicine (Goswami, 2013). However, the discovery of small molecule drugs targeting αβ1 and αβ1 integrins has been modest. In those studies, preclinical attempts were made to inhibit αβ1 with antibody (Rilikonen et al., 1995) and αβ1 with peptides (Ivaska et al., 1999; Raynal et al., 2006; Lambert et al., 2008) and small molecules (Funahashi et al., 2002; Choi et al., 2007) in the search for potential therapy of cardiovascular and inflammatory diseases and cancer.

Snake venom natural toxins, such as disintegrins (Marcinkiewicz, 2005) and C-type lectins (Arlinghaus and Eble, 2012) are important pharmacological tools because they inhibit integrins with relative selectivity. The disintegrins obtustatin, isolated from the venom of Viperera labugata obtusa (Marcinkiewicz et al., 2003), and its more potent natural analog viperistatin, isolated from the venom of Vipera xantina palestinae (Kiesiel et al., 2004), containing 41-residue monomeric polypeptides cross-linked by four conserved disulfide bonds, are the shortest snake venom disintegrins described to date (Calvete et al., 2007). They are typical members of the disintegrin family with the characteristic three amino acid KTS motif, which is responsible for their selective binding to αβ1 integrin. These KTS-containing disintegrins bind to αβ1 integrin only, in contrast with RGD disintegrins that block αβ1, αβ1, αβ1, αβ1, αβ1, αβ1, and αβ1 integrin (Ruoslahti, 1996; Calvete et al., 2002). The potency of the KTS disintegrins is strictly dependent on the KTS motif, because mutations of individual amino acids in this motif decreased potency by 10- to 80-fold (Brown et al., 2009). Nuclear magnetic resonance solution structure followed by computer modeling of KTS disintegrins indicate that the KTS motif, like RGD, is present in the loop conformation required for integrin binding (Paz Moreno-Murciano et al., 2003; Calvete et al., 2007; Brown et al., 2009). On the basis of these considerations, we used those disintegrins as lead compounds for the synthesis of peptides containing the KTS motif in conformational constraint by cyclization via disulfide bridges.

In this study, we describe the synthesis and modeling of linear and cyclic KTS-containing peptides that inhibited αβ1/αβ1-mediated adhesion and blocked angiogenic activity. Viminoc (compound 6) and vidapin (compound 10) fulfilled basic pharmacological criteria, which can be further exploited for drug development or diagnosis and can serve as a tool for investigations into αβ1/αβ1 biologic function.

Materials and Methods
Collagen IV (from bovine placentia villi) was purchased from Chemicon (Temecula, CA). Collagen I (from rat tail) and Matrigel were purchased from BD Biosciences (Bedford, MA). We obtained 96-well polystyrene plates from Nunc (Roskilde, Denmark). Bovine serum albumin (BSA), Hanks’ balanced salt solution (HBSS) sulfate, alkaline phosphatase–conjugated anti-rabbit antibody, p-nitrophenyl phosphate, human recombinant basic fibroblast growth factor (bFGF), and vascular endothelial growth factor 165 kDa (VEGF) were purchased from Sigma-Aldrich (St. Louis, MO). CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA) was purchased from Invitrogen/Molecular Probes (Eugene, OR). Rabbit polyclonal antibodies against glutathione S-transferase (GST) were purchased from Molecular Probes (Nijmegen, The Netherlands). The bromodeoxyuridine (BrdU) kit was purchased from Roche (Mannheim, Germany). Fertilized Japanese quail eggs (Coturnix coturnix japonica) were purchased from Boyd’s Bird Co (Pullman, WA). The lactate dehydrogenase (LDH) reagent set was purchased from Pointe Scientific Inc. (Canton, MI).

Disintegrins. Viperistatin was obtained from the venom of V. xantina palestinae, as previously described (Staniszewska et al., 2009). Obtustatin was isolated and purified to homogeneity by two chromatographic steps using high-performance liquid chromatography (HPLC) as previously reported (Marcinkiewicz et al., 2003).

C-Type Lectin Protein. Vixapatin was obtained from the venom of V. xantina palestinae as previously described (Staniszewska et al., 2009). Rhodocetin was purified from Calloselasma rhodostoma venom as reported (Eble et al., 2001).

Cell Lines. Human aortic endothelial cells (HAECs) were kindly provided by Dr. Peter I. Lelkes (Temple University, Philadelphia, PA) and cultured as previously described (Dollè et al., 2005). Human umbilical vein endothelial cells (HUVECs) were obtained from human umbilical cords from healthy women who underwent normal term pregnancies, as described elsewhere (Marcinkiewicz et al., 2000). K562 cells were transfected with α1 or α2 integrin subunits (Staniszewska et al., 2009). LN18 human glialoma cells were prepared as previously described (Walsh et al., 2012).

Peptide Synthesis Reagents. All amino acids and Rink resin were purchased from GL Biochem Ltd. (Shanghai, China). DIPEA (N,N-diisopropylmorpholine), HBTU [n-HO-benzotriazole-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate), and HOBt (1-hydroxybenzotriazole) were purchased from BioLab Ltd. (Jerusalem, Israel). All coupling reagents, chemicals, and solvents were purchased from Sigma-Aldrich (Rehovot, Israel).

General Procedure for Peptide Preparation and Characterization. The peptides were synthesized on a solid phase by standard fluorenylmethyloxycarbonyl (Fmoc) chemistry. The synthesis was carried out manually on a Rink amide resin using Fmoc-protected amino acids. Coupling was performed for 1 hour with four equivalents of HBTU and one equivalent of amino acid in the presence of four equivalents of HOBt and eight equivalents of DIPEA. Fmoc groups were removed with 20% piperidine in N-methyl-2-pyrrolidinone. Cyclization of the peptides was performed using several approaches, as detailed further for each peptide.

Cleavage from the resin and full deprotection of peptides was carried out using a mixture of trifluoroacetic acid (TFA)/phenol/H2O/triisopropylsilane [88:5:5:2 (v/v/v/v)] for 3 hours at room temperature (Fields and Fields, 1993). The resin was filtered and the peptide was precipitated by addition of cold diethyl ether to the filtrate. The precipitate was separated by centrifugation at 3500 rpm for 10 minutes, solubilized in water, and lyophilized.

