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A Dumbbell-Shaped Small Molecule that Promotes Cell Adhesion and Growth

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SUMMARY

During an image-based phenotype screening of our chemical library, we noted a small molecule that boosts the adhesion and growth of human cells. Chemical and cell biological experiments suggest that the diaryldispirotripiperazine derivative (adhesamine) targets selective cell-surface glycosaminoglycans, especially heparan sulfate, for increasing cell adhesion and growth. The addition of adhesamine to the culture medium enables the adhesion of even floating lymphocytes to cell culture plates and the microinjection into them. Unlike poly-*L*-lysine, adhesamine induces apparently normal cell adhesion accompanied by organized actin structures and activation of focal adhesion kinase and ERK1/2 mitogen-activated protein kinases. Adhesamine may be useful as a cell-attaching reagent for cell engineering and basic cell biology.

INTRODUCTION

Interactions between cell and extracellular matrix are pivotal for the survival of adherent cells, both in vivo and in vitro (Howe et al., 2002). In vitro maintenance of adherent cells can be promoted by coating culture plates with artificial substrates, including native extracellular matrix molecules derived from either human/animal tissues or recombinant bacteria. However, many of these native molecules are not chemically defined and have the risk of contamination (Koide, 2005). Chemically defined synthetic polycationic peptides, such as poly(*L*/*D*-lysine), are also widely used for coating cell culture dishes. Such nonspecific adhesive coatings, however, may cause abnormal cell spreading, leading to cytotoxic or physiologically irrelevant outcomes (Bershadsky et al., 1996).

A synthetic molecule that promotes physiological cell adhesion would serve as a convenient reagent for a reproducible, safe, and biocompatible cell culture. Here, we report the discovery of the first, to our knowledge, nonpeptidic organic molecule whose simple addition induces apparently normal cell adhesion to culture plates. Chemical and cell biological experiments suggest that adhesamine is most likely to exert its cell-adhesion activity by interacting with selective sulfated glysosaminoglycans (GAGs) to modulate cell-surface properties and intercellular signaling pathways.

RESULTS

Discovery of Adhesamine

During an image-based phenotype screening of our chemical library (~30,000 synthetic molecules), we noted a small molecule that has an intriguing effect on human hepatoma (HepG2) cells. In the presence of the dispirotripiperazine derivative, which we named adhesamine (**1**), HepG2 cells seemed to attach more readily to culture plates (Figures 1A and 1B).

To confirm that the molecule promotes cell adhesion, reattachment of trypsinized HepG2 cells to a culture plate was examined in the presence of 0.6–60 μM adhesamine. A 3 hr incubation time was used, because HepG2 cells began to attach to the culture plate within 3 hr of seeding. The plates were washed with phosphate-buffered saline (PBS) to remove unattached cells, and adhered cells remaining on the plate were counted (Lutolf and Hubbell, 2005). The results showed that adhesamine enhances the adhesion of HepG2 cells by up to 2 fold and that the effect is dose dependent (Figure 1E).

HepG2 cells have an intrinsic propensity to adhere to plastic tissue-culture plates. Therefore, we tested adhesamine with the Jurkat cell line, human T lymphocytes that are usually cultured as floating cells. Remarkably, 30% and 60% of the Jurkat cells attached to a culture plate in the presence of 6 and 60 μM of adhesamine, respectively (Figure 1F). Removal of adhesamine by washing the plate twice with PBS detached the cells: 93% of Jurkat cells were released from the surface of a culture plate 24 hr after washing (see Figure S1 available online). The results indicate that the effect of adhesamine is reversible. It is noteworthy that adhesamine did not induce cell aggregation or cell-cell adhesion. The addition of adhesamine to the medium promoted adhesion of Jurkat cells to the plastic surface of culture plates but not to each other.

Characterization of Adhesamine-Induced Cell Adhesion

Adhesamine had no obvious cytotoxicity in the cell lines we tested, even at 60 μM, which is close to the highest possible concentration in aqueous solution, and it enhanced the growth of the cells in a dose-dependent manner up to this range of concentrations (Figures 2A and 2B and Figure S2). Although

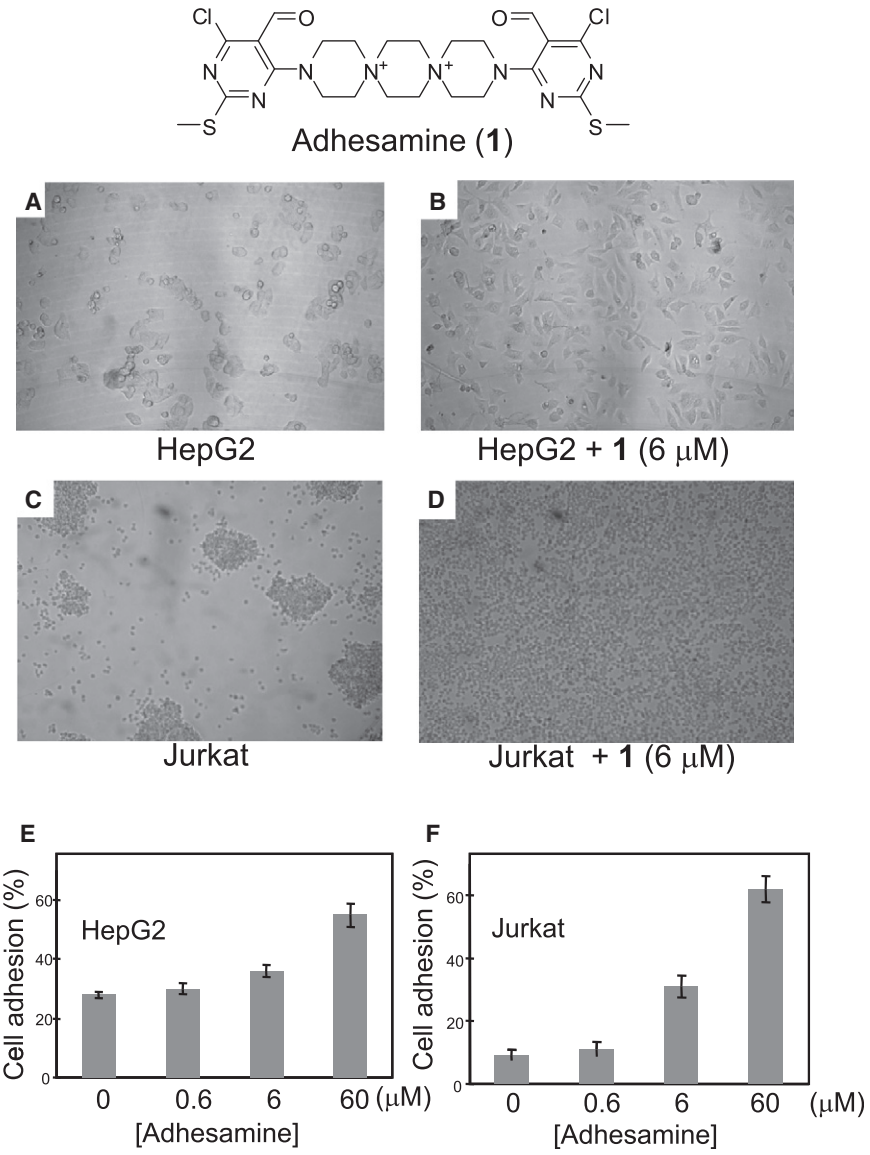

Figure 1. Promotion of Cell Attachment by Adhesamine (1)

(A and B) HepG2 cells incubated with 1% (v/v) DMSO alone (A) or 6 μM adhesamine (B).

