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FULL PAPER

Synthesis of modified RGD based peptides and their *in vitro*

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Abstract: Insert abstract text here. RGD peptides represent the most outstanding recognition motif involved in cell adhesion and binds to $\alpha_v\beta_3$ integrin that has been targeted for cancer therapy. Various RGD-containing peptides and peptidomimetics have been designed and synthesized to selectively inhibit this integrin. In this study, the synthesis of RGD based peptides through the incorporation of the short bioactive peptide FAKLF at the *C*- and *N*- terminus of RGD has been achieved using solid phase peptide synthesis approach. The peptides were purified using preparative RP-HPLC and their structures were confirmed using HR-MS (ESI). The MTT assay study displayed that RGD (Peptide **1**) and FAKLF (Peptide **2**) inhibited the proliferation of HUVECs in a dose-dependent manner, with the IC_{50} values of 3000 ng/mL and 500 ng/mL, respectively. Interestingly, a drastic improvement was observed in the antiproliferative activity of FAKLFRGD (Peptide **3**) and RGDFAKLF (Peptide **4)** combined structures, leading to the IC_{50} values of 200 and 136.7 ng/mL, respectively. Meanwhile, based on the apoptosis results, the potential of peptides for induction of apoptosis, in accordance with their antiproliferative activity, indicated that RGD (Peptide **1**) and FAKLF (Peptide **2**) peptides and the peptides synthesized based on their combinations induced cell apoptosis in a dose-dependent manner followed by inhibition of proliferation of endothelial cells. Moreover, the incorporation of D-Leucine increased the apoptosis induction by these peptides.

Introduction

Over the last decade, anticancer peptides have received great attention as alternative therapeutic agents that can overcome the limitations of the conventional approaches[1] due to several advantages, including high specificity, low toxicity, high tumor penetrating, and accessible synthesis and modifications.[2] Several anticancer peptides (e.g. leuprolide, goserelin, and octreotide) have been approved by FDA and many other antitumor peptides are currently being evaluated in various phases of preclinical and clinical trials thus reflecting the high potential of peptides as therapeutics.[3]

Meanwhile, ever since the discovery of RGD (Arg-Gly-Asp) tripeptide as an adhesion motif that is presented in many proteins of the extracellular matrix (ECM) and its role in cell-cell, integrin interaction, and cell-matrix interactions, incited the development of a broad diversity of RGD peptides and peptidomimetics for potential therapeutic applications.[4] Since RGD binds to the $\alpha_v\beta_3$ integrin, resulting in inhibition of angiogenesis, tumor growth, and metastasis, the design and synthesis of novel RGD-based peptides exhibiting high activity and selectivity is a demanding challenge for biochemistry, medicinal and synthetic chemistry.[5]

In this regard, RGD based peptides have been extensively used in many applications and have been developed to target tumor cells through different approaches. Figure **1** shows some examples of RGD based peptides. [6] Meanwhile, studies have shown that the integrin-specificity of the RGD peptide relies on a) the conformation of the RGD site and b) the nature of the neighboring amino acids where both have been well employed to design and synthesize integrin-selective peptides. [7]

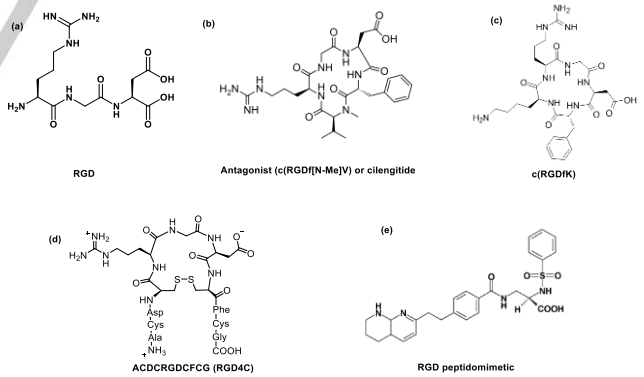


Figure 1. Some know structures of RGD peptides: (a) RGD sequence, (b) Cyclopeptide (c(RGDf[N-Me] V). (c) Cyclopeptide c(RGDfK). (d) ACDCRGDCFCG (RGD4C). (e) RGD peptidomimetic.