Synthesized peptides were purified by preparative reverse-phase (RP)-HPLC using a C18 column, with an elution gradient of 0–90% acetonitrile with 0.1% trifluoroacetic acid in water. Peptide purity was verified by Dionex Ultimate 3000 analytical HPLC (Thermo Scientific, Waltham, MA). All peptides showed 95% purity, based on the chromatographic peak area displayed at 220 nm. The peptides identities were assessed by electrospray ionization mass spectrometry using a ThermoQuest Finnigan LCQ-Duo (Conover Scientific, San Diego, CA) in the positive ion mode. The data were processed using ThermoQuest Finnigan’s Xcalibur Biomass Calculation and Deconvolution software. Masses found using electrospray ionization mass spectrometry were in the range of standard deviation (0.1% of calculated masses) (Toddi, 1991).
In addition, structures of compounds 6 and 10 were confirmed by high-resolution electrospray ionization mass spectrometry using a LTQ Orbitrap (Thermo Scientific) in the positive ion mode. Generic names of the peptides were presented with asterisk(s) representing amino acids that are involved in cyclization. Amino acids labeled with the same number of asterisk(s) are interacting and forming the bond.

**Compounds 1, 2, 3, and 4.** The peptides were synthesized as described above in the general procedure. The respective calculated and experimentally found molecular weight values were 1328.54 and 1329.67 for compound 1 (CWKTSRTSHYCY), 1326.35 and 1327.67 for compound 2 (GKWTSRTSHYCY), 1371.56 and 1372.63 for compound 3 (CWKTSRTSHYCY), and 1279.38 and 1280.23 for compound 4 (GKWTSRTSHYCY).

**Compounds 5 and 6.** The peptides were synthesized as described above in the general procedure. Disulfide bonds within these peptides were formed by oxidation of the two Cys(trityl) amino acids with 0.1 M iodine/MeOH (Kamber et al., 1980). The respective calculated and experimentally found molecular weight values were 1928.20 and 1928.01 for compound 5 (C\(^{**}\)WKTSRTSHYCY**RTSHTGKSD)**DC, and 1896.8519 and 1896.8518 for compound 6 (C\(^{**}\)WKTSRTSHYCY**PLYP). The next cyclization took place between Cys29(CH\(_2\)CH\(_2\)N=Pht) and the one at the terminal position was protected by three distinct groups, which were Lys10,19(Trt) and the one at the terminal position was removed with 3% trichloroacetic acid (TCA). The free SH group formed was allowed to react with bromoacetic acid to form Cys(CH\(_2\)COOH). Subsequent to peptide completion, Cys\(^{34}\)(Acm) was transformed into Cys\(^{34}\)(S-CH\(_2\)COOH) in the same manner as Cys34(Trt), whereas the Acm group was cleaved with 0.1 M iodine in methanol, and the Fmoc protecting group of Boc-Lys(Fmoc) was cleaved by 1 M tetrabutylammonium fluoride. The free amino and carboxylic groups formed were coupled to yield the cyclization. The respective calculated and experimentally found molecular weight values were 4251.73 and 4251.96 for compound 9 (K**TTGCP**R**SK** KLKP**GTR**TSRTSHYCY**TGKSC**DC, and 3885.36 and 3885.86 for compound 8 (C**WKTSRTSHYCY**TGKSC**G).

**Compound 10.** The peptide was synthesized as described above in the general procedure. The two intrachain disulfide bonds of compound 10 were prepared in the same manner as in compounds 7 and 8. The respective calculated and experimentally found molecular weight values were 2003.7979 and 2003.7977 for compound 10 (C\(^{**}\)WKTSRTSHYCY**TGKSC**G).

**Modeling Viperistatin Analogues in Water.** The nuclear magnetic resonance structure of obtustatin was downloaded from the Protein Data Bank (PDB; PDB ID 1MPZ) (Paz Moreno-Murciano et al., 2003) and its first model was used as a starting point for the molecular dynamics (MD) simulation. All obtustatin models of the original PDB ID 1MPZ entry were mutated to viperistatin (L24R, L38V, and P40Q) and minimized. The lowest energy structure (model 16) was used as an initial conformation for the simulation of viperistatin. This same structure was also used to derive appropriate starting points for the simulations of compounds 3, 5, 6, and 10, using residues 19–29 for compound 3, residues 19–29 and 37–41 with the mutations V38L and Q40P for compound 5, and residues 19–35 with the mutations T19C and D35N for compound 10. Peptides 5, 6, and 10 were gradually minimized, first by optimizing their disulfide bonds only, then by optimizing all side chains, and finally by optimizing the whole structures. Prior to minimization, all structures were prepared using the Prepare Protein Protocol as implemented in Discovery Studio to determine residues protonation states (Accelrys, 2005–2009).

**MD simulations** were performed using the Gromacs Molecular Dynamics package (Hess, 2008) with the AMBER99SB-ILDN force field (Lindorff-Larsen et al., 2010). Peptides were submerged in transferable intermolecular potential three point water (Jorgensen et al., 1983) in a cubic box with an extra extension along each axis of the peptide of 10 Å. Ions were added to the solution to make the system electrically neutral. Structures were minimized and then equilibrated (first under conditions in which the number of atoms, volume, and temperature are constant for 1 nanosecond and then under conditions in which the number of atoms, pressure, and temperature are constant for an additional 1 nanosecond) and finally simulated under conditions in which the number of atoms, pressure, and temperature are constant for 1 microsecond. All simulations were performed at 300 K with a time step of 2 femtoseconds using the leap-frog algorithm (Hockney et al., 1974). Long-range electrostatic interactions were computed using particle mesh Ewald summation (Essmann et al., 1995). The cutoff for van der Waals and Coulomb interactions was set to 10 Å. Periodic boundary conditions were applied. The LINCS algorithm (Hess et al., 1997) was used to constrain bond lengths.

To assess the similarity of the conformational spaces of two compounds, a matrix termed contained was developed based on the percentage of conformations of one compound that are similar [i.e., reside within a predefined root-mean-square deviation (RMSD) cutoff] to at least one of the conformations of the other compound. Contained values were calculated between the designed compounds (compounds 3, 5, 6, and 10) and the reference compounds (obtustatin/viperistatin) using a backbone RMSD cutoff of 2 Å calculated for residues 19–29 and using only the last 400 conformations obtained for each compound. These parameters were selected to allow a comparison between all compounds that share residue 19–29 backbone and to remove the effect of the starting conformations. Similar calculations were performed with a 1-Å RMSD threshold mainly resulted in zero percentages.

