Synthetic Laminin-like Peptides and Pseudopeptides as Potential Antimetastatic Agents[1]

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This paper describes our efforts to study structure–activity relationships, improve the antimetastatic potency, and limit the in vivo enzymatic degradation of YIGSR-NH_2, a synthetic peptide from the B1 chain of laminin, which reportedly has potential as an antimetastatic agent. To this end we have synthesized a series of $\psi(CH_2NH)$ peptide analogs (**5**–**9**) of YIGSR-NH_2 and a number of peptides in which the Tyr residue was replaced with D-Tyr (**1**), Phe (**2**), Phe (*p*-F) (**3**), and Phe(*p*-NH_2) (**4**). All new peptides were assayed in vitro for their ability to promote cell attachment in both B16F10 mouse melanoma cells and HT-1080 human fibrosarcoma cells. On the basis of the in vitro assay results, peptides **3**–**5**, **8**, and **9** were tested in vivo for their ability to inhibit tumor metastasis to the lungs in mice that were coinjected in the tail vein with B16F10 melanoma cells and 1 mg of peptide. In summary, of the nine new peptides only the Phe(*p*-NH_2) peptide **4** showed consistent in vitro cell attachment activity, but only low in vivo antimetastatic activity.

Introduction

Tumor metastasis is the major cause of death in patients with solid malignant tumors. The process of metastasis is comprised of sequential series of steps: the local invasion of the surrounding host tissue, entrance into vascular or lymphatic circulation and transportation to distant sites, extravasation from the circulation, and proliferation in the secondary site.[2,3] The attachment of tumor cells to, degradation of, and migration through basement membrane is a critical step in the formation of metastases.[4]

Laminin, a major glycoprotein constituent of basement membrane, and its receptors are important factors in tumor invasion and metastasis. Laminin increases tumor cell activities including adhesion, growth, migration, type IV collagenase production, plasminogen activation, and drug resistance, as well as metastasis.[5,6] Laminin, as isolated from the EHS tumor and from many cells, consists of three polypeptide chains, A (400 kDa), B1 (230 kDa), and B2 (220 kDa), which are linked together in a cruciform-like structure.[7] CDPGYIGSR, a synthetic nonapeptide from the B1 chain of laminin, has been identified as a major site for cell binding. Furthermore, YIGSR-NH_2, from within this nonapeptide, was reported to reduce the formation of lung colonies of B16F10 melanoma cells injected into the tail vein of mice and also inhibit the invasiveness of these melanoma cells, in vitro;[8] and was thus thought at the time that the pentapeptide might be useful clinically.[5]

Various amino acid substituents have defined the key amino acids in YIGSR. Removal of the Tyr residue in YIGSR was associated with a marked loss in activity, indicating that the Tyr residue is required.[9] The replacement of the Ile residue in YIGSR-NH_2 with Cys yielded YCGSR-NH_2, whose inhibitory effect on experimental metastasis formation was comparable to that of YIGSR-NH_2, suggesting that the Ile residue is not essential.[10] Replacement of the Arg residue in YIGSR-NH_2 with D-Arg or Glu, or by Lys in YIGSR, afforded inactive analogs, showing that the Arg residue is necessary for activity.[9,10] It should be noted, as observed with active peptides derived from other proteins, that YIGSR-NH_2 and its analogs still show very low activity on a molar basis, when compared with intact laminin. Also, the very short half-life time of these peptides limits their potential therapeutic use.

This paper describes our efforts to study structure–activity relationships, improve the antimetastatic potency, and limit the in vivo enzymatic degradation of YIGSR-NH_2. For biologically active peptides, the common assumption is that their activities depend on their three-dimensional structures. On the surface where contact between ligand and receptor occurs, the relative spatial arrangements of the side chain groups on the peptide pharmacophore are critical for receptor recognition and probably determine, in many cases, the affinity, selectivity, and activity of the peptide for a particular receptor protein. Therefore, side chain modifications of YIGSR-NH_2 may provide important insights into conformational and topographical requirements for its activity.

After the demonstration by Graf et al.[9] that the Tyr residue was required for the biological activity of YIGSR, there were no further published studies regarding the importance of this residue in YIGSR. Thus, our first attempts to design YIGSR-NH_2 analogs focused on whether modification of the Tyr residue would result in more potent analogs and, specifically, on what role is played by the Tyr phenolic group. Since Tyr can behave like a charged side chain and form rather strong hydrogen bonds,[11] it is reasonable to suspect that the bioactivity of YIGSR may, at least in part, be due to the ability of the Tyr phenolic group to form hydrogen bonds.