(C and D) Jurkat cells incubated with 1% (v/v) DMSO alone (C) or 6 μM adhesamine (D).

(E and F) Adhesion rates of HepG2 cells (E) or Jurkat cells (F) when 0–60 μM of adhesamine was added to the culture media. Each point represents the mean ± SD.

60 μM of adhesamine had the highest effects on cell adhesion and growth, this concentration was too close to the solubility limit of adhesamine for practical use, and 6 μM of adhesamine exhibited clear activity in Jurkat cells. For these reasons, 6 μM was used for further investigations.

The morphology of the cells treated with adhesamine appeared normal, as well-maintained HepG2 and Jurkat cells (Figure 1). Visualization of actin cytoskeletons with rhodamine-conjugated phalloidin revealed the F-actin networks in adhered cells: actin filament bundles ran in parallel to the cell axis or through cell processes in HepG2 cells, and well-organized cortical actin structure, which is reminiscent of the survival phenotype, formed in Jurkat cells (Figure 2C and Figure S3). Although adhesamine-treated cells formed organized actin structure, those in poly-*L*-lysine-coated wells formed relatively few cytoplasmic actin fibers (Figure 2D and Figure S3). Instead, poly-*L*-lysine-treated cells showed some clumps or filamentous actin aggregates, which are often accompanied by decreased viability.

The cells were also immunostained with an antibody against vincullin, a marker of focal adhesion. Vincullin-containing focal adhesions were observed in adhesamine-treated cells immediately after seeding, when nontreated cells or those seeded onto poly-*L*-lysine-coated wells had not yet formed focal contacts (Figures 2E and 2F and Figures S4 and S5). These observations indicate that the adhesamine-induced contacts of the cells to the plates initiate a normal schedule of cell adhesion (Huo et al., 2004; Parsey and Lewis, 1993).

To further examine the adhesamine-mediated adhesion, the effects of cytoskeletal disruption were evaluated. Adhesion assays were performed after treatment with cytochalasin B or nocodazole, drugs that disrupt actin filaments and microtubules, respectively (Arroyo et al., 1992; Kinch et al., 1993). Both cytochalasin B and nocodazole inhibited the adhesamine-mediated cell adhesion in a dose-responsive manner, just as they impaired the fibronectin-mediated cell adhesion. In contrast, the two reagents had no detectable effects on the cell attachment induced by poly-*L*-lysine (Figures 2G and 2H). These data collectively suggest that adhesamine induces cell adhesion similar to that induced by native extracellular matrix.

Structure-Activity Relationship Studies

To analyze the mechanism of the adhesamine-induced cell adhesion, we conducted structure-activity relationship studies of adhesamine. One prominent structural component of adhesamine (**1**) is the positively charged, highly constrained dispirotripiperazine moiety. Substitution of the dispirotripiperazine moiety with a less rigid and tertiary amine linker, dipiperazylethane (molecule **2**), completely abolished the cell-attaching activity in Jurkat cells (Figure 3A). Two tetraammonium cations were reintroduced to **2** by methylating the two nitrogen atoms in the dipiperazylethane linker. The resulting methylpiperazium derivative **3** exhibited no activity, underscoring the importance of the fixed conformation of the positively charged dispirotripiperazine moiety.

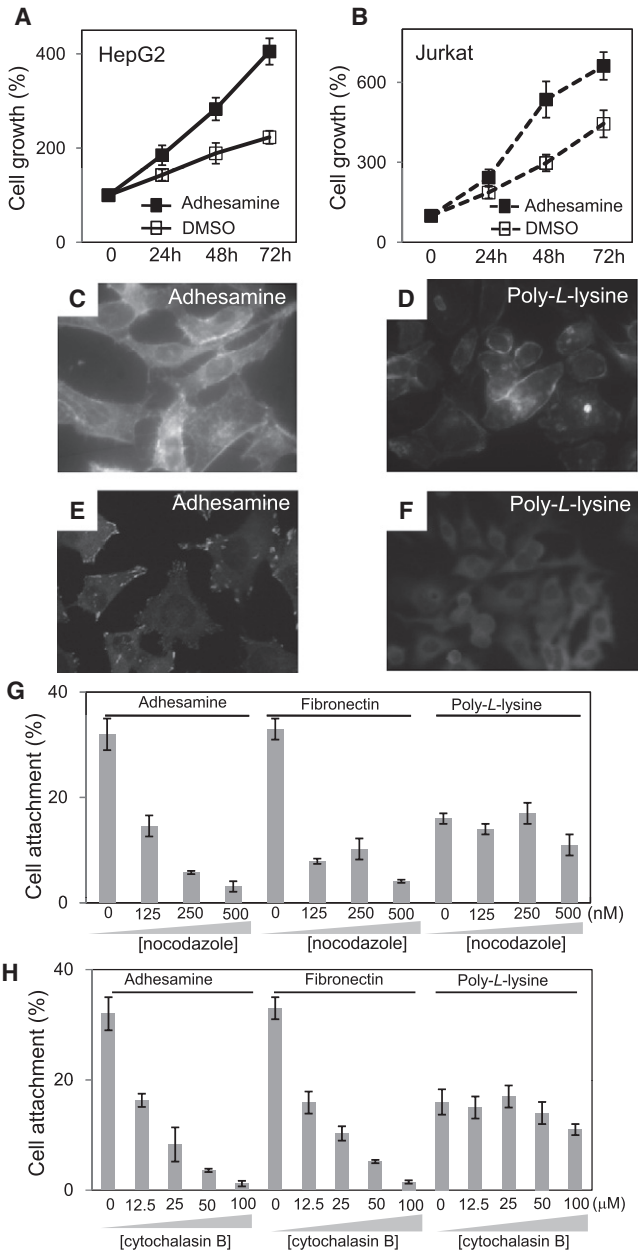

Figure 2. Effects of Adhesamine (1) on the Cell Growth and Cytoskeletal Networks

(A and B) Promotion of cell growth by **1**. The growth rates of HepG2 (A) or Jurkat (B) cells were determined 24, 48, and 72 hr after the addition of **1** (6 μM). The cell growth in the presence of 1% (v/v) DMSO was assigned as a value of 100%. The data shown are means ± SD for a minimum of three experiments.