**Cell Adhesion Assay.** The assay was carried out as previously described, with minor modifications (Staniszewska et al., 2009). The day before the experiment each well of a 96-well plate was coated with...
10 μg/ml collagen I or 1 μg/ml collagen IV in 0.02 M acetic acid and incubated overnight at 4°C. Thereafter, nonspecific binding was blocked by incubating the wells with 1% (w/v) BSA in HBSS containing 5 mM MgCl₂ at room temperature for 1 hour before use. The cells were labeled by incubation with 12.5 μM CMFDA in HBSS without 1% BSA at 37°C for 30 minutes. The labeled cells were then centrifuged at 1,000g and washed twice with HBSS containing 1% BSA to remove excess CMFDA. Labeled cells (1 x 10⁶ cells/well) were added to each well in the presence or absence of peptide and were incubated at 37°C for 60 minutes. In the presence of peptide, cells were added to the well after prior incubation with peptide for 30 minutes at 37°C. Unbound cells were removed by washing the wells three times with 1% (w/v) BSA in HBSS, and bound cells were lysed by the addition of 0.5% Triton X-100 (diluted in double-distilled water). The fluorescence in each well was quantified with a SPECTRAFluor Plus plate reader (TeCan, San Jose, CA) at λex = 485 nm and λem = 530 nm. To determine the number of adhered cells from the fluorescence values, a standard curve was generated by serial dilution of known numbers of CMFDA-labeled cells.

**GST α1α1 α2α2 Binding to Collagen IV/Collagen I Binding Assay.** The inhibition enzyme-linked immunosorbent assay was performed as previously described (Eble and Tuckwell, 2003), with the following modifications: CB3 (collagen IV fragment) or collagen I was immobilized overnight at 4°C on a microtiter plate at 10 μg/ml in Tris-buffered saline (TBS)/MgCl₂ (50 mM Tris-HCl, 150 mM NaCl, and 2 mM MgCl₂, pH 7.4) and 0.1 M acetic acid, respectively. After blocking the plate with 1% (v/v) BSA in TBS/MgCl₂, the GST-tagged α1α1/GST-tagged α2α2 domain was allowed to bind to collagen IV/collagen I in the presence or absence of different peptides for 2 hours at room temperature. The bound GST α1α1/GST α2α2 was quantified with rabbit polyclonal antibodies against GST, followed by alkaline phosphatase-conjugated anti-rabbit antibody, which served as the primary and secondary antibodies, respectively, each diluted in 1% BSA in TBS/MgCl₂. The conversion of p-nitrophenyl phosphate was measured at 405 nm in an enzyme-linked immunosorbent assay reader (BioTek, Bad Friedrichshall, Germany).

**Cell Migration Assay.** HAEC and HUVEC proliferation was assessed using a BrdU kit according to the manufacturer’s instructions.

**Cell Migration Assay.** HUVEC migration was measured using a wound healing assay (Lecht et al., 2010). In brief, 1 x 10⁶ cells/well were added to a 24-well culture plate. Upon formation of a confluent monolayer, cell migration was initiated by scratching the HUVEC monolayer with a sterile pipette tip, thus generating a cell-free area (wound) of approximately 1 mm in width. The wounded cells were washed three times with 1% FCS supplemented endothelial cell growth medium 2 and the photos of the wounds at time 0 hours were taken. Thereafter, the culture medium was changed to 2% fetal bovine serum medium as the cell migration stimulator, in the presence or absence of synthetic peptides (50 μg/ml) or obutstatin (5 μg/ml) and the cultures were allowed to migrate for 24 hours. At the end of the migration experiment, another set of photos was taken of the same regions. Images were analyzed using ImageJ software (NIH, Bethesda, MD). To assess cell migration at the wound edge and to calculate the area covered by migrating cells, the cell-free areas of the wounds at 24 hours after wounding were subtracted from the area of the wounds at the 0-hour time point and calculated as a percentage of untreated (control) cultures (Lecht et al., 2010).

**HAE Cell Tube Formation in the Matrigel Assay.** The assay was performed using a 96-well plate coated with growth factor reduced Matrigel. Briefly, 1 x 10⁶ HAEcs or HUVECs per well (in complete endothelial growth basal medium 2 or 2% fetal bovine serum) were added in the presence or absence of the peptides, and the plate was incubated overnight at 37°C in 5% CO₂. Images were captured under an inverted microscope (Olympus IX81; Olympus Corporation, Tokyo, Japan), with 35 x magnification (Momic et al., 2012a).

**Angiogenesis in the Chorioallantoic Membrane Quail Embryonic Model.** The assay of VEGF-induced and tumor-induced angiogenesis in the quail embryonic chorioallantoic membrane (CAM) system was performed as previously described (Lazarovici et al., 2006).

Fertilized Japanese quail eggs (*C. coturnix japonica*) were cleaned with ethanol, and maintained at 37°C until embryonic day 3 in an incubator without CO₂. The shells were then opened with a razor blade. Using minced sterile scissors, the contents were transferred to six-well tissue culture plates and returned to the 37°C incubator. At embryonic day 7, the compounds were applied under sterile conditions to the CAM surface, and the effect on the aortic tree was evaluated after 24 hours. The embryos were divided into experimental groups, each containing at least 10 embryos. The control group was treated with vehicle (phosphate-buffered saline).

In the experiments using LN18-induced tumors under the CAM, LN18 glioma cells (1 x 10⁵/750 μl) were injected at day 7 under the CAM and tumor-induced angiogenesis was measured at day 12. At the end of the experiment, the embryos were fixed with 5 ml of prewarmed 2% glutaraldehyde and 4% paraformaldehyde in phosphate-buffered saline for 48 hours at room temperature. Membranes with and without tumors were dissected and mounted on glass slides for evaluation of the fractal dimension. The area of the CAM selected as a square for analysis of the vascularization ratio was localized as previously described or, in the case of the tumor-induced angiogenesis, in the site opposite to the tumor on the membrane. For example, if the tumor developed in the right corner of the CAM, the vascularization tree for analysis was framed in the left corner of the membrane (Lazarovici et al., 2006).

**Conorneal Micropocket Assay.** The corneal micropocket assay was performed as previously detailed (Benny et al., 2008). In brief, pellets containing 80 ng carrier-free recombinant human bFGF or 160 ng VEGF (R&D Systems, Minneapolis, MN) were implanted into micropockets that were created in the cornea of anesthetized mice. Mice were treated daily by eye drops (8 mg/ml) for 5 days, and the vascular growth area was then measured using a slit lamp. The area of neovascularization was calculated as the vessel area calculated as the vessel length measured from the limbus and clock hours around the cornea, using the following equation: Vessel area (in millimeters squared) = [π x clock hours x vessel length (in millimeters)] x 0.2 mm².

