Antimetastatic Activities of Synthetic Arg–Gly–Asp–Ser (RGDS) and Arg–Leu–Asp–Ser (RLDS) Peptide Analogues and Their Inhibitory Mechanisms

Hideki FUJII,[a,b] Hiroyuki KOMAZAWA,[c] Hideto MORI,[c] Masayoshi KOJIMA,[c] Isamu ITOH,[c] Jun MURATA,[a] Ichiro AZUMA,[b] and Ikuo SAIKI*,[a]

Research Institute for Wakan-Yaku (Traditional Sino-Japanese Medicines), Toyama Medical and Pharmaceutical University,[a] 2630 Sugitani, Toyama 930–01, Japan, Institute of Immunological Science, Hokkaido University,[b] Kita-15, Nishi-7, Kita-ku, Sapporo 060, Japan and Fuji Photo Film Co., Ltd., Ashigara Research Laboratories,[c] 210 Nakanuma, Minami-ashigara 250–01, Japan. Received June 15, 1995; accepted July 26, 1995

We have investigated the inhibitory effect of the N-terminal modified Arg–Gly–Asp–Ser (RGDS) analogues, AcDRGDS and AcDRLDS, on tumor cell adhesion to the components of extracellular matrix and basement membrane, and also tested the antimetastatic effect of their conjugates with trimesic acid, Ar(DRGDS)$_3$ and Ar(DRLDS)$_3$. AcDRGDS significantly inhibited tumor cell adhesion to fibronectin, vitronectin and RGDS substrates, but not to CS1 substrate which is a ligand for the $\alpha_4\beta_1$ tumor surface integrin receptor. In contrast, AcDRLDS variant peptide significantly inhibited tumor cell adhesion to laminin, in addition to RGDS-mediated adhesion to fibronectin and vitronectin. AcDRLDS also inhibited tumor cell adhesion to CS1 as well as the RGDS sequence within the fibronectin molecule in a concentration-dependent manner, although the inhibitory effect was less than that of the CS1 (EILDV) peptide. Ar(DRLDS)$_3$ inhibited the laminin- and fibronectin-mediated invasion and migration of tumor cells, whereas Ar(DRGDS)$_3$ selectively inhibited fibronectin-mediated invasion and migration. Ar(DRGDS)$_3$ and Ar(DRLDS)$_3$ were much more effective in inhibiting experimental lung or liver metastases of various types of murine and human tumors than the original RGDS-containing peptides or Ar(COONa)$_3$. Multiple administrations of Ar(DRGDS)$_3$ or Ar(DRLDS)$_3$ potently inhibited spontaneous lung metastasis produced by intra-footpad injection of B16-BL6 cells without affecting the primary tumor size at the time of surgical excision, as compared with RGDS peptide or untreated control. Thus, Ar(DRGDS)$_3$ and Ar(DRLDS)$_3$ substantially increased the exhibiting any antimetastatic effect of the peptides without direct cytotoxicity.

Key words Arg–Gly–Asp–Ser (RGDS) analogue; Arg–Leu–Asp–Ser (RLDS) analogue; metastasis; fibronectin; invasiveness

Metastasis is one of the major causes of cancer mortality. During the sequential steps of metastasis, metastasizing tumor cells encounter various host cells (platelets, lymphocytes or endothelial cells), extracellular matrix and basement membrane components.[1–4] This encounter can lead to enhancement of survival, arrest or invasiveness of the tumor cells.[2,4–7] Specific incidents of tumor interaction with host cells or components are, therefore, fundamental events in the metastatic process. Consequently, both adhesion and detachment of cells are probably of prime importance in achieving control of cellular functions of diverse cell types, including highly metastatic tumor cells.

The interaction of cells with extracellular matrix and basement membrane components such as fibronectin, laminin and collagens plays a key role in a variety of biological processes including cell growth, differentiation, wound healing and cancer metastasis *etc.* Rapid progress has been made in the structural and functional analysis of cellular adhesion molecules involved in cell–cell or cell–extracellular matrix interactions. The primary structures of some cell adhesion proteins and their cell surface receptors, including integrins, have been identified by molecular DNA technology[8–11] and their cell surface receptors, including integrins, have been also identified. A common and characteristic Arg–Gly–Asp–Ser (RGDS) sequence in the central cell-binding domain of fibronectin has been shown to bind $\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, $\alpha_3\beta_1$ and $\alpha_v\beta_1$ integrins on the cell surface and other related adhesion molecules have been shown to contribute to cell functions including adhesion as well as cell spreading and motility.[12–14]

Several studies have suggested that some synthetic peptides derived from adhesion molecules that are present in extracellular matrices, basement membranes or plasma can modulate the mechanism involved in the metastasizing function of tumor cells. In attempts to regulate the mechanism involved in cell adhesion during the metastatic process, fibronectin-derived peptides such as RGDS,[15,16] and CS1[17,18] of alternative splicing type III connecting segment and purified 33-kDa heparin-binding fragment,[19,20] have been used to inhibit experimental tumor metastasis in murine tumor systems. Try–Ile–Gly–Ser–Arg (YIGSR) derived from laminin has also been shown to inhibit lung metastasis when co-injected i.v. with tumor cells.[21,22] Recently, some attempts to control tumor metastasis have been carried out by using synthetic cell-adhesive peptide analogues including cyclic RGDS or cyclic YIGSR peptides *etc.*[22–24] However, a high dose of RGD-containing oligopeptide was needed to obtain appreciable effects, therefore, it is important to find improved methods to control tumor metastasis. We have recently reported that the N-terminal modified RGDS peptide (AcDRGDS) and its analogue (AcDRLDS) in which the amino acid of Gly is substituted with Leu, are more effective in inhibiting lung and liver metastasis of tumor cells than the original RGDS peptide. Poly(RGD), which contains the repetitive RGD sequence, has also

* To whom correspondence should be addressed.

been shown to inhibit not only experimental but also spontaneous tumor metastasis of different tumors, as well as cell-adhesive properties more effectively than RGD-containing oligopeptides.[25-27] This suggests that chemical modification and polymerization of the RGD sequence can increase the inhibition of tumor metastasis.

