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Three Distinct D-Amino Acid Substitutions Confer Potent Antiangiogenic Activity on an Inactive Peptide Derived from a Thrombospondin-1 Type 1 Repeat

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ABSTRACT

Mal II, a 19-residue peptide derived from the second type 1 properdin-like repeat of the antiangiogenic protein thrombospondin-1 (TSP-1), was inactive in angiogenesis assays. Yet the substitution of any one of three L-amino acids by their D-enantiomers conferred on this peptide a potent antiangiogenic activity approaching that of the intact 450-kDa TSP-1. Substituted peptides inhibited the migration of capillary endothelial cells with an ED_{50} of 8.5 nM for the D-Ile-15 substitution, 10 nM for the D-Ser-4 substitution, and 0.75 nM for the D-Ser-5 substitution. A peptide with D-Ile at position 15 could be shortened to its last seven amino acids with little loss in activity. Like whole TSP-1, the Mal II D-Ile derivative inhibited a broad range of angiogenic inducers, was selective for endothelial cells, and required CD36 receptor binding for activity. A variety of end modifications further improved peptide potency. An ethylamide-capped heptapeptide was also active systemically in that when injected i.p. it rendered mice unable to mount a corneal angiogenic response, suggesting the potential usefulness of such peptides as antiangiogenic therapeutics.

In most normal adult tissue angiogenesis, the process by which new blood vessels arise from pre-existing ones is suppressed and vessels remain quiescent due in large part to the predominance of angioinhibitory molecules (Bouck et al., 1996). However, in numerous pathologic conditions such as arthritis, atherosclerosis, diabetic retinopathy, and cancer, angiostimulatory molecules can come to predominate, thus promoting the new vessel formation that supports disease progression. For example, the continued growth and efficient metastasis of all tumors is dependent on angiogenesis (Folkman, 1995b). In model systems, inhibitors of this process have proven to be remarkably effective drugs to which tumors do not develop resistance (Boehm et al., 1997). Although several antiangiogenic drugs have already entered clinical trials for the treatment of tumors and other angiogenesis-dependent diseases (Folkman, 1995a; Gradishar, 1997; Pluda, 1997), there remains a need to develop new agents with improved potency, stability, selectivity, and ease of delivery.

Naturally occurring inhibitors of angiogenesis that are secreted normally by mammalian cells provide one source for the identification of new antiangiogenic molecules. One such molecule is thrombospondin-1 (TSP-1). TSP-1 is a 450-kDa homotrimeric protein with multiple distinct structural domains that contribute to its involvement in diverse biological activities such as neurite outgrowth, platelet aggregation, and angiogenesis (Lahav, 1993; Bornstein, 1995; Dawson and Bouck, 1998). Between concentrations of 0.5 and 20 nM, TSP-1 inhibits in a variety of in vitro assays for angiogenesis (DiPietro, 1997; Tolsma et al., 1997) and blocks neovascularization in vivo (Good et al., 1990; Tolsma et al., 1993; Volpert et al., 1998). TSP-1 acts directly on microvascular endothelial cells, making them refractory to a broad range of angiogenic inducers (Tolsma et al., 1993; Volpert et al., 1995), an activity that is dependent on its interaction with CD36 receptor (Dawson et al., 1997). A central 50-kDa proteolytic fragment within TSP-1 containing its procollagen homology region and

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ABBREVIATIONS: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; IL-8, interleukin 8; PDGF, platelet-derived growth factor; TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor; HPLC, high-pressure liquid chromatography; BSA, bovine serum albumin.

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properdin type 1 repeats retains all of the angioinhibitory activity of the complete protein (Tolsma et al., 1993). Small peptides derived from each of these two domains are also able to inhibit angiogenesis both in vitro and in vivo (Tolsma et al., 1993) through a CD36-dependent mechanism (Dawson et al., 1997). Micromolar concentrations of the peptides are required to achieve the effects equivalent to those produced by low nM amounts of the intact TSP-1 molecule.

The therapeutic potential of whole TSP-1 has been demonstrated in animal models where it has been shown to block the growth and progression of malignant tumors by hindering their neovascularization (Weinstat-Saslow et al., 1994; Castle et al., 1997; Volpert et al., 1997, 1998). The use of whole TSP-1 as an antiangiogenic drug in humans is prohibitive due both to its size and its multiple other biological activities, but small peptides derived from it should provide a reasonable alternative if they are both specific and highly active.