**Stability of Compounds 3, 6, and 10 in Human Serum.** One milliliter of RPMI media supplemented with 25% (v/v) of human serum was introduced into a 1.5-mL Eppendorf tube and was temperature equilibrated at 37°C for 15 minutes before adding 5 μl peptide stock solution to make a final peptide concentration of 50 μg/ml. The initial time was recorded and at known intervals, 100 μl of the reaction solution was removed and added to 200 μl of 96% ethanol to precipitate serum proteins. The sample was cooled at 4°C for 15 minutes and then centrifuged at 18,000 g for 2 minutes to precipitate serum proteins. The supernatant was then applied to a C-18 column for separation by RP-HPLC. A linear gradient from 100% buffer A (0.1% TFA in water) to 50–50% buffer A and buffer B (0.1% TFA in acetonitrile) was applied for over 30 minutes. The flow rate was 1 ml/min and absorbance was detected at 220 nm (Jensen and Aspno, 2008).

**Cell Death Assay.** Cell death was measured by the release of LDH into the medium, in the absence and presence of different concentrations of compound 10, using LDH reagent. H₂O₂ was used as the positive control. LDH activity was determined spectrophotometrically at 340 nm by following the rate of conversion of oxidized NAD to the reduced form of NAD (NADH). LDH release was expressed as the optical density units. Each experiment was performed two times (n = 6).
Toxicity to Mice. Experiments with animals and animal care were approved by the Hebrew University Committee of Ethics and were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Male C57BL/6 mice and SABRA-M mice (n = 3) were injected intravenously with 0.2 ml of either compound 6 or compound 10, generating a dosage of 50 mg/kg. Mice were monitored for 3 consecutive weeks. The animals were examined for autonomic symptoms by measuring salivation, urinary delivery, pupillary constriction, heart rate, blood pressure, and hair contraction. Neurotoxicity was evaluated by general locomotor activity of the animals in the cage and the ability of the peptide-injected mice to maintain balance and motor coordination by crossing balance beams that were 3, 2, and 1 cm in width. Occurrences of either flaccid or spastic paralysis of the legs were also measured. Blood samples were taken from control and peptide-injected mice after 10 hours from injection and were submitted from hematocrit and biochemical analysis.

Statistical Analyses. The t test and analysis of variance were used to determine the significance of the differences between the various treatments compared with the control groups. P ≤ 0.05 was considered significant.

Synthesis of Linear Peptides. In the first approach, linear obtustatin and viperistatin peptide analogs (Fig. 1A) containing the KTS motif (Table 1) were synthesized and their biologic activity was investigated by an in vitro adhesion assay with K562 cells overexpressing the α1 integrin subunit, using the ligand collagen IV. The peptides with the highest antiadhesive activity are presented in Table 1. The most active peptide was compound 3, the viperistatin analog. Compound 1, the obtustatin analog (the sequence of which differs from compound 3 only by one amino acid at position 24 where Leu24→Arg24) had 6-fold decreased activity. This suggests that the positively charged side chains of Arg form a better productive interaction with residues within the ligand-binding pocket of the α1β1 integrin (Brown et al., 2009) than the alkyl chain of Leu. In addition, we showed that the two Cys of compound 3, at the carboxy and amino termini of the sequence, had a strong impact on its potency. Upon exchanging the two...
terminal Cys with two Gly, the α₁-mediated antiadhesive activity of compounds 4 and 2 decreased significantly by 35- and 7-fold, respectively, indicating their important role in binding α₁β₁ integrin.

**Synthesis of Cyclic Peptides.** In the second approach, conformationally constrained cyclic sequences containing the KTS motif were prepared. In the first group (Fig. 1B), we synthesized two cyclic peptides with one intradisulfide bridge between Cys18 and Cys34 (i.e., compounds 5 and 6, the structure of which differs by only one amino acid Gln<sup>33</sup>→Pro<sup>33</sup>, respectively). To generate new analogs, a second group was synthesized (compounds 7 and 8) (Fig. 1C), represented by two peptides with double intercyclization. The cyclization was achieved by the formation of two disulfide bonds between two identical strand sequences containing the modified 19–35 viperistatin sequence. In both peptides, Cys<sup>34</sup> was omitted. In compound 8, an additional modification was done by exchanging the position of Cys from position 29 to position 24. In both peptides, cyclization was achieved by linking Cys of identical protecting groups residing on two identical peptide strands. Two interdisulfide bonds were formed between Cys<sup>19,19</sup> and Cys<sup>29,29</sup> of compound 7, and between Cys<sup>19,19</sup> and Cys<sup>34,34</sup> in compound 8 (Fig. 1C). The third group of peptides is presented by compounds 9 and 10 (Fig. 1D). The structure of compound 9 is a modified viperistatin sequence, in which the amino acids at positions 37–40 are eliminated. The peptide is characterized by four intrastrand amide bonds between Lys<sup>8</sup> and Cys<sup>6</sup>, Cys<sup>7</sup> and Cys<sup>29</sup>, Lys<sup>10</sup> and Cys<sup>34</sup>, and Lys<sup>19</sup> and Cys<sup>36</sup>. Compound 10 includes the original viperistatin 19–34 sequence and one additional Cys at position 18. Its two involved intradisulfide bonds are between Cys<sup>18</sup> and Cys<sup>24</sup> and Cys<sup>35</sup> and Cys<sup>39</sup>.

**Potency and Efficacy of Viperistatin Analogs in the Adhesion Assays.** In the cellular adhesion assay, the inhibitory effect of all of the synthesized peptides was first tested using α₁-K562 transfectedants. To measure the potency and efficacy of viperistatin analogs, we performed dose-response experiments in a range of physiologic concentrations between 0.1 and 100 nM, using collagen IV–coated plates. As expected, viperistatin blocked α₁-mediated cell adhesion to collagen IV (Fig. 2A) with an apparent IC<sub>50</sub> value of 0.6 nM and 100% efficacy (Table 2). Compounds 5 and 6 with one intracyclization were characterized by very similar IC<sub>50</sub> values of 0.20 and 0.17 nM, respectively, and an equal efficacy of 20% (Fig. 2B; Table 2). All of the synthesized peptides were characterized by increased potency compared with viperistatin (Fig. 2, A–C; Table 2). By contrast, intercyclization or intracyclization of the peptides resulted in reduced efficacy to a level of 20% for compounds 5 and 6, 25% for compounds 7 and 8 and 40% for compounds 9 and 10, versus viperistatin (Fig. 2, A–C; Table 2). In conclusion, cyclization significantly increased the potency but reduced the efficacy of the synthesized peptides compared with viperistatin.

To test the specificity of the viperistatin analogs toward the α₁β₁ and α₁β₂ integrins, we used α₁β₂-K562 transfectedants and measured the potency and efficacy of the analogs in the same range of concentrations, using collagen I–coated plates and vixapatin, an α₂β₂-selective C-type lectin protein (Arlinghaus and Ebbo, 2012), as the positive control. Vixapatin generated a dose-response inhibitory effect on α₂β₂-mediated adhesion, with an apparent IC<sub>50</sub> value of 3 nM and an efficacy of 100% (Fig. 2D; Table 2). Viperistatin analogs showed a high potency of inhibition, with an apparent IC<sub>50</sub> value in the range of 0.12–0.25 nM and a very low efficacy in the range of 10–30% (Fig. 2, D–F; Table 2). These findings indicate that the synthesized peptides interfered with α₁β₁ and α₁β₂ integrin-mediated adhesion.