In the present study, we report the effect of AcDRGDS, AcDRLDS and their trimesic acid conjugates, $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$, on experimental metastasis of different types of tumors, as well as the therapeutic effects on spontaneous lung metastasis in mice. We have also tested these compounds in an *in vitro* tumor cell adhesion, migration or invasion assay to characterize their inhibitory mechanisms.

MATERIALS AND METHODS

Chemicals and Analysis Unless otherwise stated, all commercially available solvents and reagents were used without further purification. *N,N*-Dimethylformamide (DMF) was stored over molecular sieves (3A) for several days before use. *tert*-Butoxycarbonyl (Boc) amino acids were purchased from Kokusan Chemical Works Ltd. Arg–Gly–Asp–Ser tetrapeptide (RGDS) was purchased from Peptide Institute Inc.

^{1}H-NMR spectra were recorded with a Bruker AC-200 (200 MHz) spectrometer in chloroform-*d*, dimethyl-d_6 sulfoxide or deuterium oxide, using tetramethylsilane (TMS) or 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (DSS) as an internal standard. Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of Silica gel $60F_{256}$ (Merck).

Synthesis of RGDS Peptide Analogues The synthetic peptides used in this study and their abbreviations (based on the single-letter amino acid code) are given in Fig. 1. Chart 1 shows the synthetic procedure for $Ar(DRGDS)_3$. RGDS protected peptide was synthesized by the conventional liquid phase method. Boc, mesitylenesulfonyl (Mts), and benzyl (Bzl) groups were used as protecting groups for α-amino, guanidino and β-carboxyl groups, respectively. The purity of the peptides were confirmed by

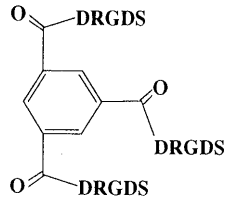
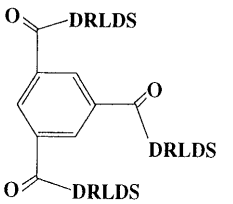
	Peptide Abbreviation (single letter amino acid code)
H-Arg-Gly-Asp-Ser-OH	RGDS
Acetyl-Asp-Arg-Gly-Asp-Ser-OH	AcDRGDS
Acetyl-Asp-Arg-Leu-Asp-Ser-OH	AcDRLDS
H-Glu-Ile-Leu-Asp-Val-OH	EILDV
	$Ar(DRGDS)_3$
	$Ar(DRLDS)_3$

Fig. 1. Peptides Used in This Study

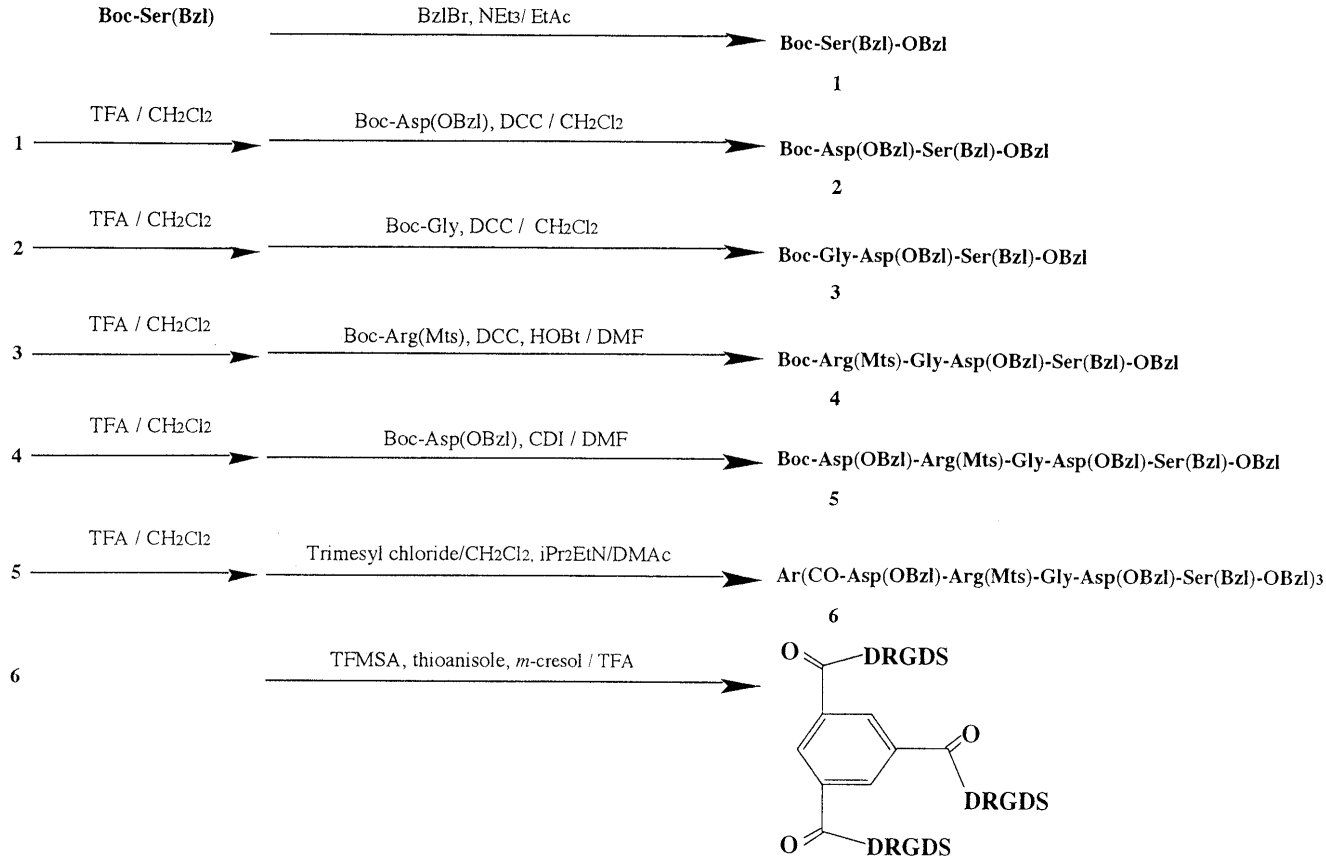


Chart 1. Synthetic Procedure for $Ar(DRGDS)_3$

TLC. This complete synthetic procedure for RGDS protected peptide has been described elsewhere.[28,29] Ar(DRGDS)$_3$ was synthesized as descrived below, and other RGDS peptide analogues were synthesized in the same way.

