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Cyclic RGD β-Lactam Peptidomimetics Induce Differential Gene Expression in Human Endothelial Cells

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Angiogenesis is a fundamental step in the transition of solid tumors from a dormant state to a malign one. Many of the low-molecular-weight anti-angiogenic drug candidates mimic the short peptide epitope Arg-Gly-Asp (RGD),[1] disrupting the extracellular matrix/integrin adhesion and, ultimately, leading to tumor cell apoptosis. In contrast to the detailed structural information available for the extracellular adhesion inhibition of endothelial cells through the recognition of integrins (typically $\alpha_V\beta_3$) by RGD peptidomimetics,[2] most aspects of possible intracellular angiogenic gene regulation caused by peptidomimetics remain unexplored.[3] Importantly, dysfunction of this signaling system is suspected to be behind the resistance phenomena developed in anti-angiogenic therapies.[4]

Inside the endothelial cell, dozens of proteins mediate or control the signaling pathways of angiogenesis after integrin activation, but only a couple of kinases (JNK, ERK) and transcription factors (NFκB, FoxO) are able to promote gene regulation[5] (Figure 1). In addition, large environmental ligands, such as vascular endothelial growth factors (VEGFs)[6] or peptide hormones,[7] are required to elicit proangiogenic gene regulation. In this context, we set out to design alternative low-molecular-weight RGD probes for interaction with $\alpha_V\beta_3$ integrin and gene regulation in HUVECs. Ideally, these peptidomimetics should: 1) contain a minimal scaffold to prevent undesired scaffold/integrin interactions, 2) have a uniform and predictable scaffold conformation, 3) bear a maximum of recognition groups, including hydrophobic or hydrophilic ones, and 4) permit the deletion of selected residues from the RGD triad without global shape change.

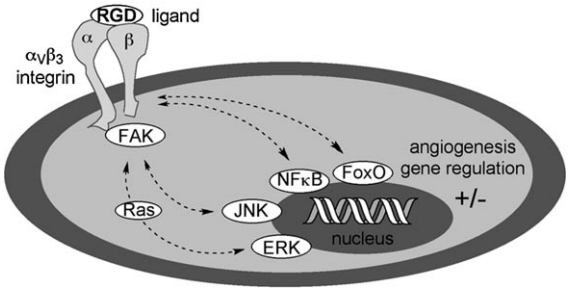

Figure 1. Signaling pathways initiated by integrins affecting the gene regulation of angiogenesis. ERK = extracellular-signal-regulated kinase, FAK = focal adhesion kinase, FoxO = forkhead box O, JNK = c-Jun N-terminal kinase, NFκB = nuclear factor κ of B-cells.

We selected five-membered and four-membered small cyclic peptides for development. The residual flexibility of these cyclopeptides can be further constrained by incorporating lactam bridges between neighboring amino acids to stabilize protein secondary structure motifs characterized by combinations of β-turns and/or γ-turns.[8] Several lactam pseudopeptides resulting from such an extension of Freidinger's concept[9] (Scheme 1; **1**→**2**) have been explored with the goals of mimicking receptor-bound conformations of bioactive peptides and of providing pharmacophore information for non-peptide drug design.[10] However, despite its apparent simplicity, this design is not always reliable for the precise positioning of a maximum number of recognition groups around the pseudopeptide cyclic core. Because the interresidual lactam bridge created by modification of the side chain (R^1) shares recognition and constraint functions, the design of mimetic libraries becomes difficult and non-general, when synthetically achievable. An alternative way to constrain cyclic peptides is based on the incorporation of a D-amino acid and an *N*-alkyl-amino acid into the macrocycle, as illustrated by the remarkable $\alpha_V\beta_3$ antagonist cilengitide (**3**, *cyclo*-[Arg-Gly-Asp-D-Phe-N(Me)Val]) developed by Kessler et al.[11]

Here we report an alternative, more versatile solution to the above design problem, by introducing α-amino-β-lactam scaffolds originating from α*C*H/N*H* proton mimicry (Scheme 2; **1**→**4**). This "β-lactam scaffold-assisted design" (β-LSAD) approach is based on the separation of recognition and constraint groups, and has previously proven efficient for promotion of unusually stable type-II (II′) β-turn peptidomimetics from extended open native peptides.[12] Accordingly, the straight application of the β-LSAD principle to RGD cyclic mimetics led us to the β-lactam pseudopeptides **4a**–**d**, which fulfilled several structural requirements to trigger quite different signaling interactions with $\alpha_V\beta_3$ integrin. The hydrophobic benzyl group in the D-Phe residue of cilengitide (**3**), for instance, was replaced by the strongly hydrophilic 1,2-dihydroxyethyl moiety in the mimetic **4a** or by the poorly hydrophobic methyl group in compound **4b**. Conversely, the hydrophobic R^1–R^3 substituents