Four YIGSR-NH_2 analogs, **1**–**4**, were prepared (Table 1). A common approach to enhancing the potency of biologically active peptides (hormones), as well as their duration of action, is to create analogs in which unusual

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Table 1. Peptides Synthesized

no.	structure
1	D-Tyr-Ile-Gly-Ser-Arg-NH_2
2	Phe-Ile-Gly-Ser-Arg-NH_2
3	Phe(*p*-F)-Ile-Gly-Ser-Arg-NH_2
4	Phe(*p*-NH_2)-Ile-Gly-Ser-Arg-NH_2
5	Tyr-$\psi(CH_2NH)$-Ile-Gly-Ser-Arg-NH_2
6	Tyr-Ile-$\psi(CH_2NH)$-Gly-Ser-Arg-NH_2
7	Tyr-Ile-Gly-$\psi(CH_2NH)$-Ser-Arg-NH_2
8	Tyr-Ile-Gly-Ser-$\psi(CH_2NH)$-Arg-NH_2
9	Tyr-$\psi(CH_2NCHO)$-Ile-Gly-Ser-Arg-NH_2

or D-amino acids have been incorporated at various regions of the peptide.[12,13] In peptide **1**, we therefore substituted D-Tyr for the natural L-Tyr, for the reasons stated above. In peptide **2**, Phe was chosen as a replacement for Tyr in order to evaluate the necessity of retaining the phenolic group. In order to further determine the role of the phenolic group, the Phe(*p*-F) analog **3** was prepared. The Phe(*p*-F) residue was chosen because (1) the fluorine atom is isoelectronic with, has an electronegativity similar to (4.0 vs. 3.5 for oxygen), and can be considered to be a bioisostere of the hydroxyl group,[14] (2) fluorine can be a useful probe of hydrogen-bonding interactions since it can act as an electron pair donor in forming hydrogen bonds,[15] and (3) it has been incorporated into several peptide hormone analogs which have high biological activity. For example, the substitution of Phe(*p*-F) for Tyr in physalaemin$_{6-11}$ affords a peptide with approximately 100, 675, and 220% of physalaemin's activity in the hypotensive, large intestine, and ileum assays, respectively.[16] The Phe(*p*-NH_2) analog **4** was prepared to probe further the importance of the Tyr phenolic group.

Many structure–activity studies of peptides have involved modifications of the peptide backbone. In this regard there has been great interest in the replacement of some peptide bonds with pseudopeptide (ψ) bonds,[13] with the $\psi(CH_2NH)$ moiety being a more widely used one.[17] Besides its potential to modify the peptide backbone conformation, to limit enzymatic degradation, and to increase hydrophilicity, another advantage of the $\psi[CH_2NH]$ replacement is that the procedure for introducing this "reduced peptide bond" during conventional solid phase peptide synthesis (SPPS) is well established and relatively easy.[18,19] Studies with the $\psi(CH_2NH)$ substitution have led to a number of new classes of receptor antagonists for bombesin,[17] LH-RH,[20] substance P,[21] GRF,[12] and secretin.[22] The introduction of a $\psi(CH_2NH)$ bond into a biologically active peptide, however, may result in an agonist, antagonist or inactive peptide.[12,17,20-22] For these reasons, we have replaced, individually, each of the peptide bonds in YIGSR-NH_2 with the $\psi(CH_2NH)$ bond, thus affording peptides **5**–**8** (Table 1).

Results and Discussion

Chemistry. All of the peptides were synthesized with a manual synthesizer using Merrifield solid-phase conditions and the *p*-methylbenzhydrylamine (MBHA) resin.[23] All amino acids were coupled as their N^{α}-Boc-derivatives.

Each amino acid residue was added to the growing peptide using single or double coupling, the choice determined by the results of a quantitative ninhydrin assay.[24] All of the residues, except Gly and Arg(Tos), were singly coupled as their symmetrical anhydrides (SA) in dichloromethane (DCM). The second couplings, if needed, were performed as hydroxybenzotriazole (HOBt) esters in dimethylformamide (DMF). Boc-Gly[25] and Boc-Arg(Tos)[26,27] were initially coupled as their HOBt esters in DMF/DCM (2/1), while the second coupling, if necessary, was performed in DMF as the HOBT ester.