Work presented here describes the optimization of one TSP-1 peptide as an antiangiogenic agent through the serendipitous discovery of an L- to D-amino acid racemization that occurred as an active contaminant in a peptide preparation. When any one of three distinct L-amino acid residues was changed to D- form, this peptide became 100- to 1000-fold more active than any previously described antiangiogenic TSP-1 peptide. The modified peptides demonstrated antiangiogenic activity both in vitro and in vivo at low nanomolar concentrations, and one of them was able to inhibit angiogenesis in vivo after systemic administration to mice, demonstrating the usefulness of these peptides as lead compounds for the development of new antiangiogenic agents.

Materials and Methods

Materials. Recombinant human acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) were purchased from R & D Systems, Inc. (Minneapolis, MN). FA6-152 monoclonal antibody against CD36 was purchased from Immunotech, Inc. (Westbrook, ME). The original Mal II peptide was derived from the second properdin repeat of TSP-1 and contained amino acid residues 424 to 442 of TSP-1 (SPWSSA*SVTA*GDGVITRIR, where A* indicates alanines in place of cysteines occurring in the natural sequence (Tolsma et al., 1993). It is similar to Mal III, which contains amino acid residues 481 to 499 (SPWDIA*SVTA*GGVQKRSK; Tolsma et al., 1993). Unless indicated by a "D" prefix, the stereochemistry of the α-carbon of amino acids listed in this article are of the natural or "L" configuration. New peptides were synthesized from side chain-protected FMOC-amino acids by solid state methods using a Synergy peptide synthesizer (Perkin-Elmer/Applied Biosystems, Foster City, CA) and purified by reversed-phase-high-pressure liquid chromatography (RP-HPLC) using a 7-μM preparative C_{18} column (VYDAC, Hesperia, CA) with increasing linear gradients of acetonitrile in water containing 0.1% trifluoroacetic acetic acid. Peaks detected by UV were isolated and products identified by fast atom bombardment mass spectrometry. All analytical RP-HPLC separations were performed at a flow rate of 1 ml/min with a 5-μM C_{18} column with an initial linear gradient from 10 to 18% acetonitrile at 15 min followed by a second linear gradient increasing to 22% acetonitrile at 45 min.

Binding Studies. Cell-binding assays were performed with Bowes melanoma cells stably transfected with human CD36 cDNA as described previously (Silverstein et al., 1992). Radiolabeled ^{125}I-TSP-1 (20 μg/ml) was mixed with increasing concentrations of peptide (0.1–1000 nM) and then incubated for 2 h at 4°C with CD36-transfected cells suspended in phosphate-buffered saline/bovine serum albumin (BSA) (0.5%). Cells were then washed five times with cold phosphate-buffered saline and lysed with 0.1 N NaOH. The amount of bound radioactivity was determined by γ-counting.

For solid-phase binding studies, a glutathione *S*-transferase-CD36 fusion protein (CD36 residues 67–157) was immobilized to plastic in a detachable 96-well microtiter plate as previously described (Dawson et al., 1997) at a final protein concentration of 200 to 300 ng per well. Wells were washed three times with 20 mM Tris, 150 mM NaCl, pH 7.4, and 0.05% Tween 20 (TBS-T), blocked with 0.5% BSA in TBS-T, and then incubated with ^{125}I-TSP-1 (20 μg/ml) along with increasing concentrations of peptide (0.1–1000 nM) for 2 h at 22°C. Wells were washed thoroughly with TBS-T and dried and bound radioactivity was measured by a gamma counter. IC_{50} values for both solid-phase and cell-binding assays were calculated using the curve-fitting software program Enzfitter (Elsevier Biosoft, Cambridge, UK).

Migration Assays. The in vitro endothelial cell migration assay provides a reproducible and quantitative measurement of the angiogenic or antiangiogenic potency of compounds and consistently parallels antiangiogenic activity seen in vivo (Folkman and Klagsbrun, 1987; Klagsbrun and D'Amore, 1991; Bouck et al., 1996). To determine angioinhibitory activity, peptides were tested for their ability to block capillary endothelial cell migration induced by the known angiogenic factor basic fibroblast growth factor (bFGF). Bovine adrenal capillary endothelial cells were provided by Judah Folkman and grown in Dulbecco's modified Eagle's medium with 10% donor calf serum (Flow Laboratories, McLean, VA) with 1% endothelial cell mitogen (Biomedical Technologies, Inc., Stoughton, MA) and used between passages 14 and 15. Human dermal microvascular endothelial cells were provided by Peter Polverini (University of Michigan, Ann Arbor, MI), grown in endothelial cell growth media (Clonetics, San Diego, CA), additionally supplemented with 10% fetal bovine serum, and used at passage 9.