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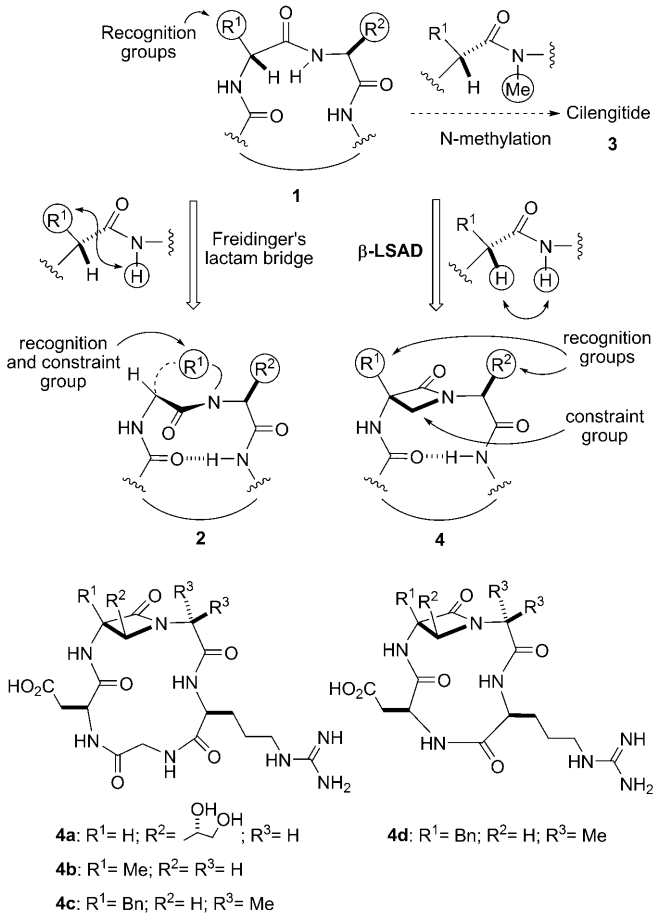
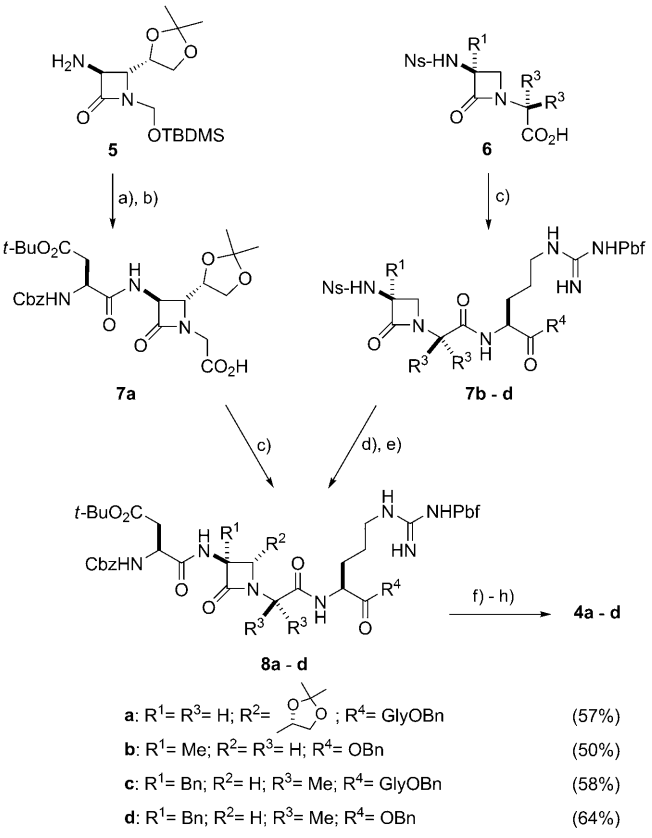


Scheme 1. Lactam pseudopeptide design in cyclic peptides. Left: Freidinger's concept: the R^1 group in **2** requires modification to fix a β-turn conformation and acts simultaneously as a recognition (functional) element. Right: β-lactam scaffold-assisted design (β-LSAD): formal insertion of a single carbon atom into **1** ($\alpha C–H+H–N\rightarrow CH_2$) mimics two hydrogen atoms and constrains the cyclic pentapeptide **4**, preserving the original R^1 group exclusively for molecular recognition. Bottom) Some β-LSAD cyclic RGD mimetics—**4a**–**d**—prepared in this work.