BocAsp(OBzl)Arg(Mts)GlyAsp(OBzl)Ser(Bzl)OBzl: To 5.52 g (18 mmol) BocAsp(OBzl) in 50 ml DMF 2.92 g (18 mmol) *N,N'*-carbonyldiimidazole (CDI) was added and stirred at 0 °C for 1 h. 18.0 g (18 mmol) TFA Arg(Mts)GlyAsp(OBzl)Ser(Bzl)OBzl and 2.33 g (18 mmol) of isoPr$_2$EtN in 50 ml DMF were added with continuous stirring at 0 °C for 2 h, then at room temperature for a further 14 h. DMF was evaporated under reduced pressure. The residue was dissolved in 300 ml $CHCl_3$ followed by washing with 200 ml water twice, 200 ml 10% citric acid twice and 200 ml 9% sodium bicarbonate solution twice. The chloroform solution was dried over Na_2SO_4 and then evaporated to dryness under reduced pressure. Ether was added to the residue to crystallize the product. 19.73 g (92.1%) BocAsp(OBzl)Arg(Mts)GlyAsp(OBzl)Ser(Bzl)OBzl was obtained in colorless solid.

TFA Asp(OBzl)Arg(Mts)GlyAsp(OBzl)Ser(Bzl)OBzl: To the pentapeptide in 50 ml CH_2Cl_2 was added trifluoroacetic acid (TFA, 100 ml) and the mixture stirred at room temperature for 1 h. After removing the solvents under reduced pressure ether was added to the residue. The crystallized colorless solid was collected by filtration. 19.76 g (quant.) TFA Asp(OBzl)Arg(Mts)GlyAsp(OBzl)Ser(Bzl)OBzl was obtained.

Ar(CO-Asp(OBzl)Arg(Mts)GlyAsp(OBzl)Ser(Bzl)OBzl)$_3$: To the 1.01 g (0.83 mmol) TFA Asp(OBzl)Arg(Mts)GlyAsp(OBzl)Ser(Bzl)OBzl (0.83 mmol) in 10 ml dry *N,N*-dimethylacetamide (DMAc), 0.26 g (2.01 mmol) dry isoPr$_2$EtN, 0.073 g (0.27 mmol) trimesyl chloride in 4 ml dry CH_2Cl_2 and a small amount of dimethylaminopyridine were added and stirred at 0 °C for 1 h, then at room temperature for a further 2 h. The reaction mixture was poured into water and the precipitate was collected by filtration followed by washing several times with water. The product was dried *in vacuo* at 40 °C. 0.94 g (quant.) trimesic acid derivative was obtained.

Ar(DRGDS)$_3$: 0.94 g trimesic acid derivative was reacted with trifluoromethanesulfonic acid (TFMSA)-*m*-cresolthioanisole/TFA solution (30 ml) at 0 °C for 2 h. The crude product was reprecipitated with ether, then dissolved in water. The pH of the solution was adjusted to 7 with 1 N NaOH; 150 ml ethanol was added to the solution, and the colorless solid was collected, dissolved in water and lyophilized. 0.34 g Ar(DRGDS)$_3$ was obtained. Elemental analysis: *Anal.* Calcd for $C_{66}H_{96}N_{24}O_{36}\cdot 10H_2O$: C, 40.00; H, 5.90; N, 16.97. Found C, 39.87; H, 5.56; N, 17.00.

Mice Specific pathogen-free female C57BL/6, BALB/c, C3H/HeN, CDF$_1$ (BALB/c × DBA/2) and BALB/c nu/nu mice, 7—10 weeks old, were purchased from Japan SLC, Inc., Hamamatsu, Japan. The mice were maintained in the Laboratory for Animal Experiments, Institute of Immunological Science, Hokkaido University, under laminar air-flow conditions.

Cells Highly metastatic B16-BL6 and K1735 melanoma cells, obtained by an *in vitro* selection procedure for invasion,[30] were kindly provided by Dr. I. J. Fidler, M. D., Anderson Cancer Center, Houston, TX. Liver metastatic L5178Y-ML25 T-lymphoma cells (partially metastasizing to the spleen), were kindly provided by Dr. A. Okura, Banyu Pharmaceutical Co., Tokyo.[31,32] Lung metastatic line of Colon26 carcinoma (Colon26 M3.1) was obtained by the *in vivo* selection method of Fidler.[33] These murine tumor cells (B16-BL6, K1735, Colon26 M3.1) and HT1080 human fibrosarcoma cells were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7.5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, nonessential amino acids and L-glutamine. L5178Y-ML25 cells were maintained in RPMI-1640 supplemented with 7.5% FBS and L-glutamine.

Assay of Experimental Lung Metastasis of Various Tumor Cells Log-phase cell cultures of B16-BL6, K1735, Colon26 M3.1 and HT1080 cells were harvested with 1 mM EDTA in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS), washed three times with serum free MEM, and resuspended to give appropriate concentrations in PBS. C57BL/6, BALB/c, C3H/HeN or BALB/c nu/nu mice were given by i.v. injection of B16-BL6 (5×10^4/100 μl), K1735 (2×10^4/100 μl), Colon26 M3.1 (3×10^4/100 μl) or HT1080 (3×10^5/100 μl) cells, respectively, admixed with compounds in PBS (100 μl). Fourteen or 21 d after inoculation of the tumor cells, the mice were killed and the lungs fixed in Bouin's solution. The number of tumor colonies in each lung was recorded under a dissecting microscope.

Assay of Spontaneous Lung Metastases of B16-BL6 Melanoma Cells Mice were given s.c. injection of B16-BL6 melanoma cells (5×10^5/50 μl), which were harvested as described above, into the right hind footpad. The compounds in PBS were administered i.v. on various days after tumor inoculation and surgical excision of the primary tumors was carried out on day 21. Mice were killed 35 d after tumor inoculation and the lung tumor colonies were manually counted.