Chen et al. reported that both linear RGDS and cyclic RGDF induced apoptosis and expression of interleukin-1 *β*-converting enzyme (ICE) in cultured glomerular mesangial cells (GMC) from adult human kidneys. [8]

Zhang et al. also described the synthesis and conjugation of tachyplesin (an antimicrobial peptide) to RGD peptide and the *in*

vitro results showed that RGD-tachyplesin inhibited the proliferation of both cultured tumor and endothelial cells and reduced the colony formation of TSU prostate cancer cells.[9] In another study, Anuradha et al. demonstrated that RGD based peptides induced apoptosis at a concentration of 1 mmol/L in HL-60 cells by activating caspase-3, leading to DNA fragmentation and cell death.[10] Boturyn et al. reported the design and synthesis of a dual RGD-ATWLPPR peptide that was able to bind with an improved tumor/background ratio to tumor cells *in vitro* and *in vivo*.[11]

On the other hand, other short bioactive peptides have been investigated for different biological activities and some showed distinguished effects with anticancer, anti-tumor and other extended biological applications.[12] Owen *et al.* registered his patent invention for the screening of a wide synthetic library of short bioactive peptides containing phenylalanine, leucine, lysine and alanine amino acid residues.[13] From his library we selected FAKLF peptide sequence for its anticancer activity.

Moreover, extensive research has been made to fulfill the demand for modified peptides with improved pharmacokinetic properties and stability profiles. One approach is through designing peptidomimetics that circumvent some of the problems associated with a natural peptide such as stability against proteolysis and poor bioavailability. Peptidomimetics have been synthesized by a) peptide cyclization, or b) replacing a peptide bond with its isostere, and/or c) incorporation of unnatural amino acids such as D-amino acids that affect the conformation of the peptide, and lead to the resistance towards proteolytic enzymes especially in circulation.[14]

Keeping in mind the vast therapeutic importance of RGD peptides and in continuation of our work herein, we report the design and synthesis of novel RGD based peptides. Phe-Ala-Lys-Leu-Phe (FAKLF), as a potent anticancer peptide, was selected to be covalently linked into RGD, so that FAKLF segment flanked by RGD in *N*- or *C*-terminus. Next, the capability of peptides to inhibit the cell proliferation and induce of programmed cell death (apoptosis) was investigated using Human Umbilical Vein Endothelial Cells (HUVECs). Furthermore, the L residue in the sequence of FAKLF was substituted with D-L and its effect on the biological activity was also investigated. Figure **2** summarizes the synthesized peptides.

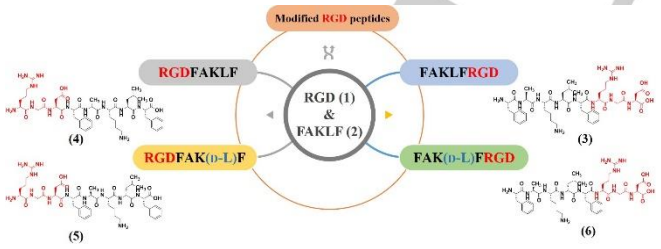
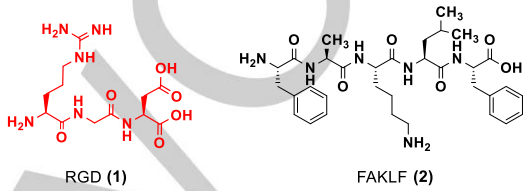