The synthesis of peptides **1**–**4** were accomplished by preparing Ile-Gly-Ser(Bzl)-Arg(Tos)-MBHA resin, as described above, and reacting a portion of this resin individually with the requisite blocked amino acid. For example, reaction of Boc-Phe(*p*-NH-Z)-SA with Ile-Gly-Ser(Bzl)-Arg(Tos)-MBHA resin gave Boc-Phe(*p*-NH-Z)-Ile-Gly-Ser(Bzl)-Arg(Tos)-MBHA resin. The Boc group was selectively removed by treatment of the peptide-bound resin with trifluoroacetic acid (TFA), followed by reaction with liquid HF in order to cleave the peptide from the resin and remove all of the blocking groups; purification via reverse phase high-performance liquid chromatography (HPLC) afforded peptide **4**. Peptides **1**–**3** were similarly prepared.

The introduction of the $\psi(CH_2NH)$ peptide bond isostere into peptides **5**–**8** was accomplished by solid-phase reaction of the N-terminal amino group of the resin-bound peptide with the requisite Boc-protected amino acid aldehyde, in the presence of sodium cyanoborohydride ($NaBH_3CN$) in DMF containing 1% AcOH.[18,19] The aldehydes Boc-Tyr(2BZ)-H, Boc-Ile-H, Boc-Gly-H, and Boc-Ser(Bzl)-H (necessary for the synthesis of peptides **5**, **6**, **7**, and **8**, respectively) were prepared by $LiAlH_4$ reduction[19,28] of their corresponding *N*,*O*-dimethyl hydroxamates.[20] The synthetic protocol used in the preparation of peptides **5**–**8** were essentially the same as used above for peptides **1**–**4**, except that Boc-Gly-ONp was used in the synthesis of peptide **8** after the introduction of the $\psi(CH_2NH)$ bond.[18]

In the synthesis of peptide **5**, semipreparative HPLC purification uncovered the presence of a second component which we have assigned as peptide **9**, the *N*-formyl derivative of **5**. Analysis of peptide **9** by fast atom bombardment mass spectrometry (FABMS) showed a protonated molecular ion ($[M + H]^+$) 28 atomic mass units (amu) higher than expected for peptide **5**, suggesting the presence of a formyl (CHO) group. In order to confirm the position of the CHO group, we subjected peptides **5** and **9** to collision-induced decomposition–mass spectrometry (CID–MS) in a tandem mass spectrometer system (MS/MS).[29,30]

The fragment ion nomenclature used here has been summarized by Biemann.[30] The CID spectra of peptides **5** and **9** showed $(M + H)^+$ ions of *m/z* 580.2 and 608.3, respectively. The difference of 28 amu was not reflected in the C-terminal ions of the CID spectrum of peptide **9** until one reached ion y_4, observed at *m/z* 459.4 (see Figure 1). The absence in peptide **9** of ion v_4 at *m/z* 373, which is abundant in peptide **5**, also supports our assignment of the modification site existing at the nitrogen atom of Ile.[31] Furthermore, a prominent immonium ion was observed at *m/z* 114 in the CID spectrum of peptide **9**. Thus, these data allow us to place the CHO group on the Ile nitrogen atom, $\psi(CH_2N-CHO)$, in peptide **9** (see Figure 1).

We have previously reported a similar formylation of another $\psi(CH_2NH)$ peptide and were unable, at that

Table 2. Cell Attachment Assay Using HT-1080 and B16F10 Cells[a,b]

peptide	exp. 1, 50 μg	exp. 2, 50 μg	exp. 3, 100 μg	exp. 4, 100 μg	summary of activity[c]
none	188 ± 77	280 ± 31	125 ± 45	98 ± 30	0
1	193 ± 90	ND	438 ± 147	274 ± 111	+
2	214 ± 83	ND	496 ± 246	245 ± 102	+
3	ND	474 ± 8	708 ± 421	299 ± 68	++
4	ND	1164 ± 260	1466 ± 209	851 ± 248	+++
5	473 ± 126	389 ± 50	ND	ND	++
6	210 ± 60	ND	697 ± 291	ND	+
7	183 ± 75	ND	ND	233 ± 89	+
8	222 ± 64	362 ± 101	475 ± 297	ND	+
9	427 ± 78	626 ± 90	553 ± 503	ND	++
YIGSR-NH_2	ND	ND	411 ± 91	ND	+
C(YIGSR)$_3$-NH_2[d]	201 ± 44	1313 ± 135	2252 ± 455	1114 ± 177	++++
laminin	514 ± 89 at 20 μg	1196 ± 101 at 50 μg	2560 ± 184 at 25 μg	1449 ± 321 at 25 μg	++++