Assay of Liver Metastasis of L5178Y-ML25 Lymphoma Cells Log-phase cell cultures of L5178Y-ML25 T-lymphoma cells were washed three times with serum-free MEM, and resuspended to give a final concentration of 4×10^5/ml in PBS. CDF$_1$ mice were given by i.v. injection of L5178Y-ML25 lymphoma cells (4×10^4). The compounds in PBS were co-injected i.v. with tumor cells. The mice were killed 14 d after tumor inoculation and the liver weight recorded to evaluate tumor metastasis as previously described.[23,28]

Microassay for Cell Adhesion The cell attachment assay was carried out by a method similar to that previously described.[34] B16-BL6 melanoma cells in an exponential growth phase were washed twice in warm PBS, harvested by adding 1 mM EDTA in PBS, and resuspended in cold serum-free MEM to form a single-cell suspension. The tumor cell suspensions (2×10^4), with or without peptides in a volume of 50 μl/well, were added to microculture wells pre-coated with 50 μl 3 μg/ml fibronectin (UCB-Bioproducts, S.A.), laminin (Collaborative Research, Inc., MA), vitronectin (Iwaki Glass, Tokyo) or type IV collagen (Life Technologies, Inc., MA). The cultures were incubated at 37 °C for 30 min. The wells were

washed four times with PBS to remove unattached cells, then the attached cells were stained with 0.5% crystal violet in 20% methanol for 30 min. After washing with water, the residual stained cells were lyzed with 100 μl 30% acetic acid and the absorbance of the lysates measured at 600 nm in an ELISA reader. Each assay was performed on triplicate cultures. In another experiment, RGDS–IgG (0.6 peptide/IgG) or CS1–IgG (1.2 peptide/IgG) conjugates were prepared by coupling GRGDSPK or DELPQLVTLPHPNLHGPEILDVPST (CS1 region of fibronectin) to human IgG according to the conventional procedure[17] using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, and used as substrates in the adhesion assay.

Invasion Assay The invasive activity of tumor cells was assayed in a Transwell cell culture chamber (Costar No.3422, Cambridge, MA) according to methods described previously.[35] Polyvinylpyrrolidone-free polycarbonate filters with an 8.0 μm pore size (Nucleopore, Pleasanton, CA) were precoated with either 5 μg fibronectin or laminin in a volume of 50 μl on their lower surfaces, and dried at room temperature. Reconstituted basement membrane Matrigel (containing laminin, collagen type IV, heparan sulfate proteoglycan and entactin, Collaborative Research Inc., MA) was diluted to 100 μg/ml with cold PBS, applied to the upper surfaces of the filters (5 μg/filter), and dried overnight at room temperature under a hood. These filters were designated Matrigel/fibronectin-coated filters. The coated filters were washed thoroughly in PBS and then dried immediately before use. Log-phase cell cultures of tumor cells were harvested with 1 mM EDTA in PBS, washed three times with serum-free MEM, and resuspended to give a final concentration of 2×10^6/ml in MEM with 0.1% bovine serum albumin (BSA). Cell suspensions (100 μl), with or without added compounds, were added to the upper compartment and incubated for the appropriate number of hours at 37 °C in a 5% CO_2 atmosphere. The filters were fixed with methanol and then stained with hematoxylin and eosin. The cells on the upper surface of the filters were removed by wiping them with a cotton swab. The cells that had invaded through the Matrigel and filter to the lower surface were manually counted under a microscope in 5 predetermined fields at a magnification of 400 and each assay was performed in triplicate.

Haptotactic Migration Assay Tumor cell migration along a gradient of substratum-bound fibronectin or laminin was assayed in a Transwell cell culture chamber according to the methods previously reported.[35] The filters with an 8.0 μm pore size were precoated with either 5 μg fibronectin or laminin in a volume of 50 μl on their lower surfaces, as described above. The subsequent procedures were the same as those of the invasion assay.

Statistical Analysis The statistical significance of differences between groups was determined by applying Student's two-tailed *t*-test.

RESULTS

Effect of AcDRGDS or AcDRLDS on the Adhesion of B16-BL6 Melanoma Cells Our previous study has shown

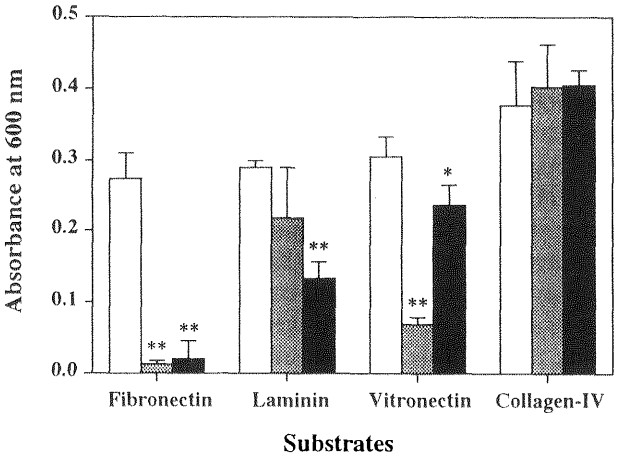


Fig. 2. Inhibition of Tumor Cell Adhesion to the Components of Extracellular Matrix by AcDRGDS or AcDRLDS Peptides

B16-BL6 cells (2×10^4) were added to wells which had been pre-coated with 50 μl 3 μg/ml of fibronectin, laminin, vitronectin or type IV collagen, and incubated with control (PBS: ▭), 1000 μg/ml of AcDRGDS (▩) or AcDRLDS (■). After 30 min incubation, nonadherent cells were washed away and the adherent cells were stained with 0.5% crystal violet in 20% methanol for 30 min. The absorbance of the stained cell lysates was measured at 600 nm in an ELISA reader. * $p < 0.01$, ** $p < 0.001$ compared with control (PBS) by Student's two-tailed *t*-test.

