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# Synthetic Molecules that Protect Cells from Anoikis and Their Use in Cell Transplantation**

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***Abstract:*** *One of the major problems encountered in cell transplantation is the low level of survival of transplanted cells due to detachment-induced apoptosis, called anoikis. The present study reports on the chemical synthesis and biological evaluation of water-soluble molecules that protect suspended cells from anoikis. The synthetic molecules bind to and induce clusters of integrins and heparan-sulfate-bound syndecans, two classes of receptors that are important for extracellular matrix-mediated cell survival. Molecular biological analysis indicates that such molecules prolong the survival of suspended NIH3T3 cells, at least in part, by promoting clustering of syndecan-4 and integrin β1 on the cell surface, leading to the activation of small GTPase Rac-1 and Akt. In vivo experiments using animal disease models demonstrated the ability of the molecules to improve cell engraftment. The cluster-inducing molecules may provide a starting point for the design of new synthetic tools for cell-based therapy.*

Cell-based therapy is an exciting new field that has shown great potential in the treatment of diseases, such as diabetes, neurodegenerative diseases, and cardiovascular diseases.[1] Cell transplantation restores lost function in damaged tissues or compromised organs by replacing damaged cells with viable, functional cells. However, despite its potential benefits, the clinical application of cell transplantation remains limited. One of the major problems encountered in cell transplantation is the low level of survival of transplanted cells, which reduces both the cell number and engraftment to the recipient tissue.[2] Various strategies have been used to increase the survival of transplanted cells: pretreatment of cells with growth factors or cytokines, genetic modifications to induce overexpression of prosurvival molecules, and transplantation together with artificial or animal-derived extracellular matrixes.[3] Each approach has limitations and potential side effects, e.g., unwanted cell proliferation or differentiation, immune attack, or mechanical stress.[4] New strategies are needed to improve the survival of transplanted cells for cell-based therapies.

Transplanted cells are particularly susceptible to anoikis, a form of detachment-induced apoptosis.[5] During injection, the cells are detached from the host matrix or cell culture substrate, resulting in loss of cell-matrix signaling and, ultimately, cell death. The association of transplanted cells with the extracellular matrix, which is primarily maintained by integrins, activates survival signaling pathways.[6] Although the signals transduced by integrins play a major role in cell survival, they alone are not sufficient for survival. It is increasingly clear that two cell membrane receptors, integrins and heparan-sulfate-containing syndecans, play a synergistic role in the generation of intracellular survival signals.[7] For example, both the integrin-binding and heparin-binding domains of fibronectin, a major extracellular matrix protein, are needed to regulate and stabilize its survival activity.[8] Syndecans act as receptors for extracellular matrix proteins and soluble ligands, and can recruit integrins at the cell surface and promote their clustering and co-localization.[9] On the other hand, binding of integrins to the extracellular matrix, independently of syndecans, is required to potentiate