Migrations were performed as described previously (Polverini et al., 1991). Endothelial cells were serum-starved overnight, harvested, resuspended in control medium (Dulbecco's modified Eagle's medium + 0.1% BSA), and plated at 3×10^4 cells/well on the bottom side of a gelatinized 5-μm (for bovine capillary endothelial cells) or 8-μm (for human microvascular endothelial cells) porous membrane (Nucleopore Corp., Pleasanton, CA) in an inverted, modified Boyden chamber and incubated at 37°C for 2 h to allow for attachment. The chamber was then reinverted, test samples were placed in the top wells, and cells were allowed to migrate toward the test samples for 4 h at 37°C. Membranes were removed, fixed, and stained, and the number of cells migrating to the top side of the membrane per 10 high-powered fields were counted. Cells migrating in control medium alone represented background resulting from random cell movement. Samples in each experiment were tested in quadruplicate and experiments were repeated at least three times. Data presented in the figures have been normalized to maximum migration, where 100% was calculated as the migration toward bFGF minus the background migration occurring in control medium alone. Negative percentages appear when test samples suppressed random cell movement. Determination of statistical significance between sample means was performed using a two-tailed *t* test on raw data before normalization.

In Vivo Angiogenesis Assays. In vivo corneal neovascularization assays were performed with Sprague-Dawley rats (Harlan, Indianapolis, IN) and C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) as described previously (Polverini et al., 1991; Kenyon et al., 1996). For rats, varying concentrations of peptide alone or in combination with 0.15 μM bFGF were incorporated into a Hydron pellet (Interferon Sciences, New Brunswick, NJ) from which a 5-μl volume was implanted into a surgically created pocket in the avascular cornea approximately 1.5 mm from the surrounding vascular limbus. At 7 days, rats were sacrificed and perfused with colloidal carbon to visualize vessels. A vigorous ingrowth of limbal vessels toward the pellet was scored as a positive response.

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For mice, 1-μl pellets of Hydron-Sucralfate (Bukh Meditec, Denmark) were formulated containing 100 ng/pellet of bFGF and implanted into the avascular cornea approximately 0.3 to 0.5 mm from the vascular limbus (Kenyon et al., 1996). Animals were divided into two groups and received i.p. injections twice a day for 5 days beginning on the day of the implant with either PBS vehicle or with the ethylamide-capped heptapeptide containing D-Ile. Vessel ingrowth was assessed by slit lamp microscopy after 5 days.

Results

Isomerization of a Serine Residue or an Isoleucine Residue Conferred Antiangiogenic Activity on the Mal II Peptide from TSP-1. We previously reported that two similar 19-mers derived from the second and third properdin repeats of TSP-1, Mal II from residues 424 to 442, and Mal III from residues 481 to 499, had antiangiogenic activity in vitro, inhibiting capillary endothelial migration with ED_{50} values of 0.6 and 5.0 μM and blocking neovascularization in vivo (Tolsma et al., 1993). These peptides were active even though the two cysteine residues that were present in the parent molecule had been replaced by alanine residues. The activity of different Mal II preparations had been somewhat variable over the years; this was attributed to stability problems. Recently, however, a newly synthesized, highly purified preparation was completely inactive. When this inactive sample was compared to an active one by mass spectrometry and Edman sequencing, the two preparations were more than 95% identical (data not shown). However, elution profiles from a C_{18} RP-HPLC column revealed minor contaminant peaks in the old active preparation (Fig. 1A). The activity of this preparation resided in one of the contaminant peaks, for when the major peak was repurified by RP-HPLC (Fig. 1B) it could no longer inhibit endothelial cell migration (Fig. 2), even when tested at concentrations up to 40-fold higher than those at which the unpurified substance was originally reported to be active (Tolsma et al., 1993).

A contaminant peak eluting just before the main peptide peak (Fig. 1A, 1) was active, for when isolated by RP-HPLC, it was able to inhibit endothelial cell migration with an ED_{50} of 10 nM (Fig. 2), based on its UV spectrum and assuming that like Mal II, the impurity contained one Trp residue per molecule. Because peak 1 represented approximately 1 to 2% of the original active Mal II preparation and had an ED_{50} roughly 60-fold better than that originally reported for Mal II, it could be responsible for most if not all of the inhibitory activity reported for Mal II in the previous study (Tolsma et al., 1993).