Figure 2. Structure of the novel modified RGD peptides

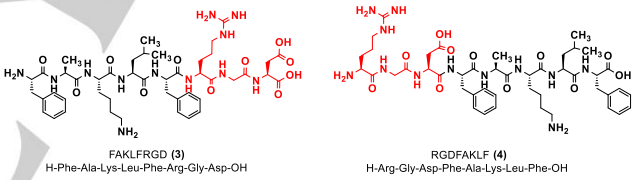
Results and Discussion

RGD peptides have been extensively employed and synthesized to target cancer. Meanwhile, desirable anticancer activity is also displayed in some short peptides such as FAKLF that cause stimulation and proliferation of human fibroblasts and lymphocytes. [13] In the beginning, peptide FALKF along with RGD were synthesized to be able to compare their biological activities with the novel modified peptides. The sequences of the two peptides are shown in scheme **1** below. These peptides were synthesized using solid phase peptide synthesis (SPPS) procedure through adding the amino acids of the desired sequence to the 2-chlorotrityl chloride (2-CTC) resin. After complete assembly of the linear peptide it was cleaved from the resin, final deprotected and then the peptide was purified using preparative RP-HPLC. The molecular composition of the synthesized peptide analogues was confirmed using mass spectrometry MS (ESI). For example, the distinguished peak of peptide **2** [M+H]$^+$ was 625.87 that confirmed the structure of the synthesized peptide.



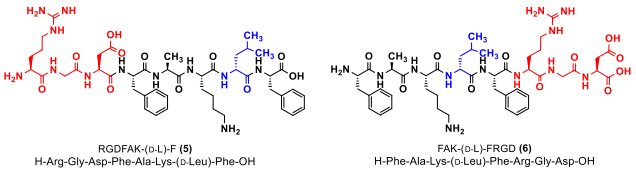
Scheme 1. Structure of RGD and FAKLF peptides

Later, the FAKLF segment was introduced to the RGD backbone at the *C*- and *N*- terminus. The sequences of the two peptides are shown in scheme **2** below. The distinguished peptide **4** [M+H]$^+$ was 953.5207 that confirmed the structure of the synthesized peptide.



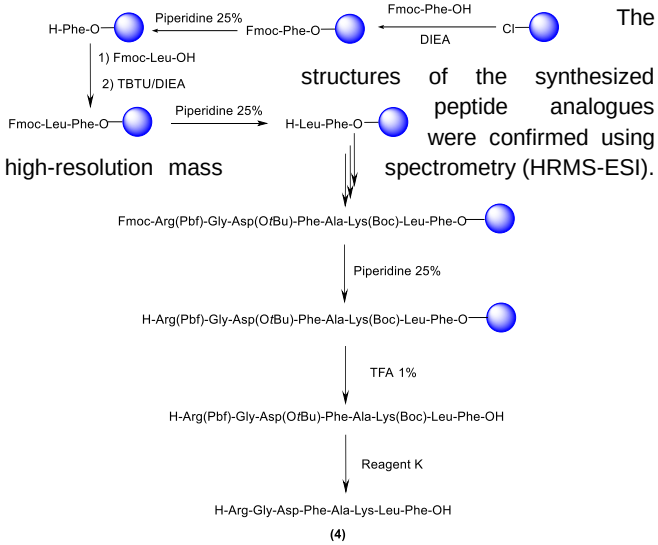
Scheme 2. Structure of RGD modified peptides

Finally, we decided to synthesize analogues through anchoring unusual amino acids to the sequence of the RGD-based peptides. The reason behind this modification is to study the effect of such modification on the activity of the peptide. Scheme **3** shows the sequences of the peptides. For example, the distinguished peak of peptide **5** [M+H]$^+$ was 953.5209 that confirmed the structure of the synthesized peptide.



Scheme 3. Structure of RGD modified peptides

The assembly and synthesis of the all above peptides FAKLFRGD, RGDFAKLF, RGDFAK(D-L)F, FAK(D-L)FRGD were also done based on the SPPS strategy. Scheme **4** illustrates the general synthesis strategy. The details of the synthetic procedure are further explained in the experimental section.



Scheme 4. The general method for the synthesis of RGD based peptide using SPPS

Antiproliferative activity of the peptide variants

To investigate the effect of peptides on the cell viability, MTT assay was performed using Human Umbilical Vein Endothelial Cells (HUVECs) after treatment with different doses (100, 200, 500, 1000, 2000, 3000, 5000 ng/mL) of the peptides. As shown in Figure **3**, Peptide **1** (RGD) and Peptide **2** (FAKLF) inhibited the proliferation of HUVECs in a dose-dependent manner, with the IC_{50} values of 3000 ng/mL and 500 ng/mL, respectively. Interestingly, a drastic improvement was observed in the antiproliferative activity of FAKLFRGD and RGDFAKLF combined structures, leading to the IC_{50} values of 200 and 136.7 ng/mL, respectively. To test whether incorporation of a D-amino acid may enhance the activity of peptides, leucine (L-L) changed to D-leucine (D-L). Whereas FAK(D-L)FRGD (Peptide **6**) exhibited a similar antiproliferative activity (IC_{50}=183 ng/mL) as FAKLFRGD (Peptide **3**), RGDFAK(D-L)F (Peptide **5**) indicated a decreased inhibitory activity (IC_{50}=476 ng/mL) compared to RGDFAKLF (Peptide **4**) (Figure **3**).