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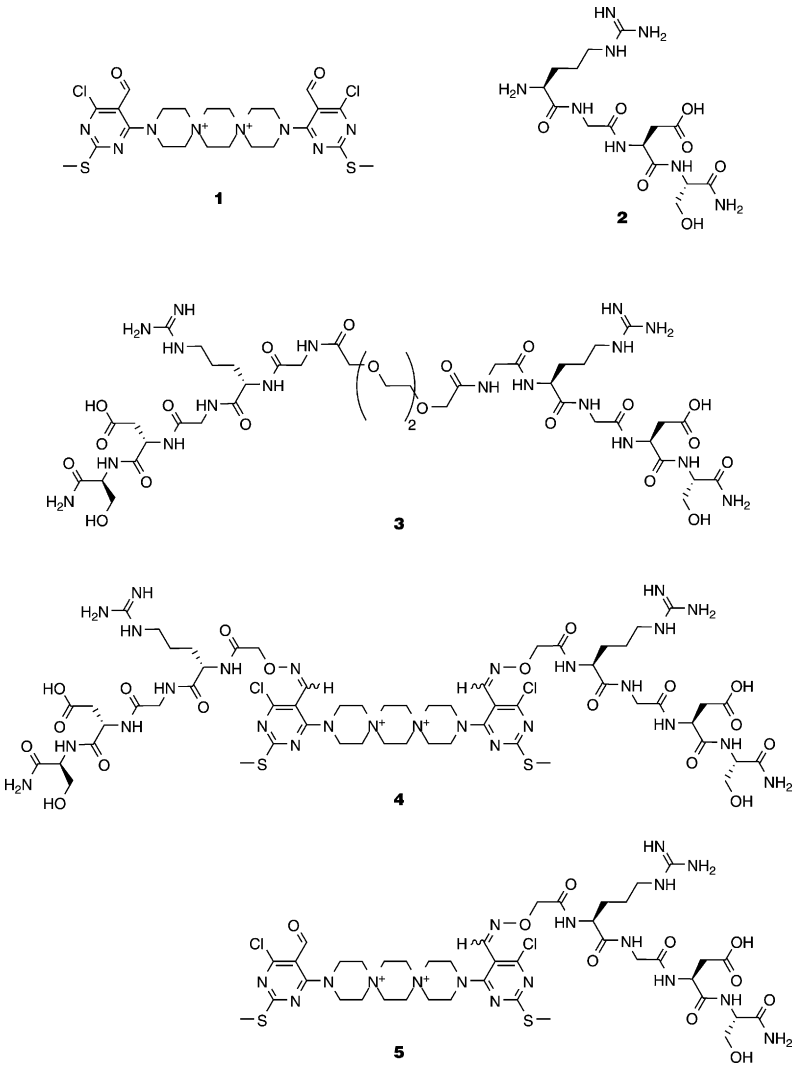
the syndecan-mediated signals.[10] Binding of the two fibronectin domains to integrin and syndecan synergistically promotes cell survival.

We took advantage of the synergistic effect of the two receptors to design a hybrid molecule that contains both integrin- and heparan-sulfate-binding/assembling modules. We introduced an RGDS peptide[11] as the integrin-binding module and adhesamine,[12] a synthetic molecule that selectively binds to and clusters the heparan sulfate of syndecans, as the heparan-sulfate-binding/assembling module. We predicted that the covalent conjugation of the highly water-soluble RGDS peptide with adhesamine would generate a synthetic soluble factor that binds simultaneously to and causes clustering of integrin and heparan-sulfate-containing syndecans, allowing suspended cells to evade anoikis. Ultimately, the higher cell viability should result in a higher engraftment efficiency and improve transplantation success. Such a synthetic molecule would also provide a relatively safe alternative for xeno-free and chemically-defined cell transplantation, which appears to be essential for the success of cell-based therapy.

Adhesamine (molecule **1**, Scheme 1) was covalently conjugated with one or two aminooxyacetic acid-functionalized RGDS peptides **2** through oxime ligation with the aldehyde groups of adhesamine, whose modifications have been shown to be well tolerated. To check if these modifications affect the ability of adhesamine to bind to heparan sulfate, we examined the affinity of molecules **4** and **5** (Scheme 1) for the heparin polymer, a model of heparan sulfate, using isothermal titration calorimetry. Molecule **5** exhibited an affinity to heparin comparable to that of adhesamine, whereas molecule **4** displayed only half of the affinity of adhesamine (Table S1 and Figure S1). Previous results suggested that the pyrimidine moieties of adhesamine stack with each other upon binding to heparin or heparan sulfate.[13] Presumably, the coupling of the bulky peptides with both of the pyrimidine moieties prevents the stacking interaction due to steric hindrance. Despite their reduced affinity, the attachment of the RGDS peptide rendered the conjugates highly water-soluble. Their solubility, in addition to their role as integrin-binding modules, makes molecules **4** and **5** suitable for inhibiting anoikis of suspended cells. The solubility in water allows the molecules to be injected together with the suspended cells, without adhering to the tubes or syringes used during cell culture and transplantation.