that, among N-terminal modified RGDS variant peptides, in which G was substituted with various amino acids (AcDR-X-DS), AcDRLDS as well as AcDRGDS significantly inhibited the metastatic and invasive potential of tumor cells *in vivo* and *in vitro* compared with the original RGDS-containing peptide.[28] To further characterize the antimetastatic properties, we first examined the inhibitory effect of AcDRGDS and AcDRLDS peptides on tumor cell adhesion to the components of extracellular matrix and basement membrane such as fibronectin, laminin, vitronectin or type IV collagen. Figure 2 shows that both AcDRGDS and AcDRLDS significantly inhibit the adhesion of B16-BL6 melanoma cells to fibronectin- and vitronectin-coated substrates. In addition, tumor cell adhesion to laminin-coated substrate was inhibited by the addition of AcDRLDS, but not by AcDRGDS. Neither AcDRGDS nor AcDRLDS exhibited any anti-cell adhesive activity to type IV collagen-coated substrate. It is of major interest that the AcDRLDS variant peptide was effective in inhibiting tumor cell adhesion to laminin, in addition to RGDS-mediated adhesion to fibronectin or vitronectin, although its inhibitory mechanism is not fully understood. Since fibronectin contains the RGDS sequence and CS1 region which are recognized by $\alpha_5\beta_1$ and $\alpha_4\beta_1$ respectively integrin receptors of the tumor surface, we next investigated the effect of AcDR-X-DS peptides on cell adhesive interactions. To do this, we coated RGDS–IgG or CS1–IgG as a substrate in the adhesion assay. As shown in Figs. 3 and 4, both AcDRGDS and AcDRLDS inhibit tumor cell adhesion to RGDS–IgG-coated substrate in a concentration-dependent fashion. AcDRGDS at concentrations ranging from 100 to 2000 μg/ml shows no inhibitory effect on tumor cell adhesion to CS1–IgG. On the other hand, AcDRLDS, as well as CS1-derived peptide (EILDV) as a positive control, inhibit cell adhesion to CS1–IgG in a concentration-dependent manner. However, the inhibitory effect of AcDRLDS was less marked than that of the EILDV

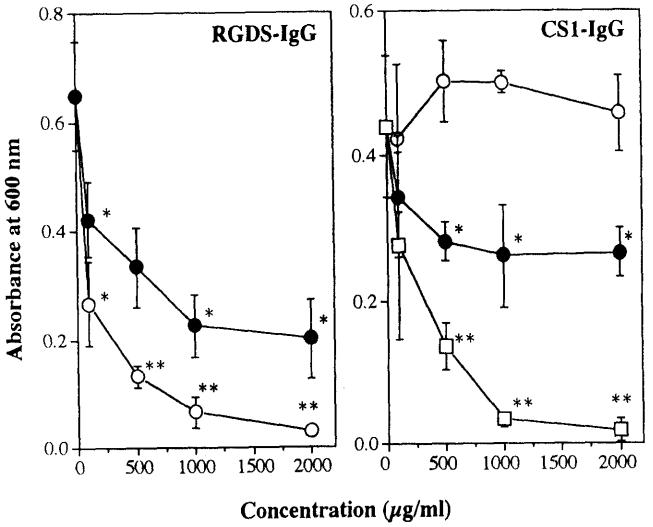


Fig. 3. Inhibition of Tumor Cell Adhesion to RGDS–IgG or CS1–IgG by AcDRGDS or AcDRLDS Peptides

B16-BL6 cells (2×10^4) were added to wells which had been pre-coated with 50 μl of 10 μg/ml RGDS–IgG or CS1–IgG, and incubated with various concentrations of AcDRGDS (○), AcDRLDS (●) or EILDV (□). After 30 min incubation, nonadherent cells were washed away and the adherent cells were stained with 0.5% crystal violet in 20% methanol for 30 min. The absorbance of the stained cell lysates was measured at 600 nm in an ELISA reader. * $p<0.01$, ** $p<0.001$ compared with controls (PBS) by Student's two-tailed t-test.

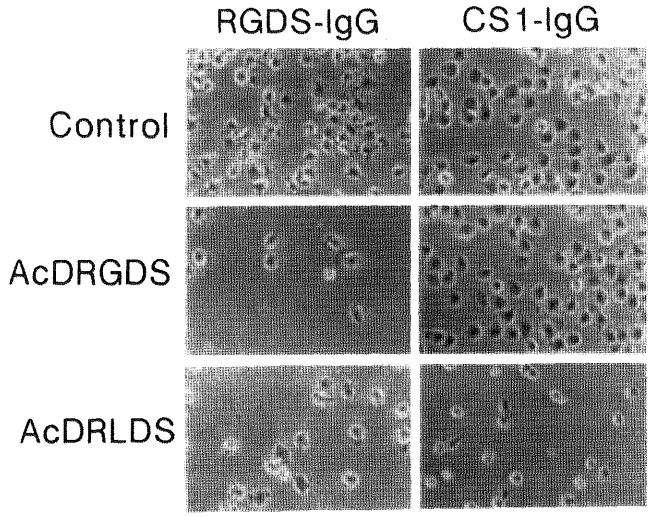


Fig. 4. Adhesion of B16-BL6 Melanoma Cells on RGDS–IgG or CS1–IgG in the Presence or Absence of AcDRGDS or AcDRLDS Peptides

B16-BL6 cells (2×10^4) were incubated in wells which had been pre-coated with 50 μl of 10 μg/ml RGDS–IgG or CS1–IgG, in the presence or absence of 1000 μg/ml AcDRGDS or AcDRLDS for 30 min, and then nonadherent cells were washed away.

peptide. These results indicate that AcDRLDS significantly inhibits tumor cell interaction with both the RGDS and CS1 sequences within the fibronectin molecule.

Inhibition of Tumor Metastasis by Ar(DRGDS)$_3$ and Ar(DRLDS)$_3$ We previously showed that RGDS–polymeric carrier conjugation can lead to an increased antimetastatic effect of RGDS peptide. We therefore investigated the effect of peptide–trimesic acid (Ar) conjugates, Ar(DRGDS)$_3$ or Ar(DRLDS)$_3$, on experimental lung metastasis produced by i.v. co-injection of various murine or human tumor cells. These compounds dissolved in PBS were admixed with tumor cells and the

Table 1. Effect of Ar(DRGDS)$_3$ or Ar(DRLDS)$_3$ on Experimental Lung Metastasis Produced by i.v. Injection of Various Tumor Cells