(C–F) The rearrangement of actin fibers (C, D) and the formation of focal adhesion (E, F) in HepG2. Trypsinized HepG2 cells were plated for 3 hr on adhesamine-added or poly-*L*-lysine-coated coverslips. The concentrations of adhesamine and poly-*L*-lysine were 6 μM and 5 μg/ml, respectively. Actin cytoskeleton was visualized with rhodamine-conjugated phalloidin. Focal adhesions were visualized with anti-vinculin antibody.

(G and H) Effects of cytoskeletal disruption on adhesamine-induced cell adhesion. Jurkat cells were attached to a plastic plate by adding adhesamine in the presence of nocodazole (a tubulin toxin) or cytochalasin B (an actin toxin). Both nocodazole and cytochalasin B impaired the adhesamine-induced cell attachment, just as observed in the cells attached to fibronectin-coated plates. The toxins had limited effects on the nonspecific cell adhesion to poly-*L*-lysine-coated plates. The concentration of adhesamine (**1**) was 6 μM. The fibronectin- or poly-*L*-lysine-coated plates were prepared by pretreating wells of 96-well plates with fibronectin or poly-*L*-lysine (1 ng/well).

The other structural components of adhesamine are the two terminal pyrimidine rings. Their removal completely eliminated the effect on Jurkat cells (Figure 3B, molecule **4**), indicating that these aromatic groups are essential for activity. Reduction of the aldehyde groups in the pyrimidine rings to hydroxyl groups (molecule **5**) had no impact on activity. This lack of impact and the chemical tractability of the aldehyde group permitted the introduction of a fluorescent dansyl group at the C5 position. The dansyl conjugate of adhesamine (molecule **6**) maintained activity and served as a fluorescent probe.

Cell-Surface Glycosaminoglycans as Possible Targets

Fluorescence microscopic observation of molecule **6** revealed its localization on the cell surface (Figure 3E). In contrast, a dansyl conjugate of dispirotripiperazine (molecule **7**), which exhibited little effect on cell adhesion, showed no clear localization (Figure 3F). The cell-surface localization of molecule **6** suggested that adhesamine acts on the cell surface.

Candidates of the cell-surface targets were integrins, well-known cell-surface receptors for extracellular matrix such as fibronectin. To explore this possibility, we added to the medium an Arg-Gly-Asp (RGD) peptide, which binds to and masks the fibronectin-interacting domain of integrins, and examined its ability to squelch the activity of adhesamine. The RGD peptide reduced attachment of Jurket cells on a fibronectin-coated plate but failed to inhibit the adhesamine-induced attachment of Jurkat cells even at a high concentration (100 μg/ml) (Figure S6). It is unlikely that adhesamine exerts its activity through interacting with the fibronectin-recognition domain of integrins.

Other potential targets of adhesamine are GAGs (Schmidtke et al., 2002), negatively charged cell-surface glycans that have been reported to play an important role in cell adhesion together with integrins (Bernfield et al., 1999; Gallagher, 1989; Cole and McCabe, 1991; Gupta and Datta, 1991; Oohira et al., 2000). Cell-surface GAGs include four major components—heparan sulfate, chondroitin sulfate, keratan sulfate, and hyaluronic acid. Excess amounts of each GAG were added to the medium, and their ability to squelch the activity of adhesamine was examined (Mahalingam et al., 2007). Heparan sulfate and keratan sulfate decreased adhesamine-induced cell adhesion in a dose-dependent manner, with IC_{50} values of 0.79 and 1.03 μM, respectively; IC_{50} values are defined as the concentrations of oligosaccharides required for 50% inhibition of the adhesamine (6 μM)–induced cell attachment. Chondroitin sulfate and hyaluronic acid had no detectable effect.

We also tested heparin, a soluble GAG analogous to heparan sulfate that has more defined structures. Remarkably, the addition of this soluble GAG blocked the adhesamine-induced cell attachment better than heparan sulfate did, with an IC_{50} value of 0.27 μM.

To confirm the interactions, we performed isothermal titration calorimetric (ITC) measurements of the binding of adhesamine to each GAG (Hernaiz et al., 2002). Clear exothermic interactions were observed for heparin, heparan sulfate, and keratan sulfate