***Scheme 1.*** Chemical structures of molecules **1**–**5**. Counteranions (TFA or acetic acid) are omitted for clarity.

We first examined the ability of the synthetic molecules to inhibit anoikis of NIH3T3 cells, an anchorage-dependent mouse embryo fibroblast cell line often used in anoikis studies. A round-bottom, noncoated plate was used in order to reduce or remove cell-substrate interactions, cells were strained twice prior to the assay to ensure single cell suspension, and serum-free media were used throughout the assay; these are conditions which promote anoikis. After 72 hours of incubation under anoikis conditions, the viability of suspended cells was quantified by measuring NADPH dehydrogenase activity.

No detectable live cells were found in DMSO-treated (control) samples (Figure 1). As expected, detachment from the extracellular matrix and loss of signals from cell–cell interaction or soluble factors in serum resulted in cell death. In contrast, the addition of the adhesamine–RGDS conjugates (molecules **4** and **5**) significantly reduced anoikis of floating cells (Figure 1). Molecule **5** exhibited the greatest activity, rescuing approximately 100% of the cells from anoikis. Adhesamine (molecule **1**) alone displayed no significant inhibition of anoikis. Instead, this lipophilic molecule induced cell attachment to the plastic plates, as previously reported.[12] Both the RGDS peptide (molecule **2**, Scheme 1) and RGDS dimer (molecule **3**, Scheme 1) failed to inhibit anoikis or induce cell attachment (Figure 1), which is not surprising, because soluble integrin ligands usually block the receptor function rather than activate it. Even the combina-

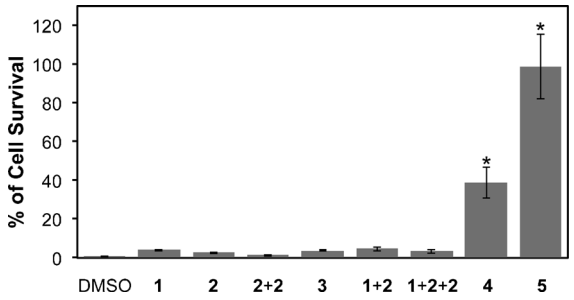



***Figure 1.*** Effects of adhesamine-RGDS derivatives on cell viability. Cell viability of suspended cells after incubation with molecules **1**–**5** (60 μM) for 72 h was determined using WST-8 assay, normalized to cells immediately after plating (100%). **2**+**2**, **1**+**2**, and **1**+**2**+**2** indicate molecule **2** (120 μM), molecules **1** and **2** (60 μM each), and molecules **1** and **2** (60 μM and 120 μM, respectively), respectively. Results from three independent experiments are shown (mean value ± standard deviation, $n=3$ for each experiment). * indicates a significant difference compared to the DMSO control ($p<0.001$).

tion of adhesamine (molecule **1**) and an RGDS peptide (molecule **2**) exhibited no significant inhibition of anoikis (Figure 1). Moreover, the addition of excess amounts of either molecule **1** or **2** impaired the activities of molecules **4** and **5** (Figure S2), suggesting that both modules of molecules **4** and **5** interact simultaneously with their receptors. These results indicate that the covalent conjugation of adhesamine with RGDS peptides is essential for preventing anoikis of suspended cells.

Time-course experiments showed that essentially all of the suspended cells in the control (DMSO) treatment underwent cell death after 48 h, while cells treated with molecules **4** and **5** had about 50 and 100% survival, respectively, after 72 h (Figure S3A). The inhibition of anoikis by molecules **4** and **5**, even at a relatively low concentration (6 μM) was greater than the inhibition by fibronectin or Y-27632, a ROCK (Rho-associated coiled-coil forming kinase) inhibitor that is often used to block anoikis (Figure S3B).[14]

We next evaluated the effect of molecules **4** and **5** on the apoptotic response of cells under anoikis conditions. The time course of annexin-V expression showed that cells alone (control) readily underwent apoptosis after 72 h (Figure S4). Treatment with molecule **4** or **5** lowered the percentages of apoptotic cells (Figure S4), consistent with the results of the cell viability assays.