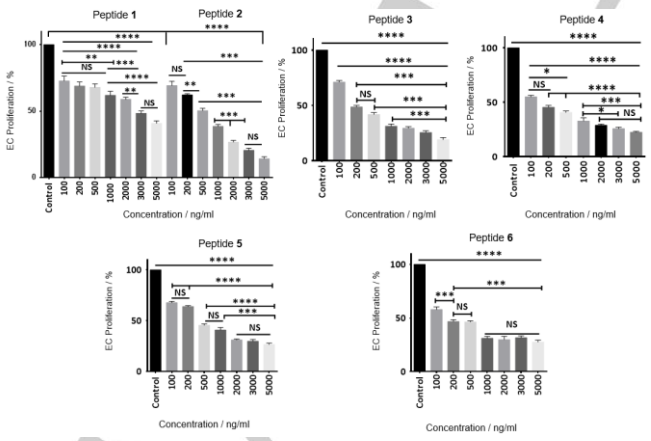
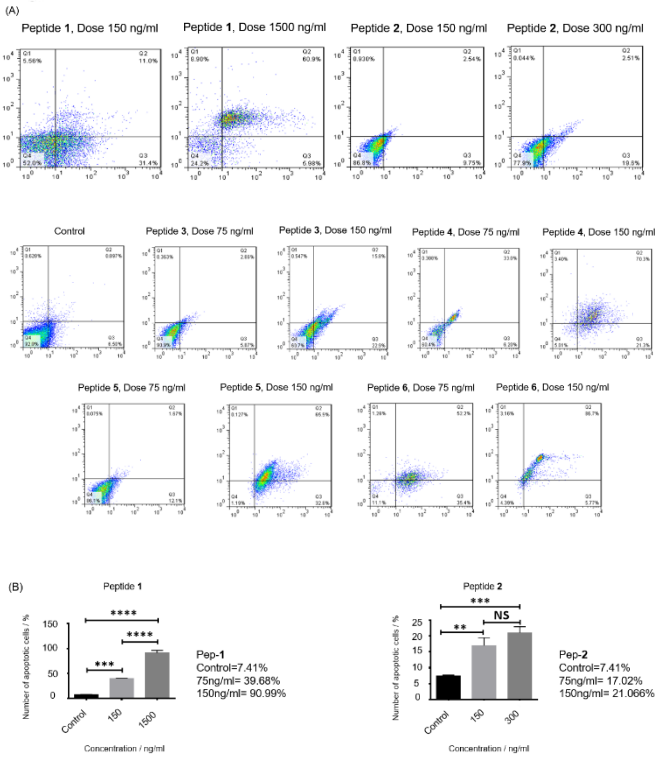


Figure 3. Effects of anti-angiogenic peptides on HUVECs proliferation. HUVECs were treated with different concentrations of RGD (peptide **1**): 150,1500 ng/mL; FAKLF (peptide **2**): 150, 300 ng/mL; and other peptides (peptide **3**, **4**, **5**, **6**): 0.75 and 150 ng/mL for 48 h. Cell proliferation was then determined by the MTT assay. The analysis was performed using Prism 6, One-way ANOVA; ± SEM. The results are representative of six independent experiments. Significant differences of each parameter between control and each concentration of peptides represent by ns, not significant, *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.