Material in peak 1 was identical with that in peak 2 by mass spectrometry and Edman sequencing (data not shown), raising suspicion that the active peak 1 might contain an L- to D-amino acid racemization, an alteration that could also explain its different mobility on RP-HPLC. To test this hypothesis, multiple Mal II peptides with single D-amino acid substitutions at each residue (except glycine) in the original 19-mer were empirically synthesized and tested in pools of three to four for their ability to inhibit endothelial cell migration. Three different substitutions conferred antiangiogenic activity: replacement of either L-Ser_4 or L-Ser_5 with D-Ser or replacement of L-Ile_{15} with D-Ile (hereafter referred to as D-Ser_4-Mal II, D-Ser_5-Mal II or D-Ile-Mal II, respectively) were antiangiogenic. They blocked the migration of capillary endothelial cells toward bFGF (Fig. 2), with ED_{50} values of 8.8 $\pm$ 0.7 nM for D-Ile-Mal II, 10 nM for D-Ser_4-Mal II and 0.8 $\pm$ 0.05 nM for D-Ser_5 -Mal II. Combining D-Ser_5 with D-Ile_{15} in a single peptide did not significantly increase peptide activity in a migration assay (data not shown).

In vivo, all three D-modified peptides inhibited neovascularization. Although neutral when tested alone, each peptide was able to suppress the growth of vessels into the rat cornea in response to bFGF (Table 1). When mixtures of modified and unmodified peptides were run in combination on RP-HPLC, only D-Ser_5-Mal II eluted just before unmodified all L-amino acid Mal II, suggesting it was likely the active contaminant in the original preparation (data not shown).

Truncation Coupled with End Modifications Enhanced the Activity of D-Ile-Mal II. To optimize D-Ile-Mal II as an antiangiogenic therapeutic, smaller peptides and peptides with end modifications were synthesized and tested for their ability to inhibit endothelial cell migration. Previous analysis of the 19-residue Mal III peptide from the third properdin repeat in TSP-1, which is very similar in sequence to Mal II but is antiangiogenic without D-amino acid substitutions (Tolsma et al., 1993), had shown that activity resided in the C-terminal eight residues (S. Tolsma, personal communication). Similarly, peptides containing either the last seven or nine amino acids of D-Ile-Mal II retained inhibitory activity (Table 2). Acetylation at the N-terminus of the heptapeptide improved activity over that of the unmodified non-

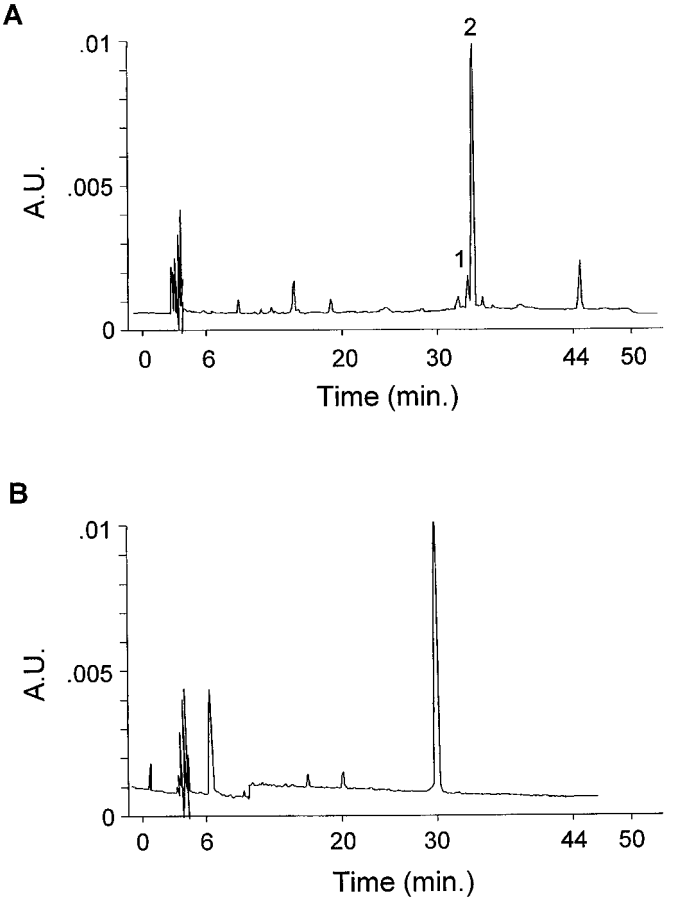

Fig. 1. RP-HPLC separation revealed minor contaminants present in an angioinhibitory Mal II preparation. Peptide profile of an active Mal II preparation separated by C_{18} RP-HPLC with an increasing linear gradient of acetonitrile before (A) and after (B) preparative separation. Peak 1, active contaminant; peak 2, Mal II 19-mer.

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apeptide (Table 2). In vivo, the acetylated heptapeptide inhibited bFGF-induced neovascularization when incorporated into a pellet implanted in the rat cornea (Table 1).