The activation of caspases, in particular caspase-8, is a major initiating event in anoikis.[15] Therefore, we investigated the effect of molecules **4** and **5** on the caspase activity. Incubation of NIH3T3 cells under anoikis conditions increased the caspase activity, measured as the luminescence of caspase-8 substrate after 12 h (Figure S5A). The treatment of cells with molecule **4** or **5** inhibited the anoikis-induced activation of caspase-8. The level of inhibition was similar to the level of caspase-3 activation in cells treated with molecule **4** or **5** (Figure S5B). Caspase-3 is an effector caspase involved in the execution of apoptotic pathways, and has been reported to be upregulated in fibroblast cells undergoing anoikis.[16] These results collectively suggest that molecules **4** and **5** are directly involved in the protection of cells from anoikis.

To determine the signaling mechanism by which molecules **4** and **5** inhibit anoikis of NIH3T3 cells, we initially examined the status of the Akt- and the ERK-dependent pathways, which are signaling cascades that have established roles in anchorage-mediated survival.[17] Western blot analysis revealed that the addition of molecule **4** or **5** resulted in a clear increase in Akt phosphorylation (Figure 2A and S6A),

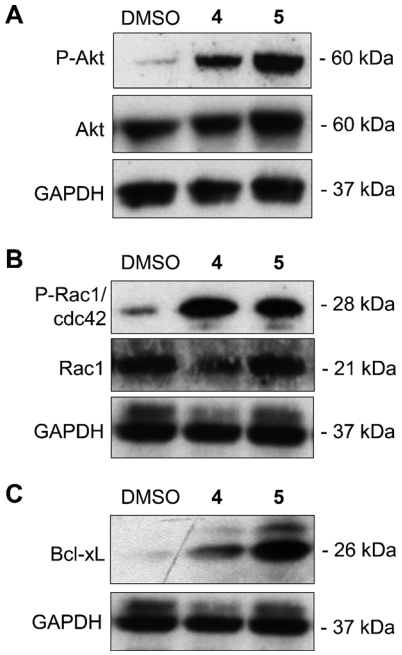



***Figure 2.*** Effects of molecules **4** and **5** on phosphorylation and expression of proteins of cells under anoikis conditions. A) Akt, B) Rac-1/cdc42, a homolog of cell division control protein 42 in the Rho family of GTPases, and C) Bcl-xL, B-cell lymphoma-extra-large. Expression of proteins after a 12 h incubation with molecule **4** or **5** was evaluated using Western blot analysis. The upper panel shows the phosphorylated form of the protein (A and B); the middle panel shows total protein (A, B, and C); and the bottom panel shows glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control (A, B, and C). Representative images from three independent experiments are shown for each protein.

but not in ERK1/2 phosphorylation (Figure S7A). Akt, serine/threonine-specific protein kinase B (PKB), plays a central role in cell survival signaling: integrin-, growth-factor- and cell–cell-mediated signal transductions converge at the activation of this kinase. Anoikis inhibition by Akt activators has been previously reported, demonstrating that Akt can suppress anoikis.[18] Our results suggest that the inhibition of anoikis by molecules **4** and **5** occurs at least partly through Akt activation.

The FAK-PI3K cascade is an upstream pathway of Akt in anchorage-mediated survival.[19] Interestingly, the addition of molecule **4** or **5** failed to increase the phosphorylation of PI3K, but upregulated the expression of total PI3K (Figure S7B). Addition of molecule **4** or **5** also failed to increase the phosphorylation of focal adhesion kinase (FAK), an even more upstream kinase,[20] but total FAK expression increased compared to the control (Figure S7C). Although the reasons

for the increased expression of PI3K and FAK by molecule **4** or **5** remain unknown, the FAK-PI3K pathway does not appear to be involved in the induced phosphorylation of Akt by molecules **4** and **5**.