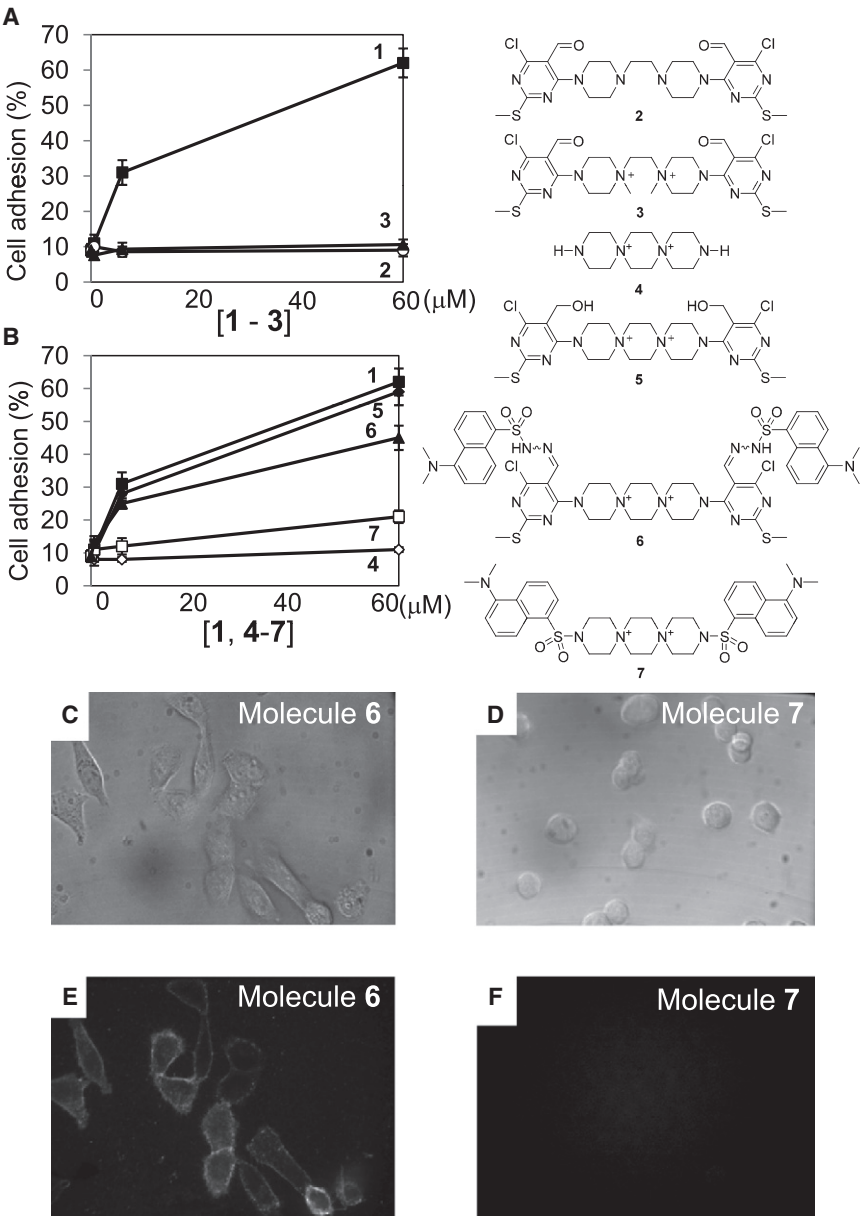

Figure 3. Adhesion-Promoting Activities of Adhesamine (1) and Its Derivatives (2–7)

(A and B) The ability of the derivatives to promote cell adhesion was evaluated with Jurkat cells.

(C–F) Subcellular localization of fluorescent probes **6** and **7**. Brightfield (C and D) and confocal (E and F) images are shown. HepG2 cells incubated with molecule **6** (6 μM, C, E) or molecule **7** (6 μM, D, F) for 3 hr after seeding. We used HepG2 cells for imaging analysis (C–F) and Jurkat cells for adhesion experiments (A, B). HepG2 cells were more suited for imaging analysis than were Jurkat cells because of their size and flatter shapes, whereas Jurkat cells were more suited for adhesion analysis than were HepG2 cells because of their nonadherent properties. Each point represents the mean ± SD.

with K_D (dissociation constant) values of 0.39, 4.67, and 5.85 μM, respectively (Table 1). Titrations with chondroitin sulfate and hyaluronic acid failed to display signals strong enough for K_D estimation. These data suggest that adhesamine interacts selectively with heparin, heparan sulfate, and keratan sulfate. The interaction with heparin is the most tight, raising the possibility that adhesamine binds to heparin-like segments of heparan sulfate on the cell surface.

For detailed analyses of the interaction, we focused on heparin as a model. Heparin is a linear polysaccharide essentially composed of repeating disaccharide units (sulfated uronic acid-*D*-glucosamine) (Gatti et al., 1979; Noti and Seeberger, 2005). To define a minimal binding unit, interactions of adhesamine with heparin oligosaccharides of various sizes (disaccharide, tetrasaccharide, hexasaccharide, octasaccharide, and decasaccharide) were examined by both squelching assays and ITC. In both experiments, the hexasaccharide displayed the highest affinity for adhesamine (Table 1). ITC results also revealed that two molecules of adhesamine bind to a hexasaccharide unit of heparin with a K_D value of 0.12 μM.

We also examined the interaction of heparin or heparan sulfate with the adhesamine derivatives that failed to promote cell adhesion (molecules **2**, **3**, and **4**). Titration with heparin or heparan sulfate generated no detectable heat release for each molecule under the same conditions as used for adhesamine (**1**). The inability of these three molecules to bind to heparin or heparan sulfate supports our model in which adhesamine promotes cell adhesion through interacting with cell-surface heparan sulfate or its heparin-like sequences.

Cell-Surface Heparan Sulfate as a Target

To confirm that cell adhesion with adhesamine is mediated by cell-surface heparan sulfate, Jurkat cells were treated with GAG-degrading enzymes. Degradation of cell-surface heparan sulfate chains with heparinase, heparitinase I, and heparitinase II (0.02 units/ml) reduced the adhesamine-induced cell attachment. In contrast, a 5-fold higher concentration (0.1 units/ml) of chondroitinase ABC had no detectable effect on the adhesamine-induced cell adhesion (Figure 4A). Digestion of heparan sulfate also affected the attachment of cells to fibronectin-coated plates, although the influence was less than it was for adhesamine-treated cells (Figure 4A). This result is consistent with the previous finding that fibronectin binds both integrins and heparan sulfate to mediate cell adhesion.

We also examined the effects of these GAG-degrading enzymes on the intact or adhesamine-enhanced attachment of HepG2 cells. The intact attachment of HepG2 cells was slightly impaired by treatment with GAGs-degrading enzymes: 13%

Table 1. Results of ITC Measurements and Squelching Assays with GAGs

GAGs	K_d (μM)	*n* (no. of adhesamine per GAGs)	IC_{50} (μM)[a]
Heparin (54 mer)	0.39 ± 0.09	20.34 ± 2.53	0.27 ± 0.05
Heparan sulfate (59 mer)	4.67 ± 0.49	6.38 ± 0.49	0.79 ± 0.19
Keratan sulfate (128 mer)	5.85 ± 4.75	33.28 ± 3.2	1.03 ± 0.11
Chondroithin sulfate (218 mer)	ND[b]	—	>120
Hyaluronic acid (600 mer)	ND[b]	—	>120
Disaccharide	ND[b]	—	>120
Tetrasaccharide	ND[b]	—	>120
Hexasaccharide	0.12 ± 0.03	2.16 ± 0.43	2.09 ± 0.13
Octasaccharide	0.45 ± 0.14	2.29 ± 0.17	2.18 ± 0.29
Decasaccharide	2.09 ± 0.14	3.42 ± 0.72	2.3 ± 0.2

Standard derivation is used for error bars. All data were collected in 50 mM phosphate buffer (pH 6.0) containing 100 mM NaCl and 1% DMSO at 25°C.

[a] IC_{50} values are defined as the concentrations of GAGs required for 50% inhibition of the adhesamine (6 μM)–induced cell attachment.

[b] Not determined because of insufficient heat release.