[a] Experiments 1 and 2 were done using HT-1080 cells and experiments 3 and 4 used B16F10 cells. Experiments 1 and 2 were also done at 10, and 10 and 25 μg, respectively, but the data are not shown. Data are shown for the maximal amount of peptide used and adhesion observed in each experiment. A dose response was observed with the active peptides when the lower concentrations were included. [b] Values are the number of cells attaching to the test peptides, generally using counts from eight fields/well (mean ± SD). ND signifies that, at this concentration, adhesion to the peptide was not determined. [c] Assessment of biological activity: + indicates some, but very low, attachment activity; ++ indicates moderate attachment activity; +++ indicates high, but not maximal, attachment activity; ++++ indicates maximal activity with about 70% of cells attached. [d] See Yamamura et al.[35]

time, to explain how the CHO group was incorporated.[32] However, the DMF solvent used in both syntheses was from the same manufacturer and of the same lot number.[32] We now believe that perhaps the formylation occurred because of contamination of the DMF with formic acid and contamination of the aldehyde used, Boc-Tyr(2BZ)-H, with dicyclohexylcarbodiimide (DCC). The literature procedure we followed in the preparation of the *N,O*-dimethyl hydroxamate of Boc-Tyr(2BZ), necessary for preparing the corresponding aldehyde, used excess DCC; the product was isolated as an oil after appropriate washings.[20] Since the aldehyde Boc-Tyr-(2BZ)-H is not stable, it was prepared fresh and was used without further purification and thus it probably contained some DCC.[28] Therefore, after introduction of the ψ(CH_2NH) bond, formylation could occur if the two contaminants, formic acid and DCC, were present. It should be noted that this formylation could not be duplicated when either a different brand of DMF, or a different lot of DMF from the same manufacturer, was used.[32]

Biological Results. One of the many biological activities of laminin is an ability to promote cell attachment. Kleinman and co-workers have demonstrated that the ability of synthetic peptides to promote attachment correlates with their antimetastatic activity.[33] Therefore, the peptides were assayed, in vitro, for their ability to promote cell attachment in both B16F10 mouse melanoma cells and HT-1080 human fibrosarcoma cells (Table 2). The cell attachment assays were generally performed as previously described,[34] with laminin and C(YIGSR)$_3$-NH_2[35] as positive controls. Replacement of Tyr in YIGSR-NH_2 with D-Tyr (peptide **1**), or by Phe (peptide **2**), gave two peptides which did not bind to the HT-1080 cells and were only weakly active in binding to B16F10 cells, when compared to their controls with no peptide. However, replacement of Tyr with a Phe containing an electronegative group in the para position (peptides **3** and **4**) afforded peptides with the ability to attach to both types of malignant cells; the Phe(*p*-NH_2) derivative (**4**) showed activity approaching that of C(YIGSR)$_3$-NH_2 (Table 2). In the cell attachment assay of the ψ(CH_2NH) peptides **5**–**9** (Table 2), only peptides **5** and **9** showed reasonable attachment to the malignant cells. It is interesting to note that the latter two peptides both contain the ψ-

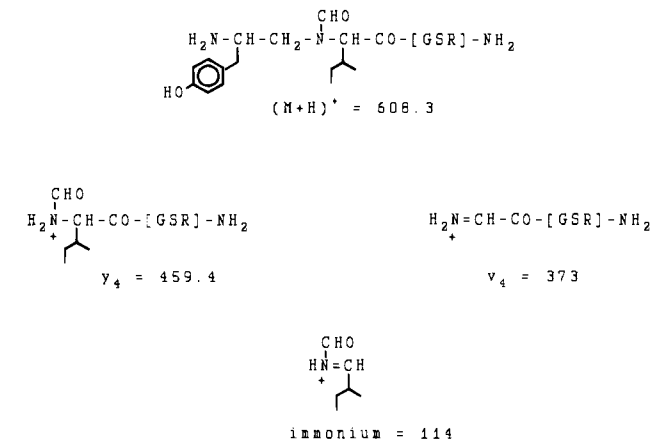

Figure 1. Ions $[M + H]^+$, y_4, v_4, and immonium from the CID-MS fragmentation of peptide **9**.