The Rho family of small GTPase Rac-1 is another upstream stimulator of Akt activity.[21] Phosphorylation of Rac-1 is strongly involved in attachment-dependent cell survival, especially through syndecans.[22] The phosphorylation of Rac-1 increased in cells treated with molecule **4** or **5** (Figure 2B and S6B). The induced phosphorylation of Akt and Rac-1 supports a model in which the binding of molecules **4** and **5** to both integrin and heparan sulfate leads to the activation of small GTPase Rac-1, in turn stimulating Akt phosphorylation.

We next examined the expression levels of Bcl-xL, a key anti-apoptotic protein that is upregulated by Akt.[23] The overexpression of Bcl-xL has previously been shown to abolish anoikis. Western blot analysis showed, as previously reported,[24] that incubation under anoikis conditions down-regulated the expression of Bcl-xL in untreated cells (Figure 2C and S6C). The addition of molecule **4** or **5** resulted in a marked increase in Bcl-xL expression (Figure 2C and S6C), consistent with the stimulation of Akt phosphorylation by molecules **4** and **5**.

Previous studies demonstrated that adhesamine (molecule **1**) induces a clustering of heparan-sulfate-bound syndecans, most significantly syndecan-4, on the cell surface.[13] We used immunostaining to determine if molecules **4** and **5** promote similar clustering of syndecan-4. Suspended NIH3T3 cells incubated with molecule **4** or **5** (60 μM) for 24 h in a serum-free medium were collected and fixed immediately after re-attachment to glass plates. Microscopic observation showed that molecule **4** or **5** did promote clustering of syndecan-4 at the cell surface (Figure 3).

The addition of an RGDS moiety was expected to allow molecules **4** and **5** bind to integrins and promote their co-clustering with syndecans. Among a number of clustering combinations of syndecans and integrins, we examined that of syndecan-4 and integrin β1, which is considered to be the most important combination for the cell survival mediated by the extracellular matrix.[7] Immunostaining images of cells treated with molecule **4** or **5** did indeed show co-localization of syndecan-4 and integrin β1 at the cell surface (Figure 3). The extent of co-localization at the cell surface was quantified using Pearson's correlation coefficient (Figure S8). These results suggest that adhesamine-RGDS derivatives act as soluble ligands, whose activity promote syndecan-4 and integrin β1 recruitment to the cell surface, and induce their clustering and co-localization. Presumably, this clustering leads to an activation of the downstream signaling survival pathway that involves Rac-1 activation, leading to Akt activation and Bcl-xL upregulation.

Finally, we examined the ability of molecule **4** to enhance cell engraftment in cell transplantation procedures. Due to the potential degradation of the peptide segments in vivo, we selected molecule **4**, which is equipped with two peptides and is more readily synthesized than molecule **5**.

Molecule **4** was first tested for its ability to promote the engraftment of bone-marrow-derived cells (BMDCs) in a type 2 diabetic (db/db) mouse model, which displays delayed and poor wound healing.[25] Wound healing required 28 days or more in the control group, and was accelerated by injection of BMDCs (Figure 4). Co-injection of cells with 6 μM of molecule **4** further improved the BMDC-mediated wound healing (Figure 4 and S9). Note that the administration of

