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New Analogues of Laminin Active Fragment YIGSR: Synthesis and Biological Activity *In vitro* and *In vivo*

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Abstract: Eleven analogues of the laminin pentapeptide amide fragment Tyr-Ile-Gly-Ser-Arg-NH_2 (YIGSR-NH_2) corresponding to a B1 chain fragment of the glycoprotein laminin have been synthesized by the solid phase method, and their biological activity has been studied *in vitro* by a cell adhesion assay: all of them inhibited the adhesion of LLC tumor cells to laminin. The analogues were found to be more resistant to enzymatic degradation in human serum than YIGSR-NH_2 itself. Analogue DatIGSHar-NH_2 was selected for an experimental pulmonary metastasis assay *in vivo*: it had higher antimetastatic activity than YIGSR-NH_2.

Keywords: laminin YIGSR-NH_2 fragment; peptide synthesis; tumor cell adhesion assay; enzymatic degradation; antimetastatic activity; melanoma

INTRODUCTION

Cellular adhesion plays pivotal roles in morphogenesis, cell migration and intercellular communication. Many adhesion proteins taking part in these processes have been identified and structurally analysed. This has allowed an understanding of their role in the pathophysiology of diseases, including carcinogenesis and tumor metastasis.

Investigations on the inhibition of angiogenesis, which enables tumor development and metastasis, have indicated that peptides from the adhesive protein laminin have an inhibitory effect on metastasis.

Laminin, the major component of the basement membrane, plays an important role in tumor invasion and metastasis. The binding of tumor cells to laminin enables their anchorage in the basement membrane and starts invasion. Hence, cell migration through the basement membrane is the critical step of metastasis formation [1].

Laminin is a multidomain glycoprotein composed of three polypeptide chains: A (~400 kDa), B1 (~230 kDa) and B2 (~220 kDa) [2]. The YIGSR peptide, corresponding to the 929–933 sequence of B1 chain [3], has been found to inhibit lung tumor colony formation by competing with laminin for its receptor on tumor cells, thus blocking the binding of these cells to basement membranes [4–6]. This finding suggested that laminin–derived peptides like YIGSR might be useful as a tool to prevent and inhibit tumor metastasis. However, the very short half-life time of these peptides *in vivo* limits their potential therapeutic use.

Several modifications of YIGSR peptide have been reported to enhance their activity and clinical utility. On the basis of biological assay it was found that Arg and Tyr or Phe containing an electronegative moiety in the *para* position might be essential for the activity [7,8]. Various YIGSR peptides which show a stronger inhibitory effect on experimental

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metastasis have been presented: amide YIGSR [4,7], cyclic YIGSR [9], poly(YIGSR) [10,11], analogues containing Tyr or Thr instead of Ser [12], a YIGSR hybrid with amino-polyethylene glycol [13,14] and multimeric forms in which the active peptides are placed on a 'lysine tree' [15].

In the present study YIGSR-NH_2 analogues were modified in order to limit their enzymatic degradation while preserving their adhesive activity. In the native sequence the tyrosine residue was replaced by desaminotyrosine (Dat), 5-carboxymethyluracil or 6-carboxymethyluracil (U5, U6); the arginine residue was replaced by homoarginine (Har), 4-guanidine-2-aminobutyric acid residue (Gab) or 3-guanidine-2-aminopropionic acid residue (Gap). A cyclic analogue in which 20-membered ring formation was achieved *via* an ureido group incorporating both the ε-amino group of *C*-terminal lysine and α-amino group of tyrosine residue and the analogue containing *N*-terminal hydantoin grouping were also obtained.

The analogues obtained were tested for their ability to inhibit tumor cell adhesion. Degradation of these peptides in human serum was also studied. The inhibitory effect of peptide **DH** on experimental melanoma pulmonary metastasis was examined.