(CH_2NH) linkage at the N-terminus, between Tyr and Ile. Ostheimer et al.,[36] using 2D NMR, NOESY, and molecular dynamics, studied the structures of CD-PGYIGSR-NH_2 and two analogs, $Gly^7 \rightarrow$ D-Ala^7 and $Gly^7 \rightarrow$ L-Ala^7. CDPGYIGSR-NH_2 and the D-Ala^7 analog, which were both active in an invasion assay, exhibited similar bends around Gly^7 or D-Ala^7 in their YIXSR segments.[36] However, the L-Ala^7 analog, which was much less capable of invasion inhibition, did not show a bend in its structure. This was in agreement with two earlier theoretical studies, which showed that the Gly residue in the YIGSR sequence is important in allowing a turn in the peptide backbone.[37,38] The replacement of CO by CH_2 affords a more flexible bond[13] and has pronounced effects on conformation due to a loss of intramolecular H-bonding sites and increased rotational freedom about the C–N bond.[12] Thus, interior ψ(CH_2NH) bond replacements in YIGSR peptides would be expected to inhibit formation of the supposed critical bend about the Gly residue, and might explain the relative inactivity of peptides **6** and **7**.

Although the in vitro results varied somewhat between experiments, it is clear that the peptide with the most consistent cell attachment activity is peptide **4**. On the basis of these in vitro assay results, peptides **3**–**5**, **8**, and **9** were tested in vivo for their ability to inhibit tumor metastasis to the lungs. Mice were coinjected in the tail vein with B16F10 (1×10^5)

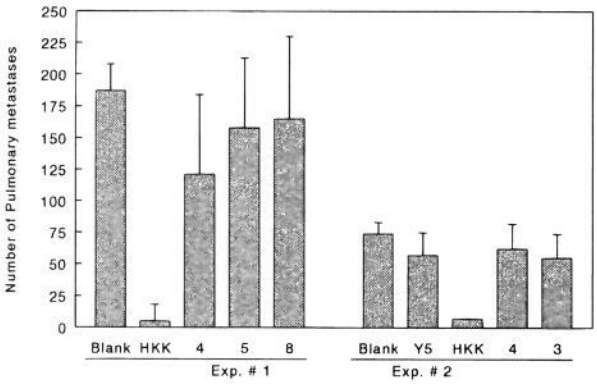

Figure 2. Inhibition of B16F10 melanoma pulmonary metastases in mice coinjected in the tail vein with either 1 mg (exp. 1) or 0.5 mg (exp. 2) of test peptide (mean ± SD). HKK = $C(YIGSR)_3$-NH_2 and Y5 = YIGSR-NH_2. HKK in exp. 2 was not tested at the same time, but has consistently effected a 90% inhibition in metastases.

melanoma cells and 1 mg of peptide (exp. 1 in Figure 2).[8,33] In the case of peptide **9**, the animals died immediately upon injection. Peptide **4** was the most active of the peptides tested, but was considerably less active than the positive control, $C(YIGSR)_3-NH_2$, in reducing the number of lung colonies observed at 3-weeks postinjection. The latter linear 16-mer peptide has been shown by Kleinman and co-workers to block lung metastasis of B16F10 cells either at the time of the inoculation or after several days when the tumors have begun to grow.[39] In a separate in vivo experiment, peptides **3** and **4** were compared to YIGSR-NH_2 and $C(YIGSR)_3$-NH_2 (exp. 2 in Figure 2). At 0.5 mg peptide per mouse, there was no significant inhibition of pulmonary metastasis by peptides **3**, **4**, or, for that matter, YIGSR-NH_2; only $C(YIGSR)_3$-NH_2 consistently showed activity.

Conclusions

We have evaluated nine analogues of the laminin fragment YIGSR-NH_2 in two biological assays. On the basis of the cell attachment assay (Table 2), we suggest that the N-terminal residue should be L-Tyr or Phe containing an electronegative moiety in the para position, in order to retain biological activity, and only replacement of the N-terminal CONH bond with $\psi(CH_2$-NH) leads to a compound with moderate activity. In the in vivo inhibition assay, the Phe(p-NH_2) derivative (**4**) had only minimal activity and was much less active in comparison to $C(YIGSR)_3$-NH_2.