Scheme 2. Reagents and conditions: a) Cbz-Asp(O*t*Bu)-OH, EDC, HOBt, Et_3N, CH_2Cl_2. b) HF·Pyr, then BAIB, TEMPO, MeCN/H_2O. c) H-Arg(Pbf)R^4, EDC, HOBt, CH_2Cl_2. d) PhSH, K_2CO_3, MeCN, RT, 2 h. e) Cbz-Asp(O*t*Bu)-OH, HATU, $KHCO_3$, DMF. f) H_2/Pd-C, MeOH. g) HATU, HOAt, $KHCO_3$, DMF. h) F_3CO_2H. BAIB = [bis-(acetoxy)iodo]benzene, EDC = 3-[(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, HATU = O-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt = 1-hydroxy-7-azabenzotriazole, HOBt = hydroxybenzotriazole, Ns = 2-nosyl, Pbf = 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl, TEMPO = 2,2,6,6-tetramethyl-1-piperidinyloxyl.

were preserved in compounds **4c**–**d**, but in **4d** the central Gly residue was removed from the RGD sequence.

The cyclic integrin probes **4a**–**d** could easily be prepared from the known α-amino-β-1,3-dioxolanyl-β-lactam **5** and the α-amino-α-alkyl-β-lactams **6**[13] (Scheme 2). Compound **5** was coupled to Cbz-Asp(O*t*Bu)-OH and the *N*-*tert*-butyldimethylsilyloxymethyl moiety was then subjected to desilylation and oxidation with the BAIB/TEMPO system to provide **7a** in 54% overall yield. Conversely, coupling of the carboxylic β-lactams **6** with the protected arginines H-Arg(Pbf)GlyOBn and H-Arg-(Pbf)OBn gave the β-lactam pseudopeptides **7b**, **7c**, and **7d** in 65, 78, and 70% yields, respectively. Next, peptide coupling at the carboxylic group of **7a** and the N termini of **7b**–**d** afforded the β-lactam macrocyclic precursors **8a**–**d** in 70–84% overall yields. These compounds were deprotected under catalytic hydrogenation conditions and cyclized under high-dilution conditions, and the resulting β-lactam cyclic peptides were deprotected again with trifluoroacetic acid to provide the target compounds **4a**–**d**. The cyclization of **8d** to **4d** is noteworthy because tetrapeptides are very reluctant to afford 12-membered macrocycles.[14] A prearranged β-turned conformation of **8d** with the β-lactam ring at the (*i*+1) position would account for the easy cyclization observed in this case.[15]

NMR/MD conformation analysis of the RGD β-lactam cyclic peptides **4a**–**d** in water solution revealed the greatly prevalent formation of highly populated single clusters lacking inner hydrogen-bonded β-turns or γ-turns. This was evident from the large thermal coefficients ($|\Delta\delta/\Delta T| > -3$ ppb K^{-1}) recorded for the amide NH protons and from the ROESY interproton distance data sets (Figure 2; see pages S18–S24 in the Supporting Information for details). These observations were in line with the reported conformation of cilengitide (**3**), which also features a peptide backbone without stable hydrogen bonds ($\Delta\delta/\Delta T$ = D-Phe: −4.1; Asp: −6.1; Gly: −4.9; Arg: −9.3 ppb K^{-1}).[11] Only the mimetic **4b**, devoid of bulky substituents in the β-lactam ring, displayed a significantly low thermal coefficient (−1.2 ppb K^{-1}) for the α-amido-β-lactam NH group, consistent with the formation of a strong inverse γ-turn around the Asp residue and stabilized by the $_{(\beta\text{-lactam})}N–H\cdots O=C_{(Gly)}$ hydrogen bond. Surprisingly, though, the strong betagenicity displayed