A cell-free assay was performed to assess whether the synthesized peptides directly interact with the A domain of α₁ or α₂. For this purpose, viperistatin and rhodocetin, another C-type lectin protein selective for α₁β₂ integrin, as well as compounds 3 and 5–10 were incubated together with the GST-linked A domains and allowed to bind to the immobilized collagen I or CB3 (collagen IV fragment). The amount of the bound recombinant A domain provided information on the inhibitory potential and biochemical recognition ability of the peptides (Fig. 3). Similarly to obtustatin (Marcinkiewicz et al., 2003), viperistatin did not inhibit binding of the GST-α₁A domain to collagen at a concentration of 1 μM (Fig. 3A). However, at a high concentrations (range 2–1000 μM), compounds 6 and 10 dose dependently inhibited binding of GST-α₁A by 50% (Fig. 3B). This finding suggests that viperistatin affects α₁ integrins outside of the A domain, most probably by conformational changes, whereas compounds 6 and 10 recognize with low potency a motif in the A domain. In the experiments with the GST-α₂A domain at 1 μM concentration, a similar lack of inhibition was observed (Fig. 3C). However, in the range of concentrations between 0.01 and 1.0 μM, as previously reported (Ebbo and Tuckwell, 2003), Rhodocetin inhibited binding of the GST-α₂A domain to collagen I in a dose-dependent manner (Fig. 3D). Surprisingly, compounds 6 and 10 increased the binding of GST-α₂A to collagen I in a dose-dependent manner, most probably by a conformational change. These results may suggest that viperistatin analogs affect α₁A and α₂A integrin domains at non-physiologic concentrations, but the mechanism requires further investigation.

**Modeling Viperistatin Analogs in Water.** MD simulations were performed to evaluate the effect of differences in amino acid composition and number of disulfide bonds on peptide conformation. MD simulations were performed for each of the reference compounds (viperistatin and obtustatin) and for compounds 3, 5, 6, and 10 as described in Materials and Methods. The selection of these compounds was done

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**TABLE 1**

Sequence of synthesized linear peptides and their activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amino Acid Position</th>
<th>IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C W K T S L T S H Y C</td>
<td>0.645</td>
</tr>
<tr>
<td>2</td>
<td>C W K T S L T S H Y G</td>
<td>&gt;4</td>
</tr>
<tr>
<td>3</td>
<td>C W K T S R T S H Y C</td>
<td>0.105</td>
</tr>
<tr>
<td>4</td>
<td>C W K T S R T S H Y G</td>
<td>3.750</td>
</tr>
</tbody>
</table>

Peptides 1 and 2 are derived from the obtustatin sequence. Peptides 3 and 4 are viperistatin analogs. Inhibition of the adhesion of the cells overexpressing α₁ integrin is measured by IC<sub>50</sub> values.

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α₁β₁/α₁β₂ Peptide Antagonists with Antiangiogenic Activity
based on the results we obtained in all of the biologic experiments presented in this work. We collected 500 snapshots in each case (see Supplemental Data). The last 400 snapshots of viperistatin and obtustatin were superimposed using the backbone of residues 4–18 and 30–36 (Paz Moreno-Murciano et al., 2003) and are presented as “sausage” plots (Koradi et al., 1996) in Fig. 4, A and B, respectively. These plots show a mean structure with atom-wise error bars indicated by the thickness of the sausage. Results for viperistatin (Fig. 4A) show that the KTS loop (in pink) region is more mobile relative to the other parts of the peptide. Results of obtustatin (Fig. 4B) show that the KTS loop (in pink) and the C-terminal regions are also more mobile relative to the other parts of the peptide. To qualitatively address the conformational space sampled by the designed compounds, we present 10 superimposed, evenly spread, conformations for each compound that were collected from the last 800 nanoseconds of its trajectory. Compound 3 samples the conformational space extensively (Fig. 4C). Compounds 5 and 6 are also quite flexible, although to a lesser extent (Fig. 4, D and E, respectively). Compound 10 is clearly the least flexible one (Fig. 4F). The trend observed for compounds 3, 5, 6, and 10 is in accordance with their structures, namely, larger numbers of disulfide bonds restrict the conformational space of the designed compounds.

Furthermore, we examined the degree of similarity between the conformational spaces sampled by the designed compounds and the reference compounds (viperistatin and obtustatin) to investigate the assumption that conformational similarity translates into similar activities (Fig. 5; Table 3). Furthermore, we examined the degree of similarity between the conformational spaces sampled by the designed compounds and the reference compounds, viperistatin and obtustatin, to investigate the assumption that conformational similarity translates into similar activities (Fig. 5; Table 3). For each compound we calculated two values: (1) the fraction (in percentage) of its conformations that are “contained” within the conformational space of a reference compound, and (2) the fraction of the conformations of the reference compounds that are “contained” within the conformational space of the synthetic

Fig. 2. Inhibitory effect of the peptides on α1 and α2 overexpressor cell adhesion. Dose-response curves of the inhibition of α1 (A–C) and α2 (D–F) cell adhesion to respective collagens. Viperistatin and vixapatin were used as controls. The number of adherent cells (mean ± S.D.) is derived from three independent experiments.
peptide. The first value (Fig. 5, left) estimates the fraction of time a compound spends in the conformational space of the reference compounds (i.e., in conformations similar to those of the reference compounds), whereas the second value (Fig. 5, right) estimates what part of the conformational space of the reference compound is sampled. The two reference compounds (viperistatin and obtustatin) sample the conformational spaces of each other rather extensively, with viperistatin having 79.6% of its conformations close to obtustatin and obtustatin having 68.8% of its conformations close to viperistatin. This similarity is in accordance with the high antiadhesive activity of both compounds. Compound 3 has relatively low “contained” percentages in the conformational ensembles of the reference compounds at 11.5 and 17.0% for obtustatin and viperistatin, respectively, and high “contained” percentages of the reference compounds at 79.6 and 96.8% of obtustatin and viperistatin, respectively, in its ensemble (Table 3). The corresponding numbers are 30.4, 40.1, 34.9, and 88% for compound 5; 3.7, 10.0, 50.6, and 19.0% for compound 6; and 64.3, and 68.1% for compound 10 (Table 3). These data indicate that compound 3 samples most of the conformational space of the reference compounds, but only for a short fraction of the time. Compound 5 samples less of the conformational space of the reference compounds, but for longer periods of time. Compound 10 samples quite a large fraction of the conformational space of the reference compounds throughout most of the simulation. The fractions of time that compounds 3, 5, and 10 spend in the conformational space of the reference compounds are in accordance with their antiadhesive activity. This confirms the assumption that conformational similarity translates into similar activity. Compound 6 deviates from this pattern and apparently its disulfide bond locks its structure in a set of conformations remote from those characteristic by the reference compounds for most of the time. It is interesting to note that although compounds 5 and 6 differ only in one residue (Gln40 in compound 5 and Pro40 in compound 6, similar to viperistatin and obtustatin, respectively), they show significant differences in their conformational ensembles. Although the conformational ensemble of compound 5 is relatively similar to those of the reference compounds, that of compound 6 is clearly different. It is likely that the combination of the rigid Pro residue with the disulfide bonds locks this peptide in a unique set of conformations.