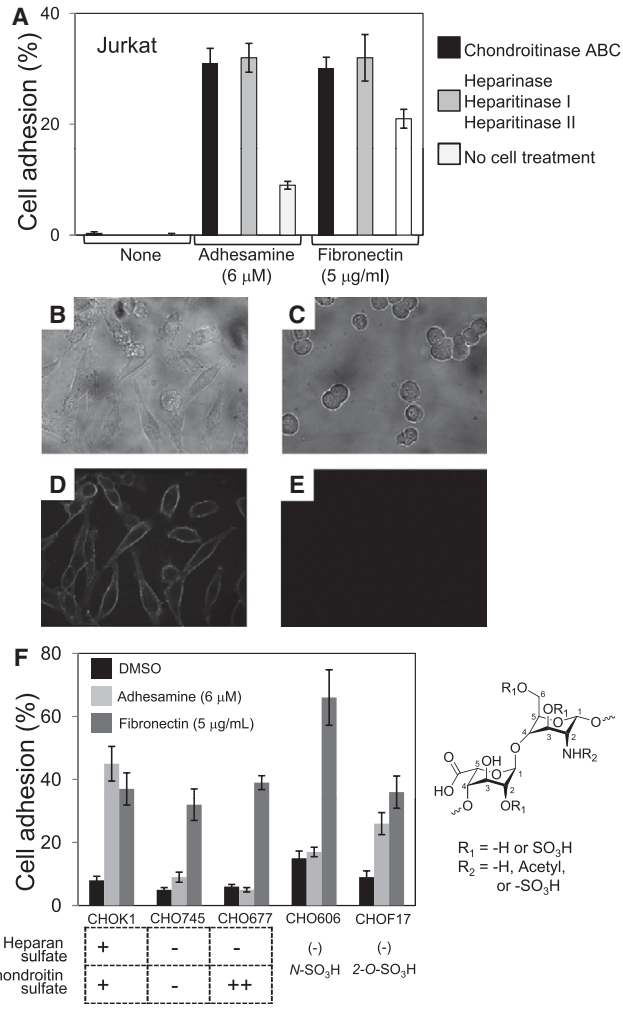

Figure 4. Validation of Cell-Surface Heparan Sulfate as a Target of Adhesamine

(A) Effects of heparan sulfate–degrading enzymes on adhesamine-induced adhesion of Jurkat cells. Cells were pretreated with 0.02 units/ml of heparinase, heparitinase I, and heparitinase II or 0.1 units/ml of protease-free chondroitinase ABC in a serum-free medium for 1 hr at 37°C before evaluating cell adhesion. Treated cells were seeded onto adhesamine-added or fibronectin-coated plastic wells, and attached cells were counted 5 hr after seeding. The concentrations of adhesamine and fibronectin were 6 μM and 5 μg/ml, respectively. Each point represents the means ± SD.

(B–E) Subcellular localization of fluorescent probe **6** in HepG2 cells treated with chondroitinase ABC (B, D) or heparinase/heparitinase I/heparitinase II (C, E). Both phase images (B, C) and confocal images (D, E) are shown.

(F) Activities of adhesamine in CHO-K1 and glycosaminoglycan-deficient mutant cell lines. Adhesion of CHO-K1 and its mutant cell lines was evaluated when 6 μM of adhesamine was added to the culture media. Cells were seeded onto adhesamine-added or fibronectin-coated plastic wells. The concentrations of adhesamine and fibronectin were 6 μM and 5 μg/ml, respectively. Attached cells were counted 5 hr after seeding. Each point represents the means ± SD. Typical structures of heparin/heparan sulfate are also shown.

and 7% inhibition was observed when cells were treated with heparinase/heparitinase I/heparitinase II (0.02 units/ml) or chondroitinase ABC (0.1 units/ml), respectively (Figure S7). As observed in Jurkat cells, adhesamine-induced cell adhesion was canceled by heparan sulfate–degrading enzymes but not by chondrotitinase ABC (Figure S7).

We also investigated the effects of the GAG-degrading enzymes on localization of the fluorescent probe of adhesamine (**6**). When cells were treated with heparan sulfate–degrading enzymes, molecule **6** was diffused from the cell surface, whereas chondroitinase treatment exhibited no impact on the cell-surface localization of molecule **6** (Figures 4D and 4E). These results supports our notion that adhesamine induces cell adhesion through its interaction with cell-surface heparan sulfate.

To further validate the interaction of adhesamine with heparan sulfate, we used well-characterized CHO-K1 cell mutants deficient in glycosaminoglycan synthesis, including CHO 745, CHO 677, CHO 606, and CHO F17. CHO 745 cells are unable to synthesize heparan sulfate and chondroitin sulfate, and CHO 677 cells exhibit reduced expression of heparan sulfate and an increase in chondroitin sulfate (Esko et al., 1985; Zhang and Esko, 1995). Both of these cells lines were less responsive to adhesamine than were parental CHO-K1 cells (Figure 4F). These data indicate that the adhesamine-induced cell adhesion is a heparan sulfate proteoglycan-dependent process and that the absence of heparan sulfate cannot be compensated for by increasing the level of expression of chondroitin sulfate.

CHO 606 cells, which lack GlcNAc N-deacetylase/*N*-sulfotransferase-1 activity, were also less responsive to adhesamine (Figure 4D) (Bame and Esko, 1989). In contrast, 2-*O*-sulfotransferase-deficient CHO F17 cells responded to adhesamine (Figure 4D) (Bai and Esko, 1996). These results suggest that *N*-sulfate groups in heparan sulfate are important elements for the interaction with adhesamine, whereas sulfate groups at C-2 uronic acid residues are less crucial for binding.

Adhesamine Induces Phosphorylation of FAK and ERK

Cell adhesion mediated by integrins and cell-surface heparan sulfate proteoglycans generates intercellular signals that stimulate a number of nonreceptor kinases. Phosphorylation of focal adhesion kinase (FAK), the most prominent kinase activated by cell adhesion to extracellular matrix, was monitored in the presence of adhesamine. Time course analysis of FAK activation in Jurkat cells demonstrated FAK phosphorylation 5 hr after adhesamine stimulation (Figure 5A). The activation was dose dependent, and 5 μg/ml of adhesamine was sufficient to phosphorylate FAK, whereas poly-*L*-lysine failed to do so at the same concentration (Figure 5B). The adhesamine-induced phosphorylation of FAK was reduced when cells were incubated with a competitive concentration of heparin (Figure 5C). The addition of the RGD peptide, in contrast, had no detectable effects (Figure 5C). Western blots performed in parallel showed no significant change in the total FAK levels in the cell lysates (Maguire et al., 1995).

Similar results were obtained with extracellular signal-regulated kinase (ERK), a downstream kinase that is activated by FAK: ERK was phosphorylated 5 hr after incubation with adhesamine, and its activation was blocked by the addition of excess amounts of heparin (Figure 5). The activation of FAK and ERK plays important roles in cell motility, cell growth, cytoskeletal organization, and adhesion-dependent cell survival (Gilmore and Burridge, 1996; Zhao et al., 1998). The phosphorylation of these two kinases may account for the cytoskeletal-organizing and growth-promoting activities of adhesamine.