Tumor	Administered i.v. with	μg/mouse	No. of lung metastasis Mean ± S.D. (Range)
B16—BL6 melanoma	Untreated (PBS)		109 ± 49 (35—201)
	GRGDS	1000	51 ± 10 (37—61)
	Ar(DRGDS)$_3$	100	133 ± 45 (64—176)
		500	21 ± 12 (3—37)**
		1000	13 ± 7 (6—20)***
	Ar(DRLDS)$_3$	100	99 ± 38 (54—144)
		500	40 ± 18 (16—53)*
		1000	36 ± 27 (2—63)**
	Ar(COONa)$_3$	1000	55 ± 8 (44—64)
K1735 melanoma	Untreated (PBS)		65 ± 24 (39—89)
	RGDS	1000	13 ± 12 (0—30)**
	Ar(DRGDS)$_3$	500	9 ± 8 (3—22)***
		1000	0 ± 0 (0)***
Colon26 M3.1 carcinoma	Untreated (PBS)		219 ± 28 (180—250)
	RGDS	1000	241 ± 54 (141—285)
	Ar(DRGDS)$_3$	1000	41 ± 25 (14—74)***
HT1080 fibrosarcoma (BALB nu/nu)	Untreated (PBS)		18 ± 12 (6—47)
	RGDS	1000	13 ± 9 (4—31)
	Ar(DRGDS)$_3$	1000	2 ± 2 (0—7)**

Five or seven mice per group were inoculated i.v. with B16-BL6 (5×10^4), K1735 (2×10^4), Colon26 M3.1 (4×10^4) or HT1080 (3×10^5) with or without peptides. Mice were killed 2 or 3 weeks after tumor inoculation and tumor colonies in the lungs were counted manually. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared with untreated controls by Student's two tailed t-test.

Table 2. Effect of Ar(DRGDS)$_3$ or Ar(DRLDS)$_3$ on Experimental Liver Metastasis Produced by i.v. Injection of L5178Y-ML25 Cells

Administered i.v. with	μg/mouse	Mean weight (g) ± S.D. Liver	Spleen
Untreated (PBS)		3.81 ± 1.03	0.24 ± 0.04
Ar(DRGDS)$_3$	100	4.02 ± 1.11	0.24 ± 0.04
	500	2.46 ± 1.67	0.17 ± 0.06*
	1000	2.27 ± 1.47*	0.15 ± 0.07**
Ar(DRLDS)$_3$	100	3.84 ± 0.81	0.24 ± 0.03
	500	2.35 ± 0.65*	0.17 ± 0.03**
	1000	1.64 ± 0.52***	0.13 ± 0.03***
(Normal)		1.00 ± 0.05	0.08 ± 0

Five CDF1 mice per group were inoculated i.v. with L5178Y-ML25 lymphoma cells (5×10^4) with or without peptides. Mice were killed 2 weeks after tumor inoculation and the weight of liver and spleen were measured. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared with controls by Student's two tailed t-test.

mixtures were immediately injected i.v. into C57BL/6 mice (B16-BL6 melanoma), C3H/HeN (K1735 melanoma), BALB/c (Colon 26 M3.1 carcinoma) or BALB/c nu/nu (HT1080 fibrosarcoma). Both Ar(DRGDS)$_3$ and Ar(DRLDS)$_3$ dramatically inhibited lung metastasis of B16-BL6 cells in a dose-dependent manner ranging from 100 to 1000 μg (Table 1). GRGDS or sodium trimesic acid Ar(COONa)$_3$, even at a high dose of 1000 μg, did not exhibit any significant inhibition of lung metastasis. Similarly, Ar(DRGDS)$_3$ reduced the number of tumor colonies in the lungs following its co-injection with K1735, Colon26 M3.1 or HT1080 cells, whereas 1000 μg RGDS tetrapeptide showed no or slight inhibitory effect. As shown in Table 2, the co-injection of L5178Y-ML25 T lymphoma cells with either Ar(DRGDS)$_3$ or Ar(DRLDS)$_3$ resulted in significant inhibition of liver metastasis

Table 3. Effect of $Ar(DRGDS)_3$ or $Ar(DRLDS)_3$ on Spontaneous Lung Metastasis Produced by Intra-footpad Injection of B16-BL6 Melanoma Cells

Treatment	Timing on day	Primary tumor size Mean±S.D. (mm)	No. of lung metastasis on day 35 Mean±S.D. (Range)
Exp. 1:	8, 10, 12, 14, 16, 18, 20, 22, 24, 26		
Control		10±2	61±22 (25—103)
RGDS		11±2	67±13 (53—88)
$Ar(DRGDS)_3$		10±2	24±13 (5—43)*
$Ar(DRLDS)_3$		11±2	27± 7 (16—37)*
Exp. 2:	7, 9, 11, 13, 15, 17, 19, 22, 24, 26		
Control		10±2	116±27 (83—150)
RGDS		11±2	102±22 (75—139)
$Ar(DRGDS)_3$		10±2	47±16 (23—70)**
$Ar(DRLDS)_3$		10±2	35±10 (21—49)**

Seven C57BL/6 mice per group were administered i.v. with peptides (100 μg/mouse) at the indicated time after intra-footpad injection (5×10^5 per 50 μl) of B16-BL6 melanoma cells. Primary tumors were surgically removed on day 21. Mice were killed 2 weeks after tumor excision and tumor colonies in the lungs were counted manually. * $p<0.01$, ** $p<0.001$, compared with untreated controls by Student's two tailed t-test.

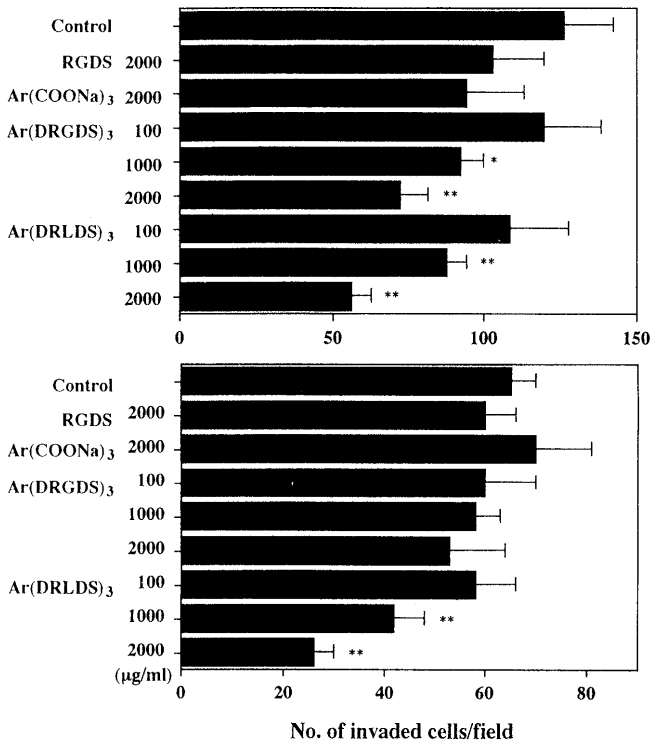


Fig. 5. Effect of $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$ on the Invasion of B16-BL6 Melanoma Cells into Matrigel/Fibronectin- or Matrigel/Laminin-Coated Filters