Apoptosis/Necrosis Analysis

To explore whether the inhibitory effects of the peptides on endothelial cells was attributed to the induction of apoptosis, HUVECs were treated with the peptides at two doses below IC_{50} obtained from the MTT assay (RGD: 150, 1500 ng/mL; FAKLF: 150, 300 ng/mL; and other peptides: 0.75 and 150 ng/mL) for 48 h. The disruption of cell membrane phospholipid asymmetry, evidenced by phosphatidylserine externalization, was identified using Annexin V-FITC/propidium iodide (PI) double staining flow cytometry. As shown in Figure **4** (**A, B**), the number of the apoptotic cells in peptide-treated groups were significantly increased compared to the untreated control group in a dose-dependent manner. Treatment with the peptides resulted in a significant increase in apoptosis of endothelial cells (Peptide **1**: 40.0 % at 150 ng/mL and 91 % at 1500 ng/mL; Peptide **2**: 17.0 % at 150 ng/mL and 21.1 % at 300 ng/mL; Peptide **3**: 11.8% at 75 ng/mL and 45.9 % at 150 ng/mL; Peptide **4**: 47.8 % at 75 ng/mL and 86.6 % at 150 ng/mL; Peptide **5**: 10.9 % at 75 ng/mL and 91.0 % at 150 ng/mL; Peptide **6**: 74.9 % at 75 ng/mL and 95.6 % at 150 ng/mL) relative to control cells (7.41 %). Based on these results, the potential of peptides for induction of apoptosis, in accordance with their antiproliferative activity, indicated that RGD and FAKLF peptides and the peptides synthesized based on their combinations induced cell apoptosis in a dose-dependent manner followed by inhibition of proliferation of endothelial cells.



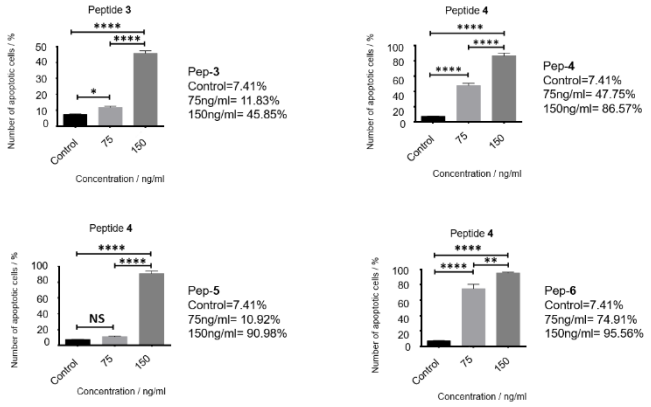


Figure 4. Induction of apoptosis by the peptides. HUVECs treated by different concentrations of peptides (peptides **3, 4, 5, 6**: 0.75 and 150 ng/mL) against RGD (peptide **1**: 150, 1500 ng/mL) and FAKLF (peptide **2**: 150, 300 ng/mL) for 48 h were identified using (A) Annexin V-FITC/PI double stained flow cytometry. (B) The number of apoptotic cells (%) in HUVECs are shown by a histogram on prism 6.0 (ns, not significant, *P≤0.05, ** P≤0.01, ***P≤0.001, ****P≤0.0001). The data are representative of six independent experiments.

Conclusions

In conclusion, this study demonstrates the design and synthesis of novel combined RGD and FAKLF peptides. Based on our functional analyses, either FAKLF (Peptide **2**) or especially RGD (Peptide **1**) showed moderate potency in inhibiting the proliferation and induction of apoptosis in endothelial cells. In comparison, the combined structures, including FAKLFRGD (Peptide **3**) and RGDFAKLF (Peptide **4**) were more effective in suppressing endothelial cells. In addition, our in vitro studies showed that incorporation of D-Leucine increased the apoptosis induction by these peptides.

Experimental Section

General Information

Commercially available materials were used without further purification. 2-Cl-Trt resin and the amino acids were purchased from Iris Biotech. *N, N*-Diisopropylethylamine (DIEA) from Sigma-Aldrich. Organic Solvents (DMF, DCM, MeOH, and CH_3CN) were purchased from Merck. RP-HPLC quality acetonitrile and water were used for RP-HPLC analyses and purification.

Peptides were synthesized manually using standard Fmoc solid-phase peptide synthesis chemistry. The amino acids were used: Fmoc-Asp (O*t*Bu)-OH, Fmoc-Arg (Pbf)-OH, Fmoc-Gly-OH, Fmoc-Phe-OH, Fmoc-Ala-OH, Fmoc-Lys (Boc)-OH, Fmoc-Leu-OH, Fmoc-D- Leu-OH.