![Fig. 3](image-url)
Viperistatin Analogs Inhibit Proliferation and Migration of Endothelial Cells. To further characterize the effect of the peptides on endothelial function, we screened their influence on HUVEC and HAEC proliferation, using the BrdU assay (Fig. 6). Similarly to obtustatin, 50 \( \mu \text{g} \) compounds 6 and 10 inhibited proliferation of HAEC by 90\%. Compounds 3, 7, 8, and 9 had no effect (Fig. 6A). The effect of compound 6 on the inhibition of HUVEC and HAEC proliferation was 50–90\% (Fig. 6). Compound 10, like obtustatin, completely inhibited the proliferation of both cell lines. These results indicate the angiostatic effect of viperistatin analog compounds 6 and 10.

In another approach, we characterized the effect of 50 \( \mu \text{g} \) compounds 6 and 10, which were the most active in inhibition of endothelial cells proliferation, on HUVEC migration using a wound healing assay (Fig. 7). In untreated cells (control), 100\% wound closing was measured, and obtustatin completely blocked wound closing (100\%). Interestingly, compound 10 blocked by 26\% and compound 6 to a higher extent, inhibited by 53\% wound closing. These results also indicate anti-angiogenic effects of these compounds on HUVECs.

Viperistatin Analogs Inhibit Tube Formation in the Matrigel Assay. The Matrigel angiogenesis assay was performed to investigate the ability of the peptides to inhibit tube formation by HAECs and HUVECs. Treatment for 14 hours with 100 \( \mu \text{g} \) obtustatin, as well as with compounds 6 and 10, completely inhibited tube formation (Fig. 8). These results indicate the angiostatic effect on endothelial cells of viperistatin analog compounds 6 and 10.

Viperistatin Analogs Inhibit Angiogenesis in the CAM Assay. In the first approach, the effect of viperistatin analogs on VEGF-induced angiogenesis in the CAM quail embryonic model was measured. The binary images of the midarterial endpoint CAM fragments showed a significant overgrowth of small capillaries after 24 hours of treatment with VEGF compared with control vehicle-treated embryos (Fig. 9, VEGF, control). At 200 \( \mu \text{g} \) per embryo, compounds 6 and 10 significantly (80\%) inhibited VEGF-induced capillary formation, as found with 20 \( \mu \text{g} \) obtustatin per embryo (Fig. 9).

### TABLE 3

<table>
<thead>
<tr>
<th>Reference Compound (Conformational Space(^a)(^b))</th>
<th>Synthetic Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td><strong>Obtustatin</strong></td>
<td></td>
</tr>
<tr>
<td>( a )</td>
<td>11.5</td>
</tr>
<tr>
<td>( b )</td>
<td>79.6</td>
</tr>
<tr>
<td><strong>Viperistatin</strong></td>
<td></td>
</tr>
<tr>
<td>( a )</td>
<td>17.0</td>
</tr>
<tr>
<td>( b )</td>
<td>96.8</td>
</tr>
</tbody>
</table>

\(^{a}\)Conformational space of the compound contained within the conformational space of obtustatin or viperistatin.

\(^{b}\)Conformational space of obtustatin or viperistatin contained within the conformational space of the compound.
In the second approach, the effect of the viperistatin analogs on angiogenesis induced by glioblastoma tumor cells was estimated. LN18 glioma cells were injected into the shell-less embryonic CAM system and the effect of the viperistatin analogs on midarterial capillary sprouting was measured (Fig. 10A). The LN18 glioma tumor induced a significant increase in angiogenesis (Fig. 10A), expressed by an increase in fractal dimension values from 1.106 to 1.162, 50% greater than that of the control. Treatment with 100 μg compound 6 reduced the LN18-induced angiogenic effect by 50%, very similar to the effect caused by the same amount of compound 10 (Fig. 10). Those results characterized compounds 6 and 10 as antiangiogenic compounds.

Viperistatin Analogs Inhibit Angiogenesis in the Corneal Micropocket Assay. The antiangiogenic properties of compounds 6 and 10 were evaluated in vivo with the corneal micropocket assay (Benny et al., 2008). After 5 days of treatment with 8 mg/kg of compounds 6 and 10, the antiangiogenic effect was measured with 80 ng bFGF-induced angiogenesis in the eyes of treated mice compared with untreated mice (control) (Fig. 11, upper panel). Quantification of the angiogenesis area (Fig. 11, lower panel) showed 20% ± 5% inhibition of angiogenesis with compound 6 compared with control (P = 0.05, n = 10). Compound 10 did not show statistically significant inhibition in this assay. Similar peptide inhibitory results were obtained with 200 ng VEGF-induced angiogenesis in the cornea (data not shown).

Viperistatin Analogs Stability in Human Serum. To investigate the effects of the peptide cyclization on proteolytic susceptibility, the degradation of the intact peptides incubated in human serum at 37°C was followed by RP-HPLC. Incubations were done for different periods of time and the results are presented in Fig. 10. The linear peptide (compound 3) was degraded with a half-life of 3 hours. In contrast with the linear peptide, cyclization of peptides prolonged their stability. Compound 6 containing one cyclization was completely degraded after 24 hours with a half-life of 10 hours. Compound 10 with double cyclization showed a more complex degradation behavior, and 50% of the starting peptide amount was preserved for 30 hours in human serum.

Safety of Viperistatin Analogs. First, to verify that the antiangiogenic effect of viperistatin analogs is not due to toxicity toward endothelial cells, compounds 6 and 10 (100 μM) were incubated with HUVECs and HAECs for 7 days and the amount of LDH release in the medium was estimated. No significant release of LDH over the control (10% ± 5%) was measured for all cultures up to 7 days of treatment, indicating a lack of necrotic cell death (data not shown). Considering
that the IC₅₀ value of these compounds for inhibition of cell adhesion is 0.17 nM, we can estimate a therapeutic index in vitro of 1000, indicating high safety for endothelial cells.