MATERIALS AND METHODS

Peptide Synthesis

Peptides were synthesized by SPPS using the Boc strategy and carbodiimide (DIC) as the coupling reagent. All amino acids except glycine were of L-configuration. The side-chain functions of the Boc-amino acids were protected as follows: Arg(Tos), Lys(Fmoc), Ser(Bzl), Tyr[Z(2Br)], Dab(Fmoc) [Dab, 2,4-diaminobutyric acid], Dap(Fmoc) [Dap, 2,3-diaminopropionic acid]. Peptides were synthesized on a MBHA resin (0.62 mmol/g Applied Biosystems). TFA (55%)/DCM was used in all deprotection steps. Neutralization was carried out with 5% DIEA/DCM. Every coupling step was monitored with the Kaiser color test. When needed, the acylation was repeated. Introduction of the guanidino group (peptides **H**, **DGp**, **DGb**, **HydH**, **DH**) was performed by *N*,*N*′-bis(*tert*-butoxycarbonyl)-S-methylisothiourea after the removal of the Fmoc group from the amino function of the side chain of Lys, Dab or Dap [16]. This protection was removed after the peptide chain was assembled by treatment with piperidine and then the peptide-resin was guanidinylated [17]. This process was controlled using the Kaiser color test, and was judged finished when free amino groups were not detected.

The cyclic analogue containing the carbonyl bridge between the amino group of Tyr and the ε-group of Lys (**cLa**) was obtained by treatment of the linear peptide with the free amino group of tyrosine and the ε-amino group of lysine {Tyr[Z(2Br)]-Ile-Gly-Ser(Bzl)-Lys-NH-resin} on the solid support with bis(4-nitrophenyl)carbonate [18]. The analogue containing a hydantoin system (**HydH**) was prepared by treatment of the linear peptide with the free amino group of tyrosine {Tyr[Z(2Br)]-Ile-Gly-Ser(Bzl)-Lys(Fmoc)-NH-resin} on the solid support with bis(4-nitrophenyl)carbonate. After removal of the Fmoc group from the amino function of the side chain of Lys the guanidinylation reaction was carried out. The peptides were deprotected and cleaved from the resin by treatment with liquid HF. Samples of the peptide resins were treated with HF (0 °C, 1 h) in the presence of anisol after the removal of the Boc group. The crude peptides were purified using a Knauer HPLC system with a Vertex column Nucleosil-300 C_{18} (8 × 250 mm, 5 μm); solvent system: A, 0.05% TFA in water, B, 60% MeCN in A. Fractions were eluted from the column with a linear gradient: peptides **S, Gp, Gb, H, U5** and **U6**, 2%–10% B in 50 min; peptides **D, DGb, DGp, DH** and **cLa**, 10%–25% B in 55 min; peptide **HydH**, 10%–30% B in 55 min; flow rate 2 ml/min. Fractions were analysed with the same system, equipped with a Eurospher 100 C_{18} (4.6 × 250 mm, 5 μm) using a gradient: peptides **S, Gp, Gb, H, U5** and **U6**, 10%–40% B in 20 min; peptides **D, DH** and **cLa**, 20%–50% B in 20 min; peptides **DGb** and **DGp**, 25%–55% B in 20 min; peptide **HydH**, 30%–60% B in 20 min; flow rate 1 ml/min; detection at 220 nm. The structure of the peptides was confirmed by ESI-MS using a Mariner Biospectrometry Workstation PerSeptive Biosystem.

Biological Assays

Inhibition of tumor cell adhesion in vitro. Cells: the LLC (Lewis lung carcinoma) cell line used was received as a gift from Dr I. Wodinsky, National Cancer Institute, Bethesda, USA and maintained *in vitro* and/or *in vivo* in syngeneic mice and stored in liquid nitrogen at the Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Wrocław.

Plastic tissue culture wells were coated overnight with laminin or YIGSR-NH_2 peptide (1 μg/50 μl per well). After drying, the wells were washed

(3 times) with PBS and blocked with 3% BSA (1 h, 37 °C). Freshly trypsinized LLC cells (4×10^4/well/50 µl) were mixed with YIGSR-NH_2 analogues (5 µg/well). The cells were incubated at 37 °C for 60 min. Non-adherent cells were removed by washing and attached cells were stained with crystal violet (0.1% crystal violet aqueous solution in 20% methanol) for 1 h. After washing and drying, 100 µl of methanol was added to dissolve the dye and the optical density (OD) at 570 nm was measured in a Multiskan RC V1 spectrophotometer.