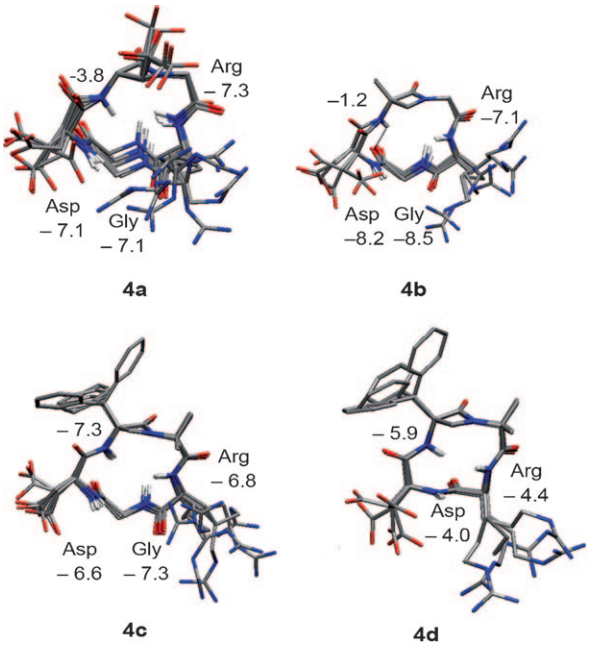


Figure 2. Five-structure overlays for compounds **4a–d** calculated from NMR interproton distance restrictions (ROESY, t_{mix} = 200 ms) determined in H_2O/D_2O (9:1). Amide thermal coefficient values are in ppb K^{-1}. All hydrogen atoms except amide NH have been omitted for clarity.

by α-amino-β-lactams in open peptides vanished in the cyclic pentapeptide and tetrapeptide counterparts, as evidenced by the absence of any $_{(Asp)}C{=}O\cdots H{-}N_{(Arg)}$ hydrogen bond in the mimetics **4a–d**. Finally, the peptide backbone strain in the β-lactam macrocycles varied with the azetidinone ring substitution position (the β-substituted compound **4c** was more rigid than the α-substituted **4a** or **4b**) and also with the number of residues (the tetrapeptide **4d** was extremely rigid). These observations confirmed the suitability of the β-LSAD design as a general scaffolding tool for positioning of different recognition groups in predefined spatial dispositions.

We next compared the antagonist affinities of the RGD β-lactam mimetics **4a–d** and of the highly active cilengitide (**3**) against $\alpha_V\beta_3$ integrin on human endothelial cells (HUVECs) by means of an adhesion inhibition assay (Figure 3).[16] All the

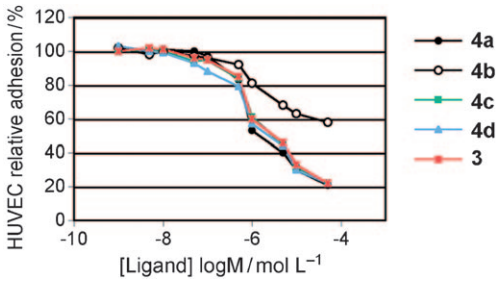


Figure 3. Adhesion inhibition of HUVECs on vitronectin-coated cell-culture plates promoted by the RGD β-lactam ligands **4a–d** and cilengitide (**3**). Adherent cells were counted after 1 h and their adhesion (%) relative to untreated control experiment is shown. HUVEC = human umbilical vein endothelial cell.

mimetics tested, except for the α-methyl-substituted compound **4b**, displayed IC_{50} values comparable to that of **3** (IC_{50} = 5 μM under the test conditions). The lower activity of **4b** was not unexpected, because the generally accepted RGD/$\alpha_V\beta_3$ integrin interaction model assumes that hydrophobic groups, such as the Bn residue of D-Phe in cilengitide, increase the affinity of the mimetic for the integrin receptor.[17] More surprisingly, the polyhydroxylic mimetic **4a** showed a slightly higher activity (IC_{50} = 1.5 μM) than cilengitide (pages S27–30 in the Supporting Information).[18] Although we cannot provide an explanation for this observation, the structural information provided by compound **4a** might be useful for the exploration of a novel, as yet unknown, hydrophilic pocket in $\alpha_V\beta_3$ integrin and its exploitation in nonpeptide mimetic design. Finally, the tetrapeptide mimetic **4d**, the product of formal deletion of the Gly residue from pentapeptide **4c**, was also a strong antagonist of $\alpha_V\beta_3$ integrin. This finding is in good agreement with the accepted model correlating the selectivity of RGD mimetics for $\alpha_V\beta_3$ integrin to a distance shorter than ≈6.0 Å between the Cβ atoms of the Asp and Arg residues.[19]