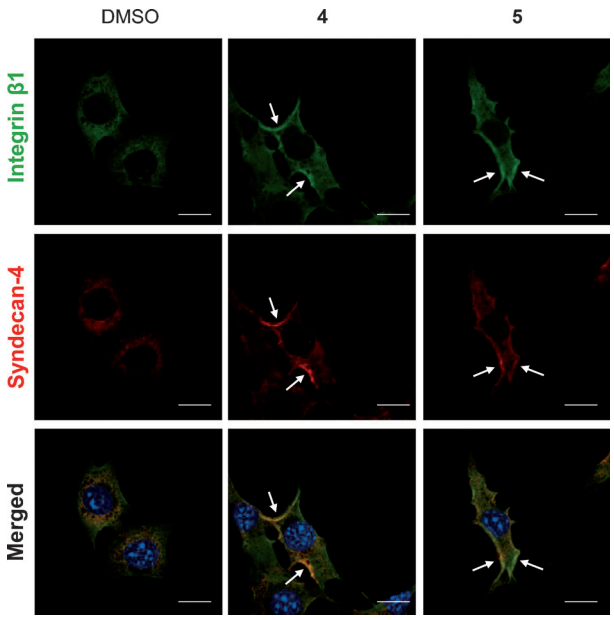



***Figure 3.*** Effects of molecules **4** and **5** on syndecan-4 and integrin β1 co-localization. Suspended cells incubated with each molecule **4** and **5** (60 μM) for 24 h in serum-free medium were collected and re-attached to glass-bottom plates for 2 h. Cells were stained with anti-integrin β1 and syndecan-4 antibodies, counterstained with DAPI, and visualized by fluorescence microscopy. The representative images for each treatment from three independent experiments are shown. (Scale bar = 10 μm)

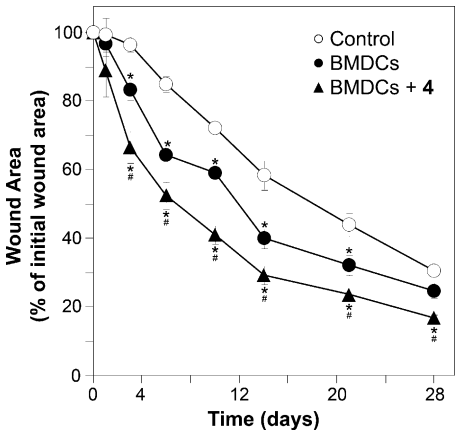



***Figure 4.*** Effects of molecule **4** on wound healing in diabetic mice. Mice received intradermal injections of BMDCs near the injury site, with or without pretreatment with molecule **4** (6 μM). Hank's balanced salt solution without cells was injected as a control. Wound size was measured from Day 0. Experiments were carried out in duplicate, and results of a representative experiment are shown, expressed as mean value ± standard deviation of 4–6 mice. $*p < 0.05$ compared to the control group; and $\#p < 0.05$ compared to the BMDCs only group.

molecule **4** alone has no significant effect on wound healing (data not shown).

The in vivo effect of molecule **4** was further investigated using a rabbit model of bullous keratopathy. The corneal endothelium maintains transparency through barrier and $Na^+$–$K^+$ transport systems. In bullous keratopathy, the dysfunction of the endothelium causes corneal cloudiness and severe visual impairment, and is usually treated by corneal transplantation. However, recent research showed that a co-injection of suspended corneal endothelial cells (CECs) with a Rho kinase inhibitor directly into the anterior chambers successfully recovered corneal transparency in rabbit and monkey models of corneal endothelial dysfunction.[26] Similarly, the co-injection of molecule **4** (10 μM) with suspended CECs in the rabbit model recovered the corneal transparency after 7 days, whereas the treatment with CECs alone exhibited hazy cornea (Figure 5). Complications related