Experimental pulmonary metastasis assay in vivo. C57BL/6 male mice 12–16 weeks old, weighing 20–25 g, were used in experiments. They were supplied by the Animal Breeding Center of the Institute of Immunology and Experimental Therapy, Wrocław, Poland and maintained in standard laboratory conditions with water and food *ad libitum*. All experiments described were performed according to *Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education* issued by the New York Academy of Sciences Ad Hoc Committee on Animal Research, and were approved by the 1st Local Committee for Experiments with the Use of Laboratory Animals, Wrocław, Poland. Tumors: the B16F-0 melanoma cell line used was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and maintained *in vitro* and/or *in vivo* in syngeneic mice and stored in liquid nitrogen at the Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Wrocław, Poland. Mice were inoculated i.v. into the lateral tail vein with 3×10^5 viable tumor cells, taken from the *in vitro* culture suspended in 0.2 ml of saline. The cells were incubated for 30 min before injection with 2.5 mg/ml of the tested peptides.

The animals were killed 21 days after inoculation with melanoma cells. Lungs were excised and metastatic nodules were counted.

Statistical evaluation: Student's *t*-test for independent samples was applied. A *p* value lower than 0.05 was considered as a significant.

Stability of peptides in human serum. A sample of peptide (1 mg) was dissolved in 100 µl of 0.16 M phosphate buffer (pH 7.4) and incubated at 37 °C for 20 min. Then the sample of peptide (75 µl) was added to 75 µl pre-heated human serum. The mixture was incubated at 37 °C. Samples of this solution (50 µl) were collected after 15, 30 and 60 min and 0.2 M TFA (50 µl) was added. After centrifugation samples were analysed for remaining substrate by RP-HPLC using the Knauer system as above (peptide synthesis). The main product of digestion was separated and identified by ESI MS using a Finnigan MAT 95S spectrometer.

RESULTS

Novel analogues of YIGSR were synthesized according to solid phase protocols on the benzhydryl amino resin using DIC as the coupling reagent. The peptides contained desaminotyrosine, 5-carboxymethyluracil or 6-carboxymethyluracil residue instead of tyrosine, and homoarginine, 4-guanidine-2-aminobutyric acid or 3-guanidino-2-aminopropionic acid residue instead of arginine. Guanidinylation was performed using *N*, *N*′-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea. An analogue containing an *N*-terminal hydantoin group and a cyclic analogue were also obtained. The peptides were cleaved from the resin by treatment with liquid HF in the presence of anisole and purified by RP-HPLC (Table 1).

The peptides were tested *in vitro* for their ability to inhibit tumor cell attachment. An assay was performed on plastic tissue culture plates (96-well) coated with laminin. The results are summarized in Figure 1. All the new tested peptides inhibited the adhesion of LLC tumor cells to laminin coated

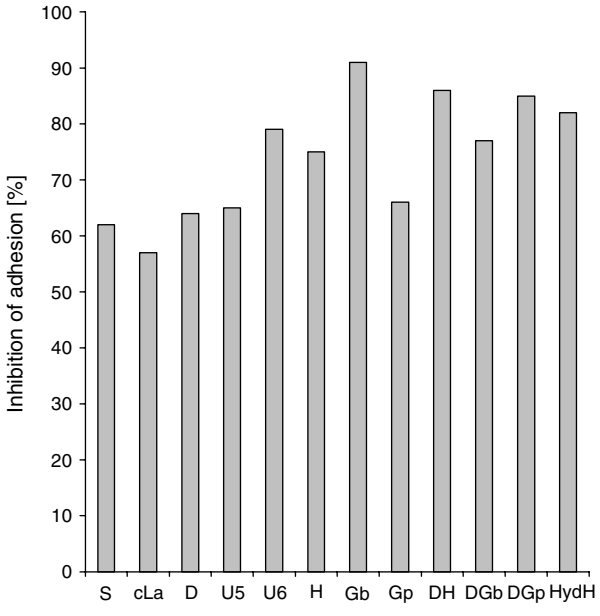
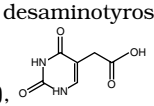