In order to study the intracellular effect of the RGD mimetics with higher affinity for integrin binding, we conducted a gene expression microarray assay. HUVECs were treated separately with a 10^{-5} M concentration of cilengitide (**3**) or of compounds **4a**, **4c**, or **4**d for 48 h. After analysis of the whole 20500 human genes, the microarray assay provided a two-color output image of normalized gene expression data (Figure 4 and pages S32–35 in the Supporting Information). Genes that were at least 1.5-fold differentially expressed on three of four arrays were scored as significant. From this transcriptomic analysis we could identify up to 225 activated genes and 64 inhibited genes for compound **3**, 221 activated and 66 inhibited for **4a**, 227 activated and 56 inhibited for **4c**, and up to 198 activated and 87 inhibited for **4d** after the RGD mimetic cell treatment. Over 90% of these genes showed a similar transcriptional pattern in cells treated with compounds **3**, **4a**, and **4c**. Less than 10% of these genes were conserved in cells treated with compound **4d**. Out of all the up- or down-regulated genes, we selected 17 genes known to be related to angiogenesis[20] for a more detailed analysis (Figure 4). After treatment with cilengitide (**3**), ten of these genes were activated and seven were blocked. The pattern of gene expression was mainly conserved (15/17 genes) for compounds **4a** and **4c** (14 of 17 genes). On the other hand, the lack of correlation in the expression of three genes (CDC7, ADAM6, and GCKR) demonstrated the highly specific "in vivo" cell activity after ligand–receptor binding.

The microarray results were further confirmed by gene-specific quantitative mRNA assays (qRT-PCR). The pattern expression of, for example, TGFBR2, TRIP12, FOXC1, and ITGA9 genes, with use of specific amplification primers and hybridization probes, was identical to that previously shown by microarray technology (pages S34–S36 in the Supporting Information). In addition, no significant changes were observed in the gene expression of the apoptosis-associated tyrosine kinase (AATK)[22] and the apoptotic regulator BAX (BL2-associated X protein)[23] genes, suggesting that apoptosis was not induced after 72 h

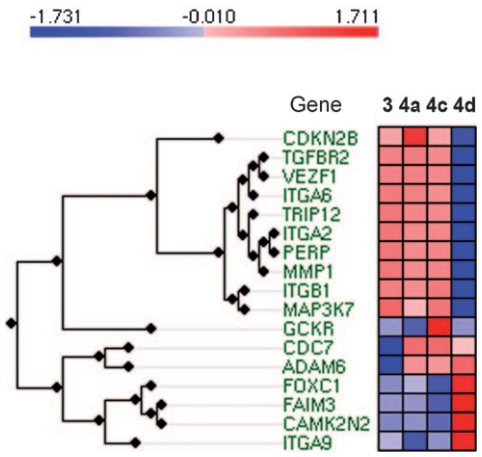


Figure 4. Angiogenesis-related gene regulation of DNA samples extracted from HUVECs after treatment with the RGD β-lactam ligands **4a**–**d** or with cilengitide (**3**). Numbers refer to a binary logarithmic scale, and colors refer to activation (red) or inhibition (blue). For gene hierarchical clustering and gene function, see page S32 in the Supporting Information.

of treatment. Surprisingly, the gene expression pattern induced after cell treatment with the cyclic tetrapeptide **4d** was almost the opposite (activation vs. inhibition) of that of cilengitide (**3**) in 15 out of 17 genes. These data suggest an "in vivo" pro-angiogenic prevalent effect of compound **4d**, which might act as an agonist ligand of the RGD receptor.[21]

In conclusion, the incorporation of α-amino-β-lactam moieties into cyclic pentapeptides and tetrapeptides prevents β-turn formation around the β-lactam residue, affording highly rigidified β-lactam cyclopeptides. This design (β-LSAD) permits the precise spatial positioning of a variety of recognition groups, as illustrated in the preparation of the cyclic RGD β-lactam mimetics **4a**–**d**. With the exception of the α-methyl-substituted mimetic **4b**, most of the RGD compounds prepared exhibited a cilengitide-like antagonist activity against $\alpha_v\beta_3$ integrin in an "in-vitro" cell assay, but they induced compound-specific intracellular angiogenesis-related gene expression. Cilengitide (**3**) and the cyclic RGD β-lactam *penta*peptides **4a**–**c** show a very similar pattern of gene expression, whereas the *tetra*peptide mimetic **4d** (with a deleted Gly residue) has the opposite effect in intracellular gene regulation. This example suggests that a very specific signaling modification of intracellular angiogenesis pathways can be achieved by using suitably designed low-molecular-weight peptidomimetics. It also outlines the potential usefulness of automated genetic tests, in combination with current adhesion assays, to establish more accurately the anti- or proangiogenic profiles of novel integrin ligands. Further studies to establish the gene-regulation effects of other glycine-deleted RGD peptidomimetics are underway in our laboratory.

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Keywords: antitumor agents · cell adhesion · gene expression · peptides · peptidomimetics

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