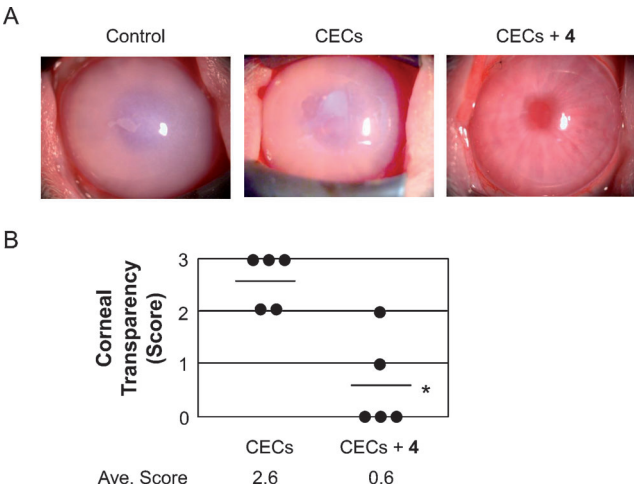



***Figure 5.*** Effects of molecule **4** on a rabbit model of bullous keratopathy. Eyes were injected with cultivated rabbit corneal endothelial cells (CECs), with or without molecule **4** (10 μM). No treatment group served as control. A) Photographs showing corneal transparency, and B) quantitative assessment of corneal transparency, one week after injection. Corneal transparency scores: 0 = clear cornea with iris details clearly visualized; 1 = partial obscuration of the iris details; 2 = iris details poorly seen with pupil margin just visible; 3 = complete obscuration of iris and pupil details. * indicates significant difference ($p < 0.01$) between mean scores of rabbits treated with molecule **4** and CECs versus CECs only ($n = 5$ for each group).

to cell injection therapy, such as the abnormal deposition of injected cells and elevation of intraocular pressure, were not observed during the experiments (Figure S10). CECs co-injected with molecule **4** covered denuded Descemet's membrane and exhibited hexagonal morphology, whereas CECs injected without molecule **4** failed to cover the Descemet's membrane 3 h past injection (Figure S11A). After 7 days, CECs injected with molecule **4** displayed characteristic staining patterns for ZO-1 (gap junction marker) and Na/K-ATPase at the plasma membranes (Figure S11B). In addition, corneal endothelial cells expressed DiI, suggesting that the corneal endothelium was regenerated by injected CECs (Figure S11B). Endothelium treated with CECs, but without molecule **4**, exhibited a stratified fibroblastic phenotype after 14 days (Figure S11C). In contrast, CECs injection with molecule **4** regenerated the hexagonal, contact-inhibited phenotype (Figure S11C). Thus, the transplantation of cultured rabbit CECs together with molecule **4** enhanced the cell engraftment and recovery of corneal transparency without the usual complications associated with corneal transplantation. Together, the positive effects of molecule **4** on the treatment of these two disease models suggest that the synthetic molecule serves as an adjuvant for cell therapy. It would be interesting to see whether molecule **4** or its derivatives can be applied to more complex cell transplantation systems.

The present study describes the chemical synthesis and biological evaluation of soluble, hybrid molecules that target two receptors essential for transmitting survival signals—syndecans and integrins. Integrin has long been recognized as a promising drug target, particularly for cancer therapy.[27] The drug, Cilengitide, is the first clinically applied integrin antagonist that uses the RGD sequence.[28] Despite progress in the development of integrin antagonists, the only soluble small-molecule integrin agonists that have been explored to date are an allosteric inhibitor-derived agonists, which stimulates ligand binding but blocks the functions of integrins, and short peptides from tenascin-C.[29] The self-assembling hybrid molecules described herein are the first soluble, synthetic dual agonists of syndecan and integrin that are useful for anoikis inhibition and, therefore, have potential applications in cell transplant therapy.

Integrins are known to be activated by both extracellular ligand binding and intracellular signaling.[30] The extracellular ligand binding alone is not sufficient to induce the integrin clustering necessary to generate significant survival signals. It has been suggested that intracellular signals induce a conformational change in integrin, to a high-affinity state that increases ligand binding. The integrin clustering caused by molecules **4** and **5** through their RGD moieties might be potentiated by intracellular signals that are generated by syndecan-4 clustering induced by the molecules' adhesamine moieties. Consistent with our results, a recent investigation showed that Rac-1 activation, a direct consequence of syndecan-4 clustering, promotes integrin clustering, which in turn activates Rac-1, establishing a positive feedback loop.[31] Because detailed three-dimensional structures of molecules **4** and **5** are not available, it is difficult to determine the contribution of each module in eliciting the biological activity. Nevertheless, molecules **4** and **5** provide a starting point for designing synthetic tools for cell-based therapy. Spatially regulated ligation of adhesamine and RGD peptides could potentially lead to the design of synthetic molecules with even more potent activities.

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