N-alkylamide formation at the C terminus of the acetylated heptapeptide to generate an ethylamide cap resulted in an approximate 4-fold increase in inhibitory activity as measured by migration (Table 2). A retro-inverso analog of the D-Ile-Mal II heptapeptide was synthesized with its amino acids in reverse order and inverted chirality in relation to α-carbon stereochemistry. Although this peptide lacked end group complementarity and failed to maintain the orientation of secondary chiral centers present in the threonine and two isoleucines, it remained active at inhibiting endothelial cell migration compared with the original heptapeptide (Table 2). None of the peptides listed in Table 2 had any significant effect on random endothelial cell migration when tested alone in the absence of inducer (data not shown).

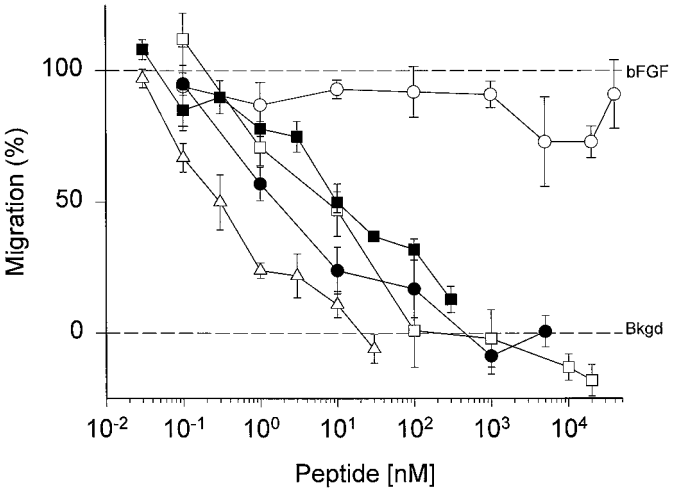

Fig. 2. D-Isomerization of either serine-4 or serine-5 or isoleucine-15 conferred inhibitory activity on Mal II. HPLC purified Mal II (Peak 2 from Fig. 1B; ○), closely eluting contaminant peak (peak 1 from Fig. 1A; ●), Mal II synthesized with a D-isoleucine at amino acid 15 (□), and Mal II synthesized with a D-serine either at amino acid 4 (■) or 5 (△) were tested at increasing concentrations for the ability to block the migration of capillary endothelial cells toward 10 ng/ml bFGF. Data are reported as a percentage of maximum migration where 100% represents the number of cells migrating in response to bFGF (----bFGF) and 0% represents the number of cells randomly migrating in the absence of any inducer (----Bkgd). Bars, S.E.

TABLE 1

D-amino acid-substituted Mal II peptides inhibited neovascularization when implanted with an inducer into the rat cornea

Peptide[a]	Concentration	Positive corneas/ no. implanted	
		Tested alone	Tested + bFGF
	μM		
D-Ser_4-Mal II, 19-mer	10	0/3	0/4
D-Ser_5-Mal II, 19-mer	1.0	1/2	1/5
D-IIe_{15}-Mal II, 19-mer	20	0/3	0/3
D-IIe_{15}-Mal II, acetylated heptapeptide	0.05	0/3	0/2
	0.1	0/3	1/3
	1.0	0/3	0/5
	20	0/4	0/2
Buffer control		nd	6/6

Indicated peptide was incorporated into a hydron pellet, implanted into rat corneas, and neovascularization was assessed 7 days later. Vigorous, sustained ingrowth of vessels was scored as a positive angiogenic response. nd, not determined.
[a] See Table 2 for peptide structures.

D-Ile-Mal II Retained the Activity and Specificity of the TSP-1 Parent Molecule. Like TSP-1 protein, D-Ile-Mal II was able to inhibit endothelial cell migration induced by all standard inducers of angiogenesis against which it was tested including aFGF, bFGF, IL-8, PDGF, and VEGF (Fig. 3). It was selective for endothelial cells as it failed to inhibit the migration of neutrophils, fibroblasts, and keratinocytes even when tested at concentrations up to 1000-fold higher than that at which it inhibited endothelial cell migration (data not shown).