Adhesamine as a New Reagent for Cell Biology

The effect of adhesamine on cell attachment of floating Jurkat cells was compared with the effects of four commonly used plate-coating reagents: type I collagen, fibronectin, poly-*L*-lysine, and poly-*L*-ornithine. The addition of adhesamine to the medium caused significantly greater attachment of Jurkat cells than did the simple addition of the coating reagents (Figure 6A). Although the addition of the two synthetic reagents (poly-*L*-lysine and poly-*L*-ornithine) had little effect, cell adhesion was enhanced by 3–6-fold by adding 5–50 μg/ml (~6–60 μM) of adhesamine. Similar activity was observed when the plastic plate surface was precoated with adhesamine: coating with adhesamine at 10 μg/well resulted in a greater enhancement of cell adhesion than did coating with poly-*L*-lysine, fibronectin, or poly-*L*-ornithine. In this case, type I collagen had a slightly greater effect than did adhesamine (Figure 6B).

One possible application of adhesamine may be its use as a reagent that boosts microinjection, since tight attachment of cells to a plate is generally required for successful microinjection. To test this possibility, we examined whether adhesamine enables microinjection into Jurkat cells, an experiment that has been considered technically difficult because of the nonadherent characteristics of the lymphocytes. In fact, microinjection of a fluorescent dye, Alexa Fluor 594, into the cells was completely unsuccessful in the absence of adhesamine. The addition of type I collagen or poly-*L*-lysine to the culture exhibited almost no effect, and that of fibronectin improved the success rates by up to ~30% (Figure 6C). The addition of the same amounts of adhesamine, however, boosted the success rates by as much as ~80% (Figure 6C) (a video clip of the microinjection is available in the Supplemental Data). Adhesamine may serve as a synthetic reagent that enables or aids microinjection into hard-to-inject cells.

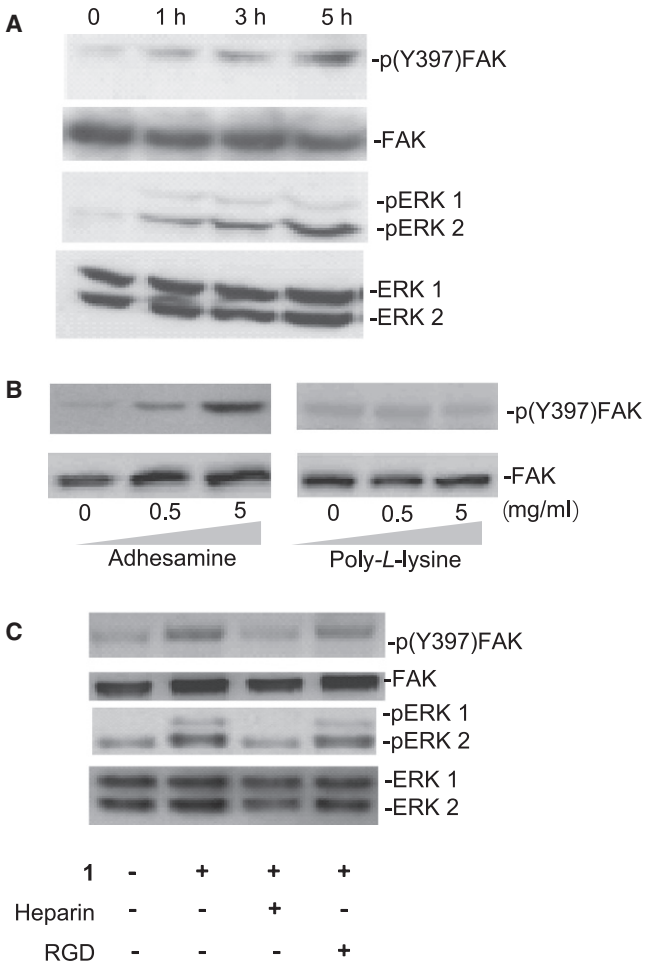

Figure 5. FAK and ERK Activation by Adhesamine

(A) Time course of FAK and ERK activation upon adhesamine treatment. Jurkat cells were treated with adhesamine (6 μM) in a serum-poor medium (0.1% FBS) for the indicated periods of time, and lysates of the treated cells were immunoblotted with antibodies against FAK phospho-tyrosine^{397} (pFAK), FAK, ERK1/2 phospho-threonine^{202}/tyrosine^{204} (pERK), or ERK.

(B) Jurkat cells were incubated for 5 hr with varied concentrations of adhesamine or poly-*L*-lysine. Cell lysates were immunoblotted with antibodies against FAK phospho-tyrosine^{397} or FAK.

(C) Effects of excess amounts of heparin or an RGD peptide (100 μg/ml) on the phosphorylation of FAK and ERK. The concentration of adhesamine (**1**) was 6 μM.

DISCUSSION

To our knowledge, adhesamine represents the first nonpeptidic organic molecule whose simple addition induces adhesion of cells to culture plates. Remarkably, adhesamine not only enhances adhesion of adherent HepG2 cells but also renders floating Jurkat cells adherent to its substrates. We tested adhesamine with a number of other cell types, including HeLa, SK-BR3, HEK293, CHO, and mouse ES cells, and found that adhesamine