Second, to investigate the safety of viperistatin analogs in mice, we intravenously injected male mice with a dose of 50 mg/kg of compounds 6 and 10 for 3 consecutive weeks. Acute tolerability was observed. At injection of this high dose of 50 mg/kg body weight, the mice did not suffer from visible weakness and/or exhaustion. No paralysis, altered motor activity, or irregular behavior was observed in mice treated with compounds 6 and 10, suggesting a lack of neurotoxicity. Cutaneous hematomas around the injection or at distant locations site within 24 hours after injection have not been observed. Furthermore, no mice sudden deaths occurred within 24 hours after the injection or during the 3 weeks of observation. After 10 hours, the blood of mice injected with compounds 6 and 10 was submitted for hematologic and biochemical analysis. The values for white blood cells, red blood cells, and platelet counts were in the normal range of 6–15 × 10⁹/µl, 7–12 × 10⁹/µl, and 200–450 × 10³/µl, respectively, for mice injected with compounds 6 and 10 similarly with the values obtained for control mice. Additional evidence on the lack of hemorrhage or anemic conditions was indicated by the similar hematocrit value in the range of 35–45% and the mean corpuscular hemoglobin of 11.1–12.7 pg/mice, between mice injected with compounds 6 and 10 compared with control mice. Lack of lymphopenia, monocytopenia, and granulocytopenia was indicated by similar values in the range of 20–40%, 3–5%, and 7–13%, respectively, between mice injected with compounds 6 and 10 compared with control mice. Alkaline phosphatase and LDH values were in the range of 100–214 units per liter and 1000–2400 units per liter, respectively, between mice injected with compounds 6 and 10 compared with control mice, suggesting no toxic effects to liver and other tissues.

Discussion

We describe the synthesis, modeling, and characterization of viperistatin analogs that inhibit α₁β₁/α₂β₁ integrin-mediated cell adhesion and angiogenic activity. These analogs represent the first attempt to generate KTS peptides using viperistatin/obtustatin as lead compounds. All of the synthesized cyclic peptides were characterized by subnanomolar potency, intermediate efficacy of binding, dual antagonism of α₁ and α₂ integrins, different stability in human serum, safety to endothelial cells and mice, and differential angiostatic effects. Summarizing all experiments, we suggest...
that compounds 6 and 10, which we named vimocin and vidapin, respectively, can serve as optimal cyclic peptides for future research and development of α1β1/α2β1 receptor antagonists.

One of the classic approaches for generating pharmacophores of pharmaceutical importance utilizing snake venom–derived disintegrins is to produce linear peptides and, subsequently, cyclic peptides (Dennis et al., 1990; Millard et al., 2011). Consistent with this approach, in this study, using the structure of viperistatin/obtustatin as a template, we first synthesized linear peptides with the KTS sequence and investigated their inhibitory potency in an adhesion cellular assay. Compound 3 was the most active linear peptide, although its potency was extremely low compared with that of viperistatin. The other, less potent linear analogs of viperistatin (Table 1) emphasize the important role of Cys at the flanking N- and C-terminal ends, as well as the important role of Arg at position 24, for their biologic activity, as previously reported for viperistatin and obtustatin (Brown et al., 2009; Momic et al., 2012b).

On the basis of these findings as well as the consideration that the integrin-binding loop supports the correct conformational presentation of the KTS sequence to α1β1 integrins (Brown et al., 2009), we synthesized cyclic viperistatin analogs in the second approach. The compound 3 linear sequence served as a basic structure for the cyclic peptides. Using single, double, and tetra intercyclization or intracyclization, we generated peptides of various sizes and structural constraints. The data we obtained in adhesion assays indicate that the number and type of cyclization had no significant effect on the potency of the cyclic compounds investigated, albeit the cyclization dramatically increased the potency compared with that of compound 3. Although all of the cyclic peptides showed reduced efficacy of binding in comparison with viperistatin, multiple intracyclization conferred 40% efficacy of compounds 9 and 10. The results we obtained by computation studies showed, as expected, an opposite correlation between the number of disulfide bonds and the conformational flexibility of the different compounds, with more bonds conferring conformational rigidity (Fig. 4) as well as stability (Fig. 12). Based on the analysis of the conformational ensembles, the conformational space sampled by compound 10 is the most similar to those of the reference peptides, obtustatin and viperistatin, being the least flexible among the modeled peptides. Compound 6 was more flexible than compound 10. This observation lends some credit to the hypothesis that active compounds have similar conformational ensembles. This hypothesis is supported by conformation-activity studies of potent RGD peptides (Gurrath et al., 1992), as well as studies on the size and shape of the integrin-binding loop as a determinant for conferring integrin specificity on cyclic RGD peptides with a known conformational rigidity (Fig. 4) as well as stability (Fig. 12). Based on the analysis of the conformational ensembles, the conformational space sampled by compound 10 is the most similar to those of the reference peptides, obtustatin and viperistatin, being the least flexible among the modeled peptides. Compound 6 was more flexible than compound 10. This observation lends some credit to the hypothesis that active compounds have similar conformational ensembles. This hypothesis is supported by conformation-activity studies of potent RGD peptides (Gurrath et al., 1992), as well as studies on the size and shape of the integrin-binding loop as a determinant for conferring integrin specificity on cyclic RGD peptides with a known conformation (McLane et al., 1996). Our findings indicating the increased potency of viperistatin cyclic analogs, compared with that of linear peptides, are supported by previous studies in which cyclic RGD analogs showed a higher potency than their linear analogs (Pierschbacher and Ruoslahti, 1987; Wong et al., 1998). In addition to the A domain, the α subunits of the integrin receptors contain a variety of other motifs, such as extracellular β-propeller, thigh, genu, calf-1, calf-2, transmembrane, and cytoplasmic (Xiong et al., 2001), to which these analogs may bind allosterically resulting in an antagonistic effect on adhesion. Furthermore, experiments with recombinant α1A and α2A domains suggest that compounds 6 and 10 interact with α integrin sequences located outside the A domain. This may suggest that partial overlap with the conformational space of the reference compounds is sufficient.