After this study was initiated, several papers appeared indicating that a single pentapeptide such as YIGSR-NH_2 has only minimal biological activity when compared to cyclic, multimeric, and polymeric forms of YIGSR-NH_2.[33,37-40] In fact, YIGSR-NH_2 shows very low activity on a molar basis when compared with intact laminin. A cyclic analog of YIGSR, $\overline{CH_2CO\text{-}GGYIGSRC}$-($NH_2$), was more potent than YIGSR-NH_2, thus suggesting that the conformation of YIGSR may play an important role in its activity.[33] Synthetic polypeptides containing repeated YIGSR sequences were found to inhibit experimental lung metastases of B16BL6 melanoma cells more effectively than the YIGSR monomer.[40,41] The linear 16-mer $C(YIGSR)_3$-NH_2 retains considerable antimetastatic activity, as seen in this paper, and previously observed by Kleinman,[35,39] and also has the ability to inhibit the formation of osteolytic metastases.[42] More recently, it has been shown that multimeric forms of YIGSR can enhance the inhibition of tumor growth and metastasis.[43] Thus, it appears that multimeric or polymeric forms of YIGSR strongly enhance YIGSR's ability to inhibit tumor growth and metastasis, and that it is these types which are potentially useful for clinical applications.[39,43] These forms may be more potent due to their conformation and/or stability in the circulation.

Experimental Section

Boc-amino acids were of the L-configuration, unless stated otherwise, and were purchased from either Bachem California or Vega Biochemicals. Symbols and abbreviations generally follow the IUPAC–IUB recommendations as published in the *International Journal of Peptide Protein and Research* (**1984**, *24*, 9–37). The other chemicals and solvents were purchased from the following sources: TFA and diisopropylethylamine (DIEA), Fisher Biotech; DCM, HPLC-grade acetonitrile (CH_3-CN), and triethylamine (TEA), Fisher; anisole, dimethyl sulfide (DMS), 1,3-diisopropylcarbodiimide (DIC), *N,O*-dimethylhydroxylamine hydrochloride, HOBt, and sodium cyanoborohydride, Aldrich; MBHA resin (1% cross-linked, 200–400 mesh), Bachem California; HF, Matheson; cobalt trifluoride (CoF_3) and lithium aluminum hydride (LAH), Alfa; DMF, Aldrich, Baxter, or B & J; dicyclohexylcarbodiimide (DCC), Chemalog; phenol, Mallinckrodt; mixed bed resin, AG501-X8 (20–50 mesh), Bio-Rad. The following were purified by distillation: DCM from anhydrous Na_2CO_3; TEA, DIEA, and pyridine were distilled from ninhydrin. Analytical (on aluminum) silica gel TLC plates were obtained from Analtech. The solvent systems used were (a) *n*-butyl alcohol:acetic acid:ethyl acetate:water (1:1:1:1), (b) ethyl acetate:pyridine:acetic acid:water (10:5:1:3), (c) *n*-butyl alcohol:pyridine:acetic acid:water (15:10:3:12). After development, spots on the TLC plates were detected by spraying with ninhydrin. All of the peptides were synthesized in a Milligen 504 synthesizer (manual). The apparatus for conducting the liquid HF cleavages was constructed as previously described.[44] The HF was dried prior to use by distilling it into the first vessel which contained CoF_3 and then distilling it from there into the reaction vessel which also contained 10% anisole and 10% DMS. Analytical and semipreparative HPLC separations were performed on a Waters Associates system, consisting of a U6K injector, M45 and 6000 pumps, a model 660 solvent programmer, and a model 481 UV detector connected to a Hitachi model D-2000 Chromato-Integrator, monitoring at 254 or 214 nm, using Vydac C_{18}, 5 μm particle size, 300 Å pore size columns, 0.46 × 15 cm and 1.0 × 25 cm, respectively. The gradients used were linear mixtures of solvent A (0.05% TFA in H_2O) and solvent B (0.05% TFA in 60% CH_3CN/H_2O); flow rates of 1 mL/min for the analytical column and 3 mL/min for the semipreparative column were used. The columns were protected with a Vydac guard cartridge containing the same packing, but of 10 μm particle size. The peptide samples, after separation from HPLC, were lyophilized on a Virtis freeze dryer. Amino acid analyses were performed, after hydrolysis of the peptides, on a Beckman model 6300 analyzer. The hydrolyses were accomplished in 6 N HCl, for 24 h, at 110 °C, in vacuo. ^1H NMR spectra were obtained on a JEOL FX90Q spectrometer at 90 MHz using TMS as an internal standard. Mass spectra were recorded on an EXTREL ELQ 400 spectrometer. Positive ion FABMS were obtained using one of three possible liquid matrixes, glycerol, thioglycerol or 3-nitrobenzyl alcohol. The instrument was equipped with a FAB gun operating at 6–8 kV and 0.5–1.0 mA using xenon bombarding gas at 1.5–2.0 × 10^{-4} Torr. The scan cycle time was 750 mass units per second (units/s). Chemical ionization mass spectra (CIMS) were obtained with an ion source temperature of 100 °C, methane as reagent gas, and an ionization energy of 70 eV.