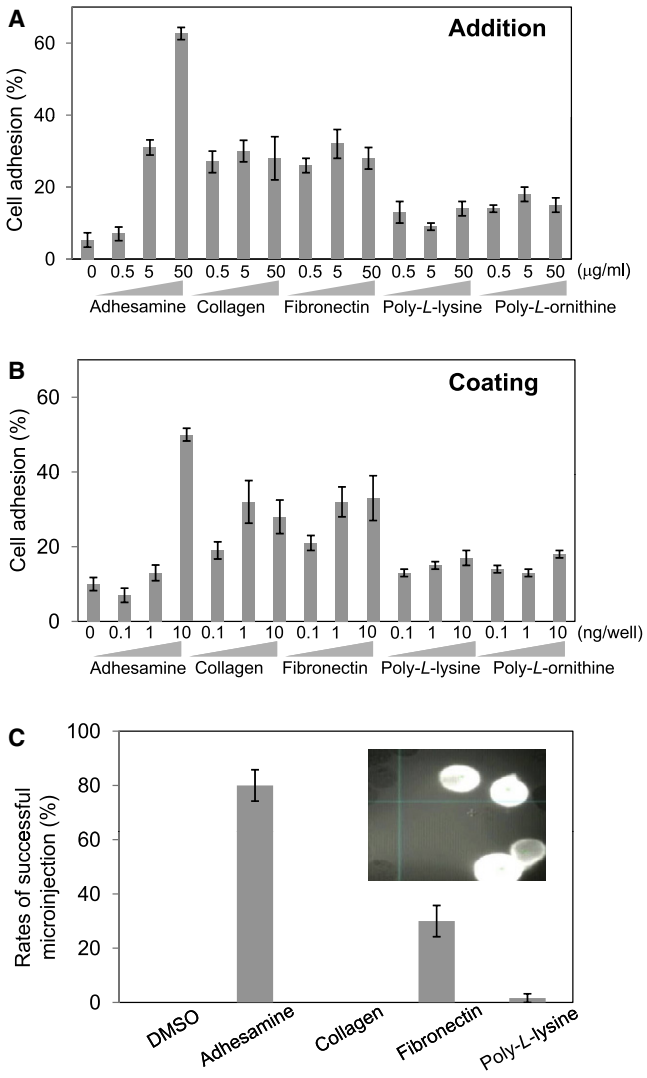

Figure 6. Utility of Adhesamine

The ability to promote attachment of Jurkat cells to plastic culture plates was compared among adhesamine (**1**) and commonly used coating materials (type I collagen, fibronectin, poly-*L*-lysine, and poly-*L*-ornithine). Adhered cells were counted when each reagent was simply added to the culture medium (A) or when the wells of plastic plates were coated with each reagent (B). The data shown are means ± SD for a minimum of three experiments. Surface area per well is 0.3 cm^2. (C) Microinjection of a fluorescent dye (Alexa Fluor 594) into Jurkat cells in the presence of adhesamine (**1**). Adhesamine increased success rates of the microinjection more than type I collagen, poly-*L*-lysine hydrochloride, and fibronectin did. The concentration of the reagents used was 5 μg/ml. Each point represents the mean ± SD.

promotes cell attachment in most cells even though different cells exhibited different levels of response.

The integrin family of cell-surface receptors has long been known to play an essential role in cell adhesion: these molecules represent the principal receptors for extracellular matrix proteins but also serve as transmenbrane bridges between extracellular matrix and actin-containing filaments of the cytoskeleton. However, it is now accepted that integrin activation is insufficient for complete cell adhesion and for maintenance of cell survival. Accumulating evidence indicates that cell-surface glycosaminoglycans mediate cell adhesion and sustained survival as well and often synergize with integrin signaling (Jeong et al., 2001; Kapila et al., 1999; Laterra et al., 1983; Schuksz et al., 2008; Sharma et al., 1999).

Chemical and cell biological analyses indicate that adhesamine binds to selected cell-surface glycosaminoglycans, especially heparan sulfate, to exert its adhesion-enhancing activity. However, it remains unclear how the interaction of adhesamine with heparan sulfate promotes cell adhesion. Adhesamine may bridge between heparan sulfate proteoglycans and plastic/glass surfaces by interacting with both of them. However, it is unlikely that adhesamine simply alters the physical properties of the cell surface through electrostatic interactions. Unlike poly-*L*-lysine, adhesamine induces apparently normal cell adhesion accompanied by organized actin structures, focal adhesion, and activation of FAK/ERK kinases. Such normal adhesion, similar to that of extracellular matrix, is usually induced by clustering of integrins and heparan sulfate. One possibility is that the dumbbell-shaped molecule acts as a bridge among heparan sulfate proteoglycans, leading to the clustering of heparan sulfate proteoglycans.

The discovery of a heparan sulfate–binding small molecule is not entirely new. It has recently been reported that surfen, a small molecule originally used as an excipient for the production of depot insulin, binds to heparan sulfate (Schuksz et al., 2008). This aminoquinoline derivative acts as an antagonist of heparan sulfate and heparin and thereby inhibits heparan sulfate–mediated cell attachment. In contrast, adhesamine behaves like an agonist of heparan sulfate, enhancing heparan sulfate–mediated cell attachment. An important implication is that it is possible to positively and negatively control heparan sulfate by small molecules. Comparison of these two molecules would facilitate mechanistic analysis of adhesamine and its analogs.

SIGNIFICANCE

Although further studies are needed for a complete understanding of the mechanism of action, adhesamine may serve as a starting point for designing chemical tools to investigate the roles of cell-surface glycosaminoglycans. This new molecule also has potential for use as a reagent for basic cell biology and cell engineering. Naturally derived, extracellular matrix components, such as fibronectin and collagen, have been extensively used as cell-adhesion matrices for hard-to-culture cells and have contributed significantly to bioengineering and basic cell biology research. The drawbacks of such animal-derived materials include the potential risk of disease transmission, relatively low purity, and poor reproducibility and stability (Koide, 2005). These limitations can be overcome by the use of synthetic materials. In fact, synthetic polycationic materials and, more recently, biomimetic materials, including those utilizing RGD peptides, antibodies, and carbohydrate, have been developed and widely used to promote cell adhesion (Hersel et al., 2003; Kobayashi et al., 1986). However, non-peptidic, completely organic molecules that promote normal cell adhesion and growth have never been reported, to our knowledge. Adhesamine or its derivatives may

provide an additional framework to complement existing naturally derived or peptidic materials.

EXPERIMENTAL PROCEDURES

Materials

Compound **4** (3,12-diaza-6,9-diazoniadispiro-(5,2,5,2)hexa-decane) was obtained as dibromide salt from Sigma-Aldrich. The TFA salts of compounds **1–7** were dissolved in DMSO before being diluted to working concentrations for the cell-adhesion assays and ITC experiments. Type I collagen from salmon skin and fibronectin from bovine plasma (Wako Pure Chemicals), poly-*L*-lysine hydrochloride (Peptide Institute. Inc.), and poly-*L*-ornithine hydrobromide (MP Biomedicals, Inc.) were dissolved in Milli-Q water and stored at −20°C until required for use. Heparin (average molecular mass [Mw], 18.0 kDa) and heparan sulfate (Mw, 13.6 kDa) were purchased as sodium salts from MP Biomedicals Inc. and Celsus, respectively. Chondroitin sulfate A (Mw, 37.5 kDa), keratan sulfate (Mw, 30.0 kDa), hyarulonic acid (Mw, 125.0 kDa), heparinase, heparitinase I, heparitinase II, and chondroitinase ABC were obtained as sodium salts from Seikagaku Corporation. Rhodamine phalloidin and Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG) were obtained from Invitrogen. Monoclonal anti-vinculin antibody was obtained from Sigma-Aldrich. Nocodazole and cytochalasin B were obtained from Wako Pure Chemicals. Rabbit anti-FAK (C-20) antibody and rabbit anti-ERK (C-16) antibody were purchased for Santa Cruz Biotechnology. Mouse anti-FAK (phospho-tyrosine397) antibody was obtained from BD Biosciences PharMingen. Rabbit anti-ERK1/2 phospho-threonine^{202}/tyrosine^{204} (pERK) antibody was purchased from Cell Signaling Technology. Anti-rabbit and anti-mouse IgG (horseradish peroxidase-linked, whole antibody) and ECL Plus Western blotting detection reagents were purchased from GE Healthcare. Synthetic RGD-specific peptide (GRGDTP) was from Sigma.