Filters were precoated with 5 μg fibronectin (upper panel) or laminin (lower panel) on their lower surfaces, and then with Matrigel (5 μg) on their upper surfaces. B16-BL6 melanoma cells (2×10^5/well) in 0.1% BSA medium were seeded with or without the indicated concentrations of compounds into the upper compartment of the Transwell cell culture chamber. After 6-h incubation, the invaded cells/field on the lower surfaces were counted visually. * $p<0.01$, ** $p<0.001$ as compared with controls (cells alone) by Student's two-tailed t-test.

(increase in liver weight) in a dose-dependent fashion. These results clearly demonstrate that $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$ of a novel synthetic RGDS analogue effectively increase the antimetastatic ability of RGDS in experimental lung or liver metastasis models using five different types of murine or human tumors.

Effect of $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$ on Spontaneous Lung Metastasis of B16-BL6 Melanoma Cells We also investigated the therapeutic effect of multiple administrations of $Ar(DRGDS)_3$ or $Ar(DRLDS)_3$ on spontaneous lung metastasis produced by intra-footpad injection of B16-BL6 melanoma cells. The first i.v. administration of the compounds began on day 8 (Exp. 1 of Table 3) or 7 (Exp. 2 of Table 3) after tumor inoculation. Ten intermittent i.v. injections of 100 μg $Ar(DRGDS)_3$ or $Ar(DRLDS)_3$ achieved a statistically significant reduction in lung tumor colonies on day 35 after tumor inoculation. However, multiple administrations of RGDS peptide showed no inhibitory effect on spontaneous lung metastasis of melanoma cells. In these experiment, i.v. administration of $Ar(DRGDS)_3$, $Ar(DRLDS)_3$ or RGDS did not affect the primary tumor growth (size) at the time of surgical excision on day 21, as compared with untreated controls (Table 3). The incubation of B16-BL6 cells with $Ar(DRGDS)_3$, $Ar(DRLDS)_3$ or RGDS at concentrations ranging from 20 to 500 μg/ml *in vitro* did not affect the incorporation of [^{3}H]thymidine into tumor cells (data not shown). These results indicate that these compounds are not directly cytotoxic nor do they inhibit cell growth.

Effect of $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$ on the Invasion of B16-BL6 Melanoma Cells Tumor cell invasion into extracellular matrices and basement membranes is a crucial step in the complex multistage process of metastasis. Therefore, we examined the effect of antimetastatic $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$ on tumor cell invasion into Matrigel (reconstituted basement membrane components) *in vitro*. B16-BL6 cells were added to the upper compartment of Transwell cell culture chambers, with or without RGDS, $Ar(COONa)_3$, $Ar(DRGDS)_3$ or $Ar(DRLDS)_3$. The invasion of tumor cells through the Matrigel/fibronectin-coated filters was inhibited by $Ar(DRGDS)_3$ or $Ar(DRLDS)_3$ in a concentration-dependent manner, whereas RGDS tetrapeptide or $Ar(COONa)_3$ alone showed no significant inhibition of tumor invasion (Fig. 5 upper panel). In contrast, while the invasion of tumor cells through the Matrigel/laminin-coated filters was significantly inhibited by $Ar(DRLDS)_3$ in a concentration-dependent manner, it was not inhibited by RGDS, $Ar(DRGDS)_3$, $Ar(COONa)_3$ (Fig. 5 lower panel).

Effect of $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$ on the Migration of B16-BL6 Melanoma Cells Since tumor cell motility on extracellular matrices and basement membranes is considered to be an important step in the invasive process for metastatic tumor cells, we investigated whether or not $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$ were able to inhibit the migration of tumor cells to the filters that had been precoated on their lower surfaces with fibronectin or laminin without the matrix barrier (Matrigel) on the upper surface. B16-BL6 cells were incubated for 4 h at 37 °C, with or without compounds in the upper compartment of a Transwell cell culture chamber. Figure 6 showed that $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$ significantly inhibited haptotactic migration of tumor cells to the fibronectin-coated filters (upper panel). In contrast, only $Ar(DRLDS)_3$

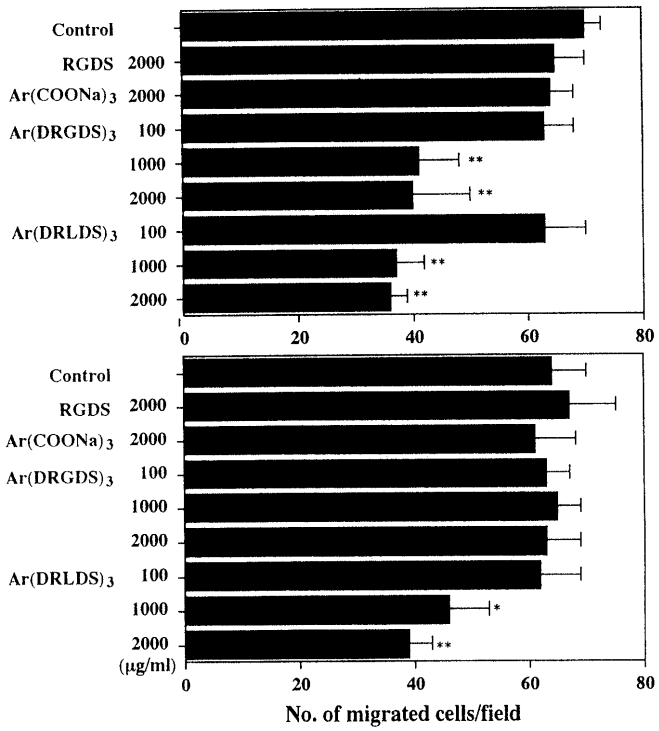


Fig. 6. Effect of $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$ on the Haptotactic Migration of B16-BL6 Melanoma Cells to Fibronectin- or Laminin-Coated Filters