The CID–MS spectra were recorded at the Massachusetts Institute of Technology Mass Spectrometry Facility.

Solid-Phase Peptide Synthesis. Boc-L-amino acids were used for all of the syntheses, protection for the side chain functionalities was Arg(Tos), Ser(Bzl), Phe(*p*-NH-Z), and Tyr-(2BZ), and where appropriate, D-Tyr(2BZ) was used. Each synthesis was begun by loading 1.0 g of MBHA resin (about 0.7 mequiv/g) into the Milligen manual synthesizer, and the details of the procedure used for the synthesis of peptides 1–**4** has been previously described.[32] All final peptides were at least 96% pure according to analytical HPLC. Correct $[M + H]^+$ ions were obtained by FABMS for all peptides synthesized: 1 (594), **2** (578), **3** (596), **4** (593). The FABMS results for the pseudopeptides are described below. Amino acid analyses were obtained for peptides **2**–**4** and agreed with theory.

Tyr-ψ(CH_2NH)-Ile-Gly-Ser-Arg-NH_2 (5) and Tyr-ψ(CH_2-NCHO)-Ile-Gly-Ser-Arg-NH_2 (9). The *N,O*-dimethyl hydroxamate of Boc-Tyr(2BZ) was obtained as a colorless oil from the DCC coupling of Boc-Tyr(2BZ) with *N,O*-dimethylhydroxylamine.[20] The resulting hydroxamate was reduced with $LiAlH_4$ in ether to give a colorless oil which was used without further purification.[19,28] A portion of the crude aldehyde (0.50 g) was dissolved in DMF (10 mL) containing 1% acetic acid and added to H_2N-Ile-Gly-Ser(Bzl)-Arg(Tos)-MBHA resin (0.30 g), which had been prepared by the SPPS procedure described above, followed by the addition of $NaBH_3CN$ (0.031 g) in 4 min.[18,19] The reaction mixture was allowed to shake overnight and then the N-terminal Boc group was removed with TFA, followed by cleavage from the resin with HF/anisole/DMS. The crude product was purified using semipreparative HPLC with 0–100% solvent B in 30 min, which afforded the desired peptide **5** [0.012 g; t_R = 12.4 min; FABMS and CID–MS (*m/z*) 580 $[M + H]^+$; TLC R_f 0.12 (b), 0.49 (a), 0.83 (c)] and also peptide **9** [0.041 g; t_R = 17.4 min; FABMS and CID-MS (*m/z*) 608 $[M + H]^+$; TLC R_f 0.14 (b), 0.74 (a), 0.83 (c)] (see Figure 1).

Tyr-Ile-ψ(CH_2NH)-Gly-Ser-Arg-NH_2 (6). Reduction ($LiAlH_4$) of the *N,O*-dimethyl hydroxamate of Boc-Ile[28] afforded Boc-isoleucinal, whose ^1H NMR spectrum agreed with that reported in the literature.[28] The latter aldehyde was used in the reductive alkylation of H_2N-Gly-Ser(Bzl)-Arg(Tos)-MBHA (0.20 g), as described above.[18,19] The peptide was further elongated by coupling with Boc-Tyr(2BZ), followed by cleavage with HF/anisole/DMS. The lyophilized crude product gave a complex HPLC chromatogram and was basified with NH_4OH to pH 9–10, in order to reverse any possible N → O acyl migration,[45] and purified via semipreparative HPLC with 0–100% solvent B in 60 min to yield Peptide **6**: 0.012 g; t_R = 12.6 min; analytical column, 0–100% solvent B in 50 min, 96% pure; TLC R_f 0.16 (b), 0.30 (a), 0.78 (c); FABMS *m/z* 580 $[M + H]^+$.