Peptide synthesis

The RGD peptide (**1**) was synthesized following the standard Fmoc-Solid Phase Peptide Synthesis (SPPS) procedure on 2-chloro-chlorotrityl resin and using TBTU as a coupling reagent. [15] At first, Fmoc-Asp (O*t*Bu)-OH was loaded to the surface of resin using diisopropylethylamine (DIEA) and after capping with acetic anhydride other Fmoc-protected amino acids were added to the peptide sequence using TBTU as coupling reagent. Coupling reactions were performed using Fmoc-amino acid/TBTU /DIEA/. Fmoc groups were deprotected by treating the resin using 25% piperidine in DMF. To confirm the coupling of the amino acids Kaiser test was performed. After successive addition of the three amino acids in the sequence, the desired fully protected tripeptide on the surface of resin was obtained. To access the RGD peptide (**1**), the following steps were made a) the protected peptide was cleaved from the resin surface using 1% TFA. b) Then, the final deprotection was done using reagent K (TFA/TES/H_2O/MeOH 88:4.8:4.8:2.4). c) The purification of the peptide was done using preparative HPLC with column (ODS-C18, 120 × 20 mm, Eurospher 100, 7 µm) and UV detector (λ = 210 nm). The elution solvent was ACN/10 mM NaH_2PO_4 buffer. The structure of RGD (**1**) was approved using MS (ESI). Table **1** summarizes the synthesized peptides and their yields. The RGD analogues were obtained with satisfactory purities (typically > 95%), as assessed by analytical HPLC.

Table 1. The synthesized peptides and their sequences

Synthesized Peptides
Peptide **1**: H-Arg-Gly-Asp-OH (RGD) (**72**%)
Peptide **2**: H-Phe-Ala-Lys-Leu-Phe-OH (FAKLF) (**69%**)
Peptide **3**: H-Phe-Ala-Lys-Leu-Phe- Arg-Gly-Asp-OH (FAKLFRGD) (**60%**)
Peptide **4**: H-Arg-Gly-Asp- Phe-Ala-Lys-Leu-Phe-OH (RGDFAKLF) (**70%**)
Peptide **5**: H-Arg-Gly-Asp- Phe-Ala-Lys-D-Leu-Phe-OH (RGDFAK-(D-L)F) (**73%**)
Peptide **6**: H-Phe-Ala-Lys-Leu-D-Leu-Arg-Gly-Asp-OH (FAK-(D-L)RGD)(**75%**)

The molecular compositions of the synthesized peptide analogues were confirmed using high-resolution mass spectrometry HR-MS (ESI). Table 2 summarizes the results.

Table 2. The molecular compositions of the synthesized peptides

Peptides	Molecular composition
Peptide **1**	HPLC analysis found that peptide (**1**) was obtained in 98 %< purity (t_R: 5.32 min). Mass (ESI): $C_{12}H_{22}N_6O_6$ *m/z* =$[M+H]^+$ Found 347.73.
Peptide **2**	HPLC analysis found that peptide (**2**) was obtained in 98 %< purity (t_R: 27.00 min). Mass (ESI): $C_{33}H_{48}N_6O_6$m/z =$[M+H]^+$ Found 625.87.
Peptide **3**	HPLC analysis found that peptide **(3)** was obtained in 98 %< purity (t_R: 34.47 min). HR-Mass (ESI): $C_{45}H_{69}N_{12}O_{11}$m/z =$[M+H]^+$ Found 953.5215, Calc. for 953.5203.
Peptide **4**	HPLC analysis found that peptide **(4)** was obtained in 98 %< purity (t_R: 42.26 min). HR-Mass (ESI): $C_{45}H_{69}N_{12}O_{11}$*m/z* =$[M+H]^+$ Found 953.5207, Calc. for 953.5203.
Peptide **5**	HPLC analysis found that peptide **(5)** was obtained in 96 %< purity (t_R: 41.32 min). HR-Mass (ESI): $C_{45}H_{69}N_{12}O_{11}$m/z =$[M+H]^+$ Found 953.5209, Calc. for 953.5203.
Peptide **6**	HPLC analysis found that peptide **(6)** was obtained in 95 %< purity (t_R: 49. 84 min). HR-Mass (ESI): $C_{45}H_{69}N_{12}O_{11}$*m/z* =$[M+H]^+$ Found 953.5194, Calc. for 953.5203.