Filters were precoated with 5 μg fibronectin (upper panel) or laminin (lower panel) on their lower surfaces. B16-BL6 melanoma cells (2×10^5/well) in 0.1% BSA medium were incubated with or without the indicated concentrations of compounds in the upper compartment of the Transwell cell culture chamber. After 4-h incubation, the migrated cells/field on the lower surfaces were counted visually. $*p<0.01$, $**p<0.001$ compared with controls (cells alone) by Student's two-tailed *t*-test.

inhibited haptotactic migration of tumor cells to the laminin-coated filters, while RGDS, $Ar(DRGDS)_3$ or $Ar(COONa)_3$ did not exhibit any inhibitory effect (lower panel). The results in Figs. 5 and 6 clearly demonstrate that the tumor cell invasion to Matrigel/fibronectin and migration to fibronectin were RGDS-dependent and were also inhibited by $Ar(DRLDS)_3$ as well as $Ar(DRGDS)_3$. In contrast, the invasion to Matrigel/laminin and migration to laminin were inhibited only by $Ar(DRLDS)_3$, but not by $Ar(DRGDS)_3$.

DISCUSSION

We have attempted to control the mechanism involved in cell functions such as adhesion, migration and invasion of tumor cells during the metastatic process. Some synthetic peptides derived from extracellular matrix components, such as fibronectin and laminin, have been used to inhibit tumor metastasis and invasion in murine tumor systems. Synthetic RGDS peptide analogues in which the amino acid of G was substituted by various amino acids, named X, *i.e.* R-X-DS, and N-terminal modified R-X-DS have been synthesized to examine their antimetastatic effects in murine lung or liver metastases models. Consequently, AcDRLDS as well as AcDRGDS have been shown to possess potent antimetastatic and anti-invasive effects compared with RGDS or R-X-DS.[28] Interestingly, AcDRLDS was able to regulate tumor cell migration and invasion mediated by laminin as well as by fibronectin in a different manner to AcDRGDS.

In general, most peptides such as RGD-containing peptides as well as some cytokines or anticancer drugs have very short half lives in the circulation, which results in a decrease in their therapeutic and biological effects *in vivo*. Therefore, an increase in the half-life of a drug in circulation without increasing its toxicity may lead to an improved biological effect. Our previous studies showed that the conjugation of RGD-containing peptides with various drug carriers such as polyethylene glycol, poly(carboxyethylmethacrylamide) and carboxymethyl chitin increased the ability to inhibit experimental and spontaneous tumor metastasis.[29,36,37]

In the present study, to further characterize the antimetastatic properties of AcDRGDS and AcDRLDS peptides, we first investigated the inhibitory effect of these peptides on tumor cell adhesion to the components of extracellular matrix and basement membrane, and also tested the antimetastatic effect of the conjugates of these peptides with trimesic acid, $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$. Since AcDRGDS inhibits tumor cell adhesion to fibronectin, vitronectin and RGDS–IgG substrates, but not to CS1–IgG substrate which is recognized by the $\alpha_4\beta_1$ integrin receptor on the tumor surface (Figs. 2—4), this inhibitory mechanism is RGDS-dependent and presumably $\alpha_5\beta_1$- or $\alpha_v\beta_1$-mediated. In contrast, the AcDRLDS variant peptide significantly inhibited tumor cell adhesion to laminin, in addition to RGDS-mediated adhesion to fibronectin and vitronectin (Fig. 2). AcDRLDS also inhibited tumor cell adhesion to CS1 other than the RGDS sequence within the fibronectin molecule in a concentration-dependent manner, although the inhibitory effect was less than that of CS1 (EILDV) peptide (Fig. 3). Since Humphries *et al.* have suggested that a new recognition sequence, X-D-Y (*e.g.* X can be glycine, leucine or glutamic acid, and Y can be serine or valine), can be recognized by $\alpha_4\beta_1$ integrin on the cell surface,[38] AcDRLDS may structurally or functionally mimic the active EILDV sequence in the CS1 peptide. Thus, the effect of AcDRLDS may retain the properties of both RGDS and EILDV.[28] We previously reported that CS1 peptide derived from fibronectin is able to modulate the adhesive interaction between tumor cells and laminin as well as fibronectin and consequently inhibits tumor invasion and metastasis.[39] Therefore, an AcDRLDS-mediated inhibitory mechanism may be associated with the hybrid properties of the RGDS and CS1 (EILDV) sequences.

In addition, trimesic acid conjugates with peptide, $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$ were much more effective in inhibiting lung or liver metastasis of various types of murine and human tumors than the original RGDS-containing peptides or $Ar(COONa)_3$ (Tables 1 and 2). In the spontaneous lung metastasis experiment, multiple administrations of $Ar(DRGDS)_3$ or $Ar(DRLDS)_3$ effectively inhibited lung metastasis produced by intra-footpad injection of B16-BL6 cells, without affecting the primary tumor size at the time of surgical excision, compared with RGDS peptide or untreated controls (Table 3). We also observed that N-terminal modified peptides (AcDRGDS

and AcDRLDS) were not effective in inhibiting lung metastasis under this administration schedule, although these peptides as well as $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$ have been shown to possess potent anti-adhesive, migrative and invasive effects *in vitro*.[28] Thus, $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$ efficiently achieved an antimetastatic effect without exhibiting direct cytotoxicity. Figures 5 and 6 show that $Ar(DRLDS)_3$ inhibits laminin- and fibronectin-mediated invasion and migration of tumor cells, and that $Ar(DRGDS)_3$ selectively inhibits fibronectin-mediated invasion and migration. These are similar to the results in Figs. 2 and 3 illustrating the inhibition of tumor cell adhesion by AcDRGDS or AcDRLDS. The above results indicate that the increased inhibitory effect by $Ar(DRGDS)_3$ and $Ar(DRLDS)_3$ may be due in part to selective interference with the cell-adhesive interaction with fibronectin or laminin, or enhancement of the affinity for cell surface receptors, as well as an improved biological availability *in vivo*, by introducing DRGDS or DRLDS into trimesic acid.

Thus, the conjugation of DRGDS or DRLDS with trimesic acid may explain the increased peptide-mediated antimetastatic effect *in vivo*. The synthetic N-terminal modified RGDS variant peptides and their trimesic acid conjugates provide a useful basis for the development of new anti-metastatic compounds.

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