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On the basis of these findings as well as the consideration that the integrin-binding loop supports the correct conformational presentation of the KTS sequence to α1β1 integrins (Brown et al., 2009), we synthesized cyclic viperistatin analogs in the second approach. The compound 3 linear sequence served as a basic structure for the cyclic peptides. Using single, double, and tetra intercyclization or intracyclization, we generated peptides of various sizes and structural constraints. The data we obtained in adhesion assays indicate that the number and type of cyclization had no significant effect on the potency of the cyclic compounds investigated, albeit the cyclization dramatically increased the potency compared with that of compound 3. Although all of the cyclic peptides showed reduced efficacy of binding in comparison with viperistatin, multiple intracyclization conferred 40% efficacy of compounds 9 and 10. The results we obtained by computation studies showed, as expected, an opposite correlation between the number of disulfide bonds and the conformational flexibility of the different compounds, with more bonds conferring conformational rigidity (Fig. 4) as well as stability (Fig. 12). Based on the analysis of the conformational ensembles, the conformational space sampled by compound 10 is the most similar to those of the reference peptides, obtustatin and viperistatin, being the least flexible among the modeled peptides. Compound 6 was more flexible than compound 10. This observation lends some credit to the hypothesis that active compounds have similar conformational ensembles. This hypothesis is supported by conformation-activity studies of potent RGD peptides (Gurrath et al., 1992), as well as studies on the size and shape of the integrin-binding loop as a determinant for conferring integrin specificity on cyclic RGD peptides with a known conformation (McLane et al., 1996). Our findings indicating the increased potency of viperistatin cyclic analogs, compared with that of linear peptides, are supported by previous studies in which cyclic RGD analogs showed a higher potency than their linear analogs (Pierschbacher and Ruoslahti, 1987; Wong et al., 1998). In addition to the A domain, the α subunits of the integrin receptors contain a variety of other motifs, such as extracellular β-propeller, thigh, genu, calf-1, calf-2, transmembrane, and cytoplasmic (Xiong et al., 2001), to which these analogs may bind allosterically resulting in an antagonistic effect on adhesion. Furthermore, experiments with recombinant α1A and α2A domains suggest that compounds 6 and 10 interact with α integrin sequences located outside the A domain. This may suggest that partial overlap with the conformational space of the reference compounds is sufficient.
for activity of compounds 6 and 10, which in turn implies that only part of the conformational space of the unbound (i.e., in solution) reference compounds is occupied by these compounds in their integrin bound state. This bioactive space is apparently shared by compounds 10 and 6 but to a lesser extent by compounds 5 and 3. These possibilities will be clarified in future experiments of characterizing the interactions between viperistatin analogs with whole α1 and α2 recombinant integrin receptor proteins.

Because both the α1 and α2 integrins provide critical support for physiologic and/or tumor angiogenesis (Senger et al., 2002), we characterized the effect of viperistatin analogs in several in vitro angiogenic assays, such as endothelial cell proliferation and migration and tube formation in Matrigel, and in vivo capillary formation in the CAM assay and corneal micropocket assay. Here we report that the novel compounds 6 and 10 efficiently inhibited endothelial cell proliferation and migration in the wound healing assay, tube formation in Matrigel, as well as VEGF and glioma-induced angiogenesis in the CAM assay. However, in the in vitro wound healing assay using HUVECs, compound 6 was more potent that compound 10; in vivo, in the mice corneal micropocket assay, mainly compound 6 was active. These different effects may be related to the different stability profiles of the peptides (Fig. 12) or their pharmacokinetic properties in in vivo assays. Furthermore, differences in antiangiogenic properties between compounds 6 and 10 obtained can be attributed to the differences in the assays used. Moreover, angiogenesis is not the same in different tissues and the cornea is naturally avascular (Staton et al., 2009). It is also known that antiangiogenic compounds, which have efficacy in vitro, may not show any activity in vivo and vice versa, with some compounds showing little efficacy in vitro having strong activity in vivo (Lievens et al., 2001). Although efficacy of binding for compounds 6 and 10 was lower than that of their natural lead structures, these findings were anticipated, because we previously found that the natural compound α1 antagonists viperistatin (Staniszewska et al., 2009) and obtustatin (Marcinkiewicz et al., 2003) and the α2 antagonist vixapatin (Momic et al., 2012a) are angiogenic inhibitors. Thus far, inhibition of angiogenesis by integrin antagonism has focused mostly on integrins αβ1 and αβ3, which serve as receptors for the extracellular matrix proteins fibronectin, vitronectin, and fibrinogen that contain an RGD motif (Cheresh, 1987). The peptides described in this article were designed based on the structure of the αβ1 integrin–inhibiting viperistatin. The integrin αβ1 binds collagen in an RGD-independent manner. To our knowledge, viperistatin is among the first highly selective and potent disintegrins to inhibit αβ1 integrin binding to collagen. The integrin αβ1 is closely related to αβ3 integrin and binds collagen similarly in an RGD-independent manner. Standing far apart from the RGD-dependent integrins, these two collagen-binding integrins have an overlapping ligand binding specificity. Hence, it is pharmacologically more challenging to test the selectivity of peptides toward αβ1 integrin versus the very similar αβ3 integrin. The original K562 cells used in our experiments as controls express only αβ1 integrin (Järvinen et al., 1993). In our hands, their binding to fibronectin-coated microtiter plates was not affected by compounds 6 and 10; therefore, the possibility that these compounds inhibit αβ1 is less plausible. Our findings further point to the involvement of αβ1/αβ3 integrins in angiogenesis, consistent with the phenotype of reduced vascularity in α1 and α2 knockout mice (Pozzi et al., 2000; Zhang et al., 2008).

In summary, this study describes the synthesis of small, cyclic, KTS peptides and dual antagonists of the αβ1/αβ3 integrins. Compound 6 (named vimocin) and compound 10 named (vidapin) are characterized by a high potency and an intermediate efficacy in αβ1 integrin binding, a conformational, tridimensional organization in water similar to viperistatin and obtustatin, as well as antiangiogenic activities. These compounds were characterized by half-lives of 12 and 30 hours, respectively, in human serum, and were not toxic to endothelial cells in culture and safe upon intravenous injection in mice. We suggest that these compounds may serve as candidates for drug development in therapy of angiogenesis disorders and cancer.

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Authorship Contributions

Participated in research design: Momic, Katzechendler, Benny, Eble, Marcinkiewicz, Lazarovici.

Conducted experiments: Momic, Katzechendler, Benny, Lahiani, Cohen, Noy, Eble, Marcinkiewicz.

Contributed new reagents or analytic tools: Benny, Noy, Senderowitz, Eble, Marcinkiewicz.

Performed data analysis: Momic, Katzechendler, Benny, Noy, Senderowitz, Eble, Marcinkiewicz, Lazarovici.

Wrote or contributed to the writing of the manuscript: Momic, Benny, Noy, Senderowitz, Eble, Marcinkiewicz, Lazarovici.

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