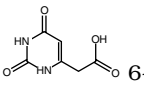
Figure 1 Inhibition of LLC cell adhesion to laminin-coated wells by YIGSR-NH_2 and analogues.

Table 1 Synthetic Analogues of YIGSR-NH_2

Code	Peptide	Formula	ESI-MS	
			Calc.	Found
S	Tyr-Ile-Gly-Ser-Arg-NH_2	$C_{26}H_{43}O_7N_9$	593.7	594.0
D	Dat[a]-Ile-Gly-Ser-Arg-NH_2	$C_{26}H_{42}O_7N_8$	578.7	579.3
U5	(U-5)[b]-Ile-Gly-Ser-Arg-NH_2	$C_{23}H_{38}O_8N_{10}$	582.6	583.5
U6	(U-6)[c]-Ile-Gly-Ser-Arg-NH_2	$C_{23}H_{38}O_8N_{10}$	582.6	583.4
Gb	Tyr-Ile-Gly-Ser-Gab[d]-NH_2	$C_{25}H_{41}O_7N_9$	579.7	580.0
Gp	Tyr-Ile-Gly-Ser-Gap[e]-NH_2	$C_{24}H_{39}O_7N_9$	565.6	566.3
H	Tyr-Ile-Gly-Ser-Har[f]-NH_2	$C_{27}H_{45}O_7N_9$	607.0	608.4
DH	Dat[a]-Ile-Gly-Ser-Har[f]-NH_2	$C_{27}H_{44}O_7N_8$	592.7	593.0
DGb	Dat[a]-Ile-Gly-Ser-Gab[d]-NH_2	$C_{25}H_{40}O_7N_8$	564.6	565.0
DGp	Dat[a]-Ile-Gly-Ser-Gap[e]-NH_2	$C_{24}H_{38}O_7N_8$	550.6	551.0
HydH	Tyr-Ile[g]-Gly-Ser-Har[f]-NH_2 CO	$C_{28}H_{43}O_8N_9$	633.7	634.3
cLa	Tyr-Ile-Gly-Ser-Lys-NH_2[h] CO	$C_{27}H_{43}O_8N_7$	591.0	592.0

[a] Dat, desaminotyrosine [3-(4-hydroxyphenyl)propionic acid].

[b] (U-5),  5-carboxymethyluracil.

[c] (U-6),  6-carboxymethyluracil.

[d] Gab, 4-guanidino-2-aminobutyric acid.

[e] Gap, 3-guanidino-2-aminopropionic acid.

[f] Har, homoarginine.

[g] HydH, an analogue with a carbonyl bridge between the α-amino group of Tyr and the α-amino group of Ile.

[h] cLa, a cyclic analogue with a carbonyl bridge between the α-amino group of Tyr and the ε-amino group of Lys.

wells. The highest inhibitory activity was exhibited by peptides **Gb, DH, DGp** and **HydH**.

Further, the peptides were subjected to digestion in human serum. The course of the digestion was monitored by RP-HPLC. The obtained results indicate that the new analogues, except peptide **H** and **cLa**, were cleaved more slowly than YIGSR-NH_2 (**S**) (Figure 2). In the case of **H** and **Gp,** showing the most advanced digestion during the 60 min hydrolysis time, one of the products corresponding to the major peak was separated and identified as tyrosine by electrospray-ionization mass spectrometry. It was observed that peptides modified at the *N*-terminal position were more resistant to enzymatic hydrolysis than those modified at the *C*-terminal position.