Material & Methods

Endothelial cell proliferation

The effects of the peptides on the proliferation of Human Umbilical Vein Endothelial Cells (HUVECs) were quantified after 48h by 3-(4,5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, Missouri, USA) assays. The absorbance was measured at 570 nm with background subtraction of 630 nm using an ELISA reader (Space Fax 2100, Awareness, USA). Briefly, 5×10^3 HUVECs were added to each well of a plate in DMEM media containing 5% FBS and incubated overnight at 37 °C. Alternatively, cells were treated with varying concentrations of peptides (100, 200, 500, 1000, 2000, 3000, 5000 ng/mL) for comparison with untreated RGD and FAKLF-treated controls for 48 h under the same conditions.

Apoptosis Assays

Apoptosis assay was performed using Annexin V/FITC microbead Kit (Miltenyi Biotec, GmbH, USA), and the fluorescence intensities of FITC-conjugated annexin-V and PI in cells were analyzed using flow cytometry (BD Biosciences Immunocytometry Systems™, USA).[16] HUVEs were cultured in medium containing different concentrations of peptides (peptides **3**, **4**, **5**, **6**: 0.75, 150 ng/mL; RGD: 150, 1500 ng/mL, and FAKLF: 150, 300 ng/mL) for 48 h, collected and washed twice with PBS, gently resuspended in 195 µL annexin V-FITC binding buffer (1x) and incubated with 5 µL annexin V-FITC in the dark for 10 min at 25 °C. The cells were then centrifuged at 3000 rpm for 5 min, gently resuspended in 190 µL annexin V-FITC binding buffer (1x) and 10 µL propidium iodide (PI) was added in an ice bath, followed by immediate analysis by flow cytometry CellQuest software (Partec GmbH). The cell distributions in flow cytometry histograms are as follow: cells in the lower right quadrant (Q3) represented early apoptotic cells; which are PI negative and Annexin positive (PI/FITC -/+), the lower left quadrant (Q4) represented live cells; which are negative to both probes (PI/FITC -/-), the upper right quadrant (Q2) represented late apoptotic cells; which are PI and Annexin positive (PI/FITC +/+) and upper left quadrant (Q1) represent necrotic cells; which are PI positive and Annexin negative (PI/FITC +/-) (Figure **4 A**). The addition of both early and late apoptotic cells (annexin V-FITC positives) was defined as the total percentage value of apoptotic cells.

Statistical analysis

All the results are represented as the mean ± SEM. One-way ANOVA followed by Tukey's post-hoc test was used to statistical significance for multiple comparisons. The Prism software (version 6.00 for Windows, GraphPad Software, La Jolla, California, USA; www.graphpad.com) was used for data analysis, for the generation of graphs, and for statistical analysis.

Acknowledgements

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Keywords: RGD peptides • peptide synthesis • apoptosis • MTT assay • *in vitro* study.

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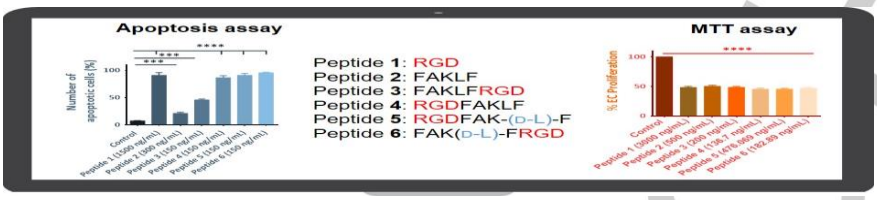

The synthesis of RGD, FAKLF and their conjugation are described. The *in vitro* study showed that the combined peptides FAKLFRGD (Peptide **3**) and RGDFAKLF (Peptide **4**) have more suppressing activity toward endothelial cells. Moreover, the incorporation of D-Leucine increased the apoptosis induction by these peptides.