The CD36 receptor is necessary for TSP-1 inhibition of endothelial cell migration (Dawson et al., 1997) and of neovascularization in vivo (see *Discussion* in Dawson et al., 1997). To determine whether D-Ile-Mal II inhibition of endothelial cell migration was also dependent on an interaction with the CD36 receptor, the peptide was tested in an endothelial cell migration assay in combination with the anti-CD36 monoclonal antibody FA6-152. This antibody binds an epitope on the extracellular domain of CD36 (Daviet et al., 1995), physically displaces TSP-1 from CD36 (Kieffer et al., 1989), and functionally blocks the inhibition of endothelial cell migration by TSP-1 and by Mal III, a peptide derived from the third properdin repeat of TSP-1 whose primary amino acid sequence is quite similar to that of inactive Mal II (Dawson et al., 1997). The anti-CD36 antibody was able to completely block D-Ile-Mal II and Mal III inhibition of bFGF-induced human microvascular endothelial cell migration (Fig. 4), whereas human umbilical vein endothelial cells that do not express CD36 were insensitive to inhibition by D-Ile-Mal II (data not shown).

D-Ile-Mal II peptides physically interacted with CD36, for they were able to competitively displace ^{125}I-TSP-1 from CD36 expressed exogenously on the surface of transfected Bowes melanoma cells. Each of the D-Ile-Mal II peptide analogues inhibited TSP-1-binding with an IC_{50} that correlated

TABLE 2

Modified Mal II peptides containing D-IIe_{15} inhibited capillary endothelial cell migration

Peptides	Sequence																		ED_{50}
																			nM
Unmodified Mal II	S	P	W	S	S	A	S	V	T	A	G	D	G	V	DI	T	R	I	R	>200
D-IIe 15-Mal II derivatives																				
19-mer	S	P	W	S	S	A	S	V	T	A	G	D	G	V	DI	T	R	I	R	8.5 ± 0.7
Nonapeptide											G	D	G	V	DI	T	R	I	R	3.0 ± 3.8
Acetylated heptapeptide													Ac-G	V	DI	T	R	I	R	7.1 ± 1.0
Ethylamide-capped heptapeptide													Ac-G	V	DI	T	R	I	R-Neth	1.8 ± 0.2
Retro-inverso													COOH-G	DV	I	DT	DR	DI	DR-NH_2	7.0 ± 0.6

The concentration at which each peptide inhibited 50% of bFGF-stimulated capillary endothelial cell migration was determined from three independent experiments with at least five concentrations of peptide. Each peptide is listed starting from its N terminus, with the exception of the retro-inverso which is listed starting from its C terminus. Ac, acetyl group; Neth, *N*-ethylamide group.

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with its ED_{50} for inhibition of endothelial cell migration. The HPLC purified Mal II containing only L-amino acids that was inactive in migration assays was unable to inhibit TSP-1 binding (Table 3). The D-Ile-Mal II peptide analogues also blocked ^{125}I-TSP-1 binding to CD36 immobilized on plastic. Similar trends were seen when comparing the solid-phase-binding assay IC_{50} values with the migration assay ED_{50} values of different peptides. IC_{50} values were greater for the solid-phase studies compared with those of the cell-binding studies in the case of the retro-inverso peptide (Table 3), a difference that may reflect unfavorable constraints placed on CD36 when immobilized to plastic.

Systemically Administered D-Ile-Mal II Peptides Inhibited Angiogenesis In Vivo. A mouse corneal neovascularization assay was used to demonstrate the potential usefulness of systemically administered D-Ile-Mal II derivatives as antiangiogenic therapeutics. Pellets formulated with the angiogenic inducer bFGF were implanted in mouse corneas where they induced vigorous vessel ingrowth by 5 days (Table 4). However, when animals additionally received daily i.p. injections of active peptide, vessel ingrowth toward bFGF was suppressed (Table 4). No such suppression was seen in animals injected with vehicle alone.

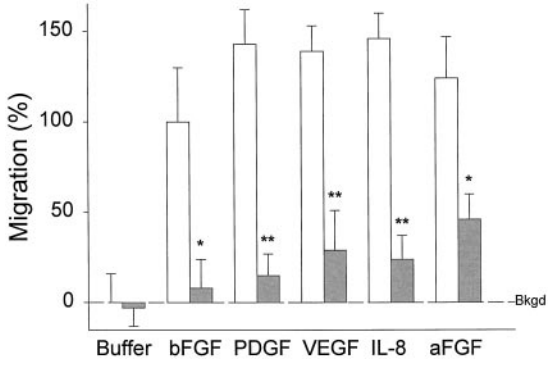

Fig. 3. D-Ile-Mal II blocked the response of capillary endothelial cells to multiple inducers of angiogenesis. Endothelial cells were allowed to migrate either in the absence (open column) or presence (shaded column) of *N*-ethylamide capped, D-Ile Mal II heptapeptide (20 nM) toward a variety of different inducers including bFGF (10 ng/ml), PDGF (250 pg/ml), VEGF (100 pg/ml), IL-8 (50 ng/ml), and aFGF (50 ng/ml) and migration quantified as in Fig. 2. Significant inhibition by peptide compared to each inducer when tested alone is indicated $*p < .05$; $**p < .005$.