Tyr-Ile-Gly-ψ(CH_2NH)-Ser-Arg-NH_2 (7). Crystalline *N,O*-dimethylhydroxamate of Boc-Gly (mp 102–103 °C, from acetone)[46] was converted to Boc-glycinal by reduction, as above, and its ^1H NMR spectrum agreed with that reported in the literature.[47] The peptide resin H_2N-Ser(Bzl)-Arg(Tos)-MBHA (0.20 g) was reductively alkylated with Boc-glycinal, as above, and then coupled sequentially with Boc-Ile and Boc-Tyr(2BZ). After Boc cleavage with TFA, the peptide was fully deblocked and removed from the resin with HF/anisole/DMS. The crude product was purified using semipreparative HPLC with 0–100% solvent B in 120 min, affording peptide **7**: t_R = 11.7 min; analytical column, 0–100% solvent B in 50 min; 97% pure; TLC R_f 0.09 (b), 0.32 (a), 0.70 (c); FABMS (*m/z*) 580 $[M + H]^+$.

Tyr-Ile-Gly-Ser-ψ(CH_2NH)-Arg-NH_2 (8). As above, the *N,O*-dimethyl hydroxamate of Boc-Ser(Bzl) was reduced to Boc-serinal, whose physical data agreed with that reported in the literature.[48]

Boc-serinal was used in the reductive alkylation of H_2N-Arg(Tos)-MBHA resin to give Boc-Ser(Bzl)-ψ(CH_2NH)-Arg-(Tos)-MBHA, which was elongated by coupling, in turn, with Boc-Gly-ONp, Boc-Ile, and Boc-Tyr(2BZ). After sequential cleavage with TFA and HF/anisole/DMS, the crude product was lyophilized, redissolved in water, and basified with NH_4-OH to pH 9–10.[45] Purification by semipreparative HPLC with 0–100% solvent B in 60 min afforded peptide **8** that was 99% pure by analytical HPLC: t_R = 11.9 min, 0–100% solvent B in 50 min; TLC R_f 0.07 (b), 0.34 (a), 0.72 (c); FABMS (*m/z*) 580 $[M + H]^+$.

Biological Studies. Cell Attachment Assay. Cell attachment was performed in 24- or 96-well Falcon tissue culture plates as previously described, with some modifications.[34] The test peptides were dissolved in water containing a drop of acetic acid, added to the tissue culture wells in a final volume of 100 μL, and dried overnight. The concentrations of peptides indicated in the text are the amounts dried onto the plastic. The peptide-coated wells were treated with 1.0 mL of Eagle's minimal essential medium (EMEM) containing 3% bovine serum albumin (BSA) for 30 min at 37 °C. The EMEM solution was removed and 1.0 mL of EMEM containing 0.02% BSA was added to each of the wells. HT-1080 human fibrosarcoma cells[34] or B16F10 cells[34] (4×10^4) in 100 μL of media containing 0.02% BSA were added to each well and incubated for 1 h at 37 °C in 5% CO_2, 95% air. The media was removed and the wells were then washed with 1.0 mL of phosphate-buffered saline (PBS) to remove unattached cells. Attached cells were either fixed, stained, and counted in an Optomax, using counts from eight fields per well, or released with trypsin–EDTA and counted in a Coulter ZBI. Each assay was carried out in duplicate. Each peptide was tested at least twice.

Experimental Metastasis. Test peptides were dissolved in PBS at a concentration of either 5.0 or 10 mg/mL and were filter-sterilized. A suspension of B16F10 cells (1×10^5) in 0.1 mL of Dulbecco's modified Eagle's medium (DMEM) was mixed with 0.1 mL of the peptide solution and then injected into the tail vein of syngeneic C57BL/6 female mice at 6 weeks of age. Each treatment and control group consisted of eight mice in experiment 1 and five mice in experiment 2. Two to three weeks after the injections, the mice were sacrificed, and the number of colonies on lung surface was counted.

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