On the basis of the results obtained peptide **DH** was selected for further *in vivo* studies for its ability to inhibit tumor lung metastasis in mice injected i.v. with B16 melanoma cells. When the melanoma cells were incubated with peptides **DH** or **S**, a reduction in the number of lung colonies was observed compared with the control mice inoculated with untreated melanoma cells. Incubation with peptide **S** caused a 19% reduction, while a 39% reduction was observed in mice treated with peptide **DH** (Figure 3). This difference was statistically significant.

CONCLUSIONS

This paper describes the synthesis and biological evaluation of 11 novel analogues of YIGSR-NH_2, synthetic peptide from the B1 chain of laminin. The aim of this work was to improve the antimetastatic potency of YIGSR-NH_2 by modification of the structure limiting the enzymatic degradation. The introduced structural modifications prevented their enzymatic degradation. All the new peptides inhibited (up to 90%) the adhesion of LLC tumor cells to laminin. The selected Dat-Ile-Gly-Ser-Har-NH_2

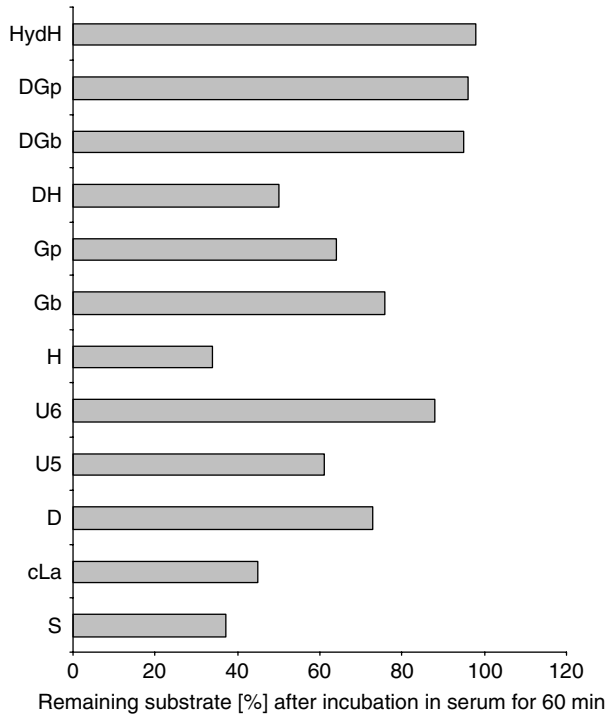


Figure 2 Stability of peptides in human serum.

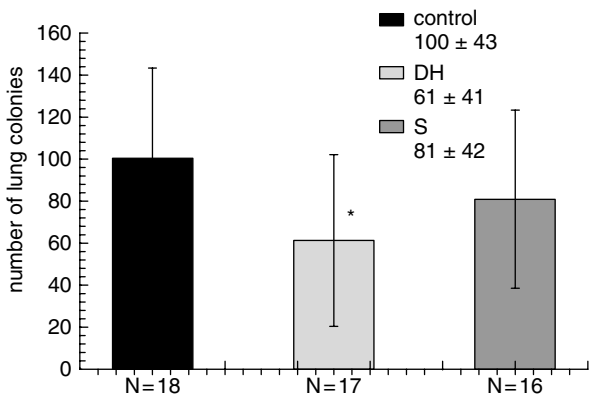


Figure 3 Antimetastatic effect of peptides in mice inoculated i.v. with B16 melanoma cells. Number of lung colonies (day 21). N, number of mice. $^*p = 0.009$ (Student's *t*-test) in comparison with control.

peptide enhanced the inhibitory effect on lung colonization by B16 melanoma cells over that observed with YIGSR-NH_2. Thus, it appears that the enhanced *in vivo* activity of the analogue **DH** is due to its increased enzymatic stability as well as increased inhibition of LLC cell adhesion to laminin.

All the analogues obtained had greater enzymatic stability and an increased ability to inhibit tumor cell adhesion to laminin compared with the standard peptide but the highest inhibitory activity could be attributed to the peptide **DH**.

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