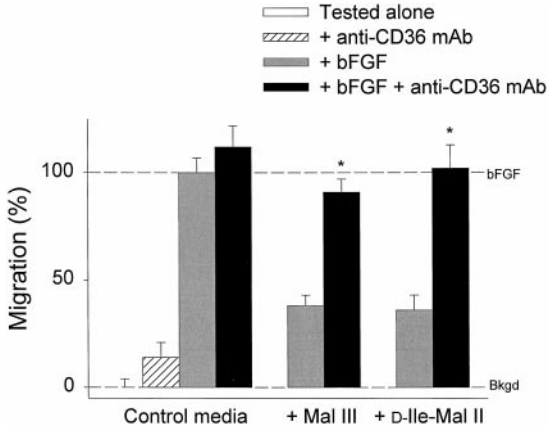

Fig. 4. D-Ile- Mal II inhibition of endothelial cell migration required the CD36 receptor. A monoclonal IgG antibody against CD36 (FA6–152; 15μg/ml) was tested for its ability to prevent the inhibition of human microvascular endothelial cell migration toward bFGF by either Mal III, an active peptide from the third properdin repeat in TSP-1, used at 30 μM, or D-Ile-Mal II used at 100 nM. Data were normalized and reported as in Fig 2. Significant differences due to addition of CD36 blocking antibody is indicated $*p < .05$.

Discussion

When synthesized with either a single D-Ser substitution at position 4 or 5 or a D-Ile at position 15, the inactive L-amino acid 19-residue Mal II peptide became potently antiangiogenic. Its activity increased over 200-fold, becoming comparable to that of the large 450-kDa TSP-1, which contains at least two antiangiogenic regions on each of its three subunits (Tolsma et al., 1993). D-amino acid substitutions in a variety of other biologically active peptides typically increase in activity from 2- to 50-fold over an already active parent peptide (Morley, 1980; Spatola, 1983; Fauchere and Thurieau, 1992). Increases in activity can be due to increased resistance to proteolytic degradation (Morley, 1980; Fauchere and Thurieau, 1992). Proteolysis is a component of angiogenesis, and migrating endothelial cells are known to secrete protease activity (Moscatelli and Rifkin, 1988) so increased stability may play a role in the rise in activity we observe, particularly in vivo where the peptides are exposed to a variety of physiological proteases.

However, enhanced stability is not sufficient to explain the dramatic increases in receptor binding observed in solid-phase-binding studies performed at low temperature in the absence of cells and their proteases. Moreover, a retro-inverso peptide with most amino acids in protease-resistant D form showed no increase in cell binding or antiangiogenic activity. It thus seems likely that the primary effect of the selective D-amino acid substitutions is to increase the like-

TABLE 3
Inhibition of TSP-1 binding to CD36 by Mal II peptides

Peptide	IC_{50}	
	Cell binding	Solid phase
	nM	
Unmodified Mal II	>10,000	1,000
D-Ser_5-Mal II, 19-mer	6.22 ± 5.02	7.4 ± 4.2
D-IIe_{15} Mal II variants		
Original 19-mer	20.8 ± 12.3	12.5 ± 3.3
Nonapeptide	15.4 ± 8.9	12.8 ± 4.2
Ethylamide-capped heptapeptide	6.8 ± 4.1	4.6 ± 3.3
Retro-inverso	11.2 ± 5.8	28.4 ± 5.4

Binding of ^{125}I-labeled TSP-1 (20 μg/ml) to a confluent monolayer of Bowes melanoma cells expressing CD36 or to solid-phase fusion protein expressing CD36 amino acids 93 to 155 immobilized on plastic was competed with increasing concentrations of hyperpurified inactive Mal II or various Mal II analogs. IC_{50} values were determined using the ENZFITTER curve-fitting program.

TABLE 4
Suppression of corneal neovascularization by systemic treatment of mice with D-IIe_{15} Mal II peptides

Peptide Injected Systemically	Dose	Corneal responses			
		+	+/−	−	% inhibited
	mg/mouse/day				
Ethylamide-capped heptapeptide	0.2	2	4	1	14
	0.75	1	2	6	67
PBS vehicle		9	0	0	<0.2

Mouse corneas were implanted with pellets releasing bFGF and the animals were treated systemically with the indicated compound for 5 days, after which corneal angiogenesis was assessed. +, vigorous ingrowth to pellet; +/−, weak response with few vessels growing inward; −, no vessel ingrowth.

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lihood that the Mal II peptide will assume a conformation that favors receptor engagement (Morley, 1980). Each of the D substituted peptides clearly acquired an ability to bind to the TSP-1 receptor that the all L-amino acid peptide did not have. They all required the receptor for biological activity and at low nanomolar concentrations could physically displace radiolabeled TSP-1 from cell surface CD36 and from CD36 immobilized on plastic. X-ray diffraction studies performed with related peptide sequences in properdin protein predict that Mal II sequence occurs as an exposed, elongated β-sheet in TSP-1 (Smith et al., 1991). A D-enantiomer substitution could both alter the position of amino acid side chains in relation to one another and change the configuration of the peptide backbone itself to favor a similar exposed structure.

The unmodified Mal II peptide failed to displace whole TSP-1 from CD36 and did not activate CD36 despite the presence of the SVTCG motif, a sequence previously shown to be a binding site for CD36 on peptides derived from the type 1 repeats of TSP-1 (Asch et al., 1992; Li et al., 1993). The SVTCG motif occurs twice in TSP-1, in both the second and third type 1 repeats. Experiments reported here used peptides from the second repeat and in this context SVTCG did not mediate binding to CD36. However, when SVTCG is present in the context of the third type 1 repeat, the one tested by Li et al. (1993), it may have binding activity and may influence biological activity. Although the SVTCG-containing Mal II peptide derived from the second type 1 repeat is inactive, the parallel peptide derived from the third type 1 repeat, Mal III, is an active antiangiogenic agent without any D-amino acid substitutions. Its SVTCG motif is not essential for its biological activity, but the presence of these amino acids does enhance antiangiogenic activity by 2- to 3-fold (Tolsma et al., 1993; Tolsma, 1995). These data are consistent with the suggestion of Li et al. (1993) that there may be more than one binding site for TSP-1 on CD36.

Presumably as a result of their ability to bind CD36, the D-substituted peptides retained biological features of the intact TSP-1 molecule. They were selective for microvascular endothelial cells and made these cells refractory to a wide variety of inducers. The peptides reported here are distinct from another set of peptides derived from the second properdin repeat in TSP-1 that show some antiangiogenic activity against heparin-binding inducers of angiogenesis in vitro (Vogel et al., 1993; Guo et al., 1997a,b). The amino acid sequence from which these peptides are derived on the intact TSP-1 is adjacent to that from which Mal II comes. The six N-terminal residues of the 19-amino acid Mal II are shared between the two peptides, but these shared residues are not sufficient to confer activity on the peptides studied by Vogel et al. (1993) and Guo et al. (1997a,b) and the shared residues could be deleted from D-Ile-Mal II derivatives without significant loss of activity.

The substitution of D amino acids has also been shown to improve by 3- to 4-fold the activity of these other TSP-1 peptides that derive their activity from their heparin-binding motif (Guo et al., 1997b). But unlike the current work, where only single D-amino acid substitutions were made, resulting in inactive peptides becoming active due to alterations in their conformation, all of the L-amino acids in the heparin-binding peptides were changed to D-isomers in a retro-inverso format and their enhanced activity seemed to be due solely to improved stability.

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When the quantitative endothelial cell migration assay was used as a benchmark for potency, D-substituted Mal II peptides, including some as short as seven amino acids, compared favorably with other known inhibitors of angiogenesis. Their ED_{50} values, which ranged from 0.85 to 8.5 nM, are comparable to those of whole TSP-1 (0.5 nM; Tolsma et al., 1993), endostatin (3.0 nM; O.V.V. and N.P.B., unpublished data), TSP-2 (4 nM; Volpert et al., 1995), angiostatin (3.5 nM; Gately et al., 1996), and retinoic acid (15 nM; Lingen et al., 1996) and are far below those of other small TSP-1 peptides (Tolsma et al., 1993), tissue inhibitor of metalloproteinase-1 (Johnson et al., 1994), and captopril (Volpert et al., 1996), which each have ED_{50} values well into the micromolar range.

D-Ile-Mal II was quite amenable to alterations aimed at improving its clinical utility. It could be shortened to as little as seven amino acids with only minimal loss in activity, and the addition of acetyl and ethylamide end groups further increased potency. Most remarkably, i.p. delivery of one modified derivative drove mice into a systemic antiangiogenic state. The in vivo efficacy of these simple peptides and their apparent dependence on CD36 as a receptor suggest that CD36 may be a promising target for the development of drugs designed to treat pathologic neovascularization by mimicking the antiangiogenic properties of TSP-1.

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