Synthesis of Compounds 1–3, 5–7

Full experimental procedures and compound characterization data are provided in the Supplemental Data.

Cell Culture and Small Molecule Screen

HepG2 cells were cultured in MEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C under humidified 5% CO_2. Jurkat cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C under humidified 5% CO_2. For the small molecule screen, HepG2 cells (2×10^5 cells/ml) were plated into 96-well plates and were treated individually with a 20 μg/ml compound in 100 μl of the culture medium. After 3, 16, and 24 hr incubation, the cells were subjected to microscopic observation. CHO K1 cells and CHO mutant cell lines were maintained in Ham's F-12 medium supplemented with 7.5% fetal bovine serum.

Cell Adhesion Assay

Addition

One microliter of adhesamine (1), Type I collagen, poly-*L*-lysine hydrochloride, poly-*L*-ornithine hydrobromide, and fibronectin was added to 96-well plate at a concentration of 50, 500, and 5000 μg/ml. HepG2 cells were suspended in the growth medium, and 100 μl of cell suspension was added to each well at a density of 4×10^5 cells/ml. After 3 hr incubation at 37°C, nonadherent cells were removed by washing with PBS three times, and the attached cells were counted with a Neubauer counting chamber (Digital Bio) after trypsinization. For Jurkat cells, 100 μl of cell suspension in the culture medium was added to each well at a density of 1×10^6 cells/ml. After 5 hr of incubation at 37°C, nonadhered cells were removed by washing with PBS twice, and the attached cells were counted with a Neubauer counting chamber. For CHO wild-type and mutant cell lines, 100 μl of trypsinized cell suspension in the culture medium was added to each well at a density of 2×10^5 cells/ml. After 5 hr of incubation at 37°C, nonadhered cells were removed by washing with PBS twice, and the attached cells were counted with a Neubauer counting chamber. To calculate adhesion rates, the cell numbers before washing were assigned as a value of 100%. Each assay was conducted in triplicate, and the means and standard derivations were calculated for a minimum of three independent experiments.

Coating

The working solutions of adhesamine (1), Type I collagen, poly-*L*-lysine hydrochloride, poly-*L*-ornithine hydrobromide, and fibronectin were prepared by diluting with PBS containing 1% DMSO. One hundred microliters of each solution (1, 10, and 100 μg/ml) was added to 96-well plates and incubated overnight at 4°C. One hundred microliters of cell suspension in a culture medium was added to each well at a density of 1×10^6 cells/ml. After 5 hr of incubation at 37°C, nonadhered cells were removed by washing with PBS twice, and attached cells were counted with a Neubauer counting chamber.

Squelching Assay

Adhesamine (**1**, 0.6 nmol) and each GAG (heparin, heparan sulfate, chondroitin sulfate, keratan sulfate, hyaluronic acid, and heparin oligosaccharides) ranging from 0.006 to 12 nmol were preincubated in 10 μl of 50 mM phosphate buffer (pH 6.0) containing 100 mM NaCl and 1% DMSO by stirring with a vortex mixer at room temperature for 30 min. These mixtures were transferred to 96-well plates, and 90 μl of Jurkat cell suspension (1×10^5 cells/ml) in growth culture medium was added to each well. After 5 hr of incubation at 37°C, nonadherent cells were removed by washing with PBS three times, and the attached cells were counted with a Neubauer counting chamber. IC_{50} values are defined as the concentrations of GAGs required for 50% inhibition of the adhesamine (6 μM)–induced cell attachment. The results of the squelching assays were plotted as percentage of inhibition versus log [GAG], and IC_{50} values were determined from curve fits of multiple independent experiments.

Isothermal Titration Calorimetry

ITC experiments were performed at 25°C using a MicoCal VP-ITC microcalorimeter. Adhesamie (**1**, 15 μM) and its derivatives of (**2**, **3** and **4**, 15 μM) were titrated with 25 × 10 μl injections of GAGs (2.7 mM of heparan sulfate, heparin, chondroitin sulfate, hyaluronic acid, and heparin oligosaccharides). The titrations were performed in 50 mM phosphate buffer (pH 6.0) containing 100 mM NaCl and 1% (v/v) DMSO. The concentrations of GAGs are given as the concentrations of their disaccharide units.

Cell Detachment Assay

One microliter of DMSO solution of adhesamine (**1**) (6 mM) was dispensed to each well on a 96-well plate. Jurkat cells were suspended in the growth medium, and 100 μl of cell suspension was added to each well at a density of 1×10^6 cells/ml. After 5 hr of incubation at 37°C, the medium was aspirated, and the plates were washed twice with PBS; 100 μl of fresh medium was added to each well. After further 4, 8, 14, and 24 hr incubation at 37°C, nonadhered cells were removed by washing with PBS twice, and the attached cells were counted with a Neubauer counting chamber. To calculate adhesion rates, the cell numbers before washing were assigned as a value of 100%. Each assay was conducted in triplicate, and the means and standard derivations were calculated for a minimum of three independent experiments.

Subcellular Localization of 6 and 7

HepG2 cells were seeded on a 96-well microplate (Greiner) at a density of 2×10^5 cells/ml, and the cells were incubated with the solution of compounds **6** or **7** for 3 hr at 37°C at the concentration of 6 μM. After incubation, the medium was replaced with fresh growth medium, and the cells were then subjected to fluorescent microscopic observation. Fluorescence images of the cells were captured by a Yokogawa CSU22 confocal fluorescence microscope with 405 nm laser excitation.

Enzymatic Treatment of Cells

Jurkat cells (5×10^4) were cultured in 100 μl of RPMI and treated with a combination of heparinase, heparitinase I, and heparitinase II (0.02 units/ml) or chondroitinase ABC (0.1 units/ml) for 30 min at room temperature. The ability of the treated cells to respond to adhesamine was evaluated by counting the cells that were attached to plastic wells of a 96-well plate in the presence of adhesamine (6 μM).

Cell Growth Assay

One microliter of DMSO solution of adhesamine (**1**) was added to a 96-well plate at a concentration of 600 μM. HepG2 cells and Jurkat cells were suspended in the growth medium, and 100 μl of cell suspension was added to each well at a density of 2×10^5 and 4×10^5 cells/ml, respectively. After incubation for the indicated time, the cell viability was measured using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Gaithersburg, MD), which

contains a tetrazolium salt (WST-8) that produces a water-soluble formazan dye by NADH dehydrogenases in living cells. Five microliters of WST-8 assay solution was added to 100 μl of the growth medium per well and was incubated for 4 hr. The optical density (OD) of the solution was read by a microplate reader, using a test wavelength at 450 nm and a reference wavelength at 650 nm. The cell viability incubated in the growth medium containing 1% (v/v) of DMSO was assigned as a value of 100%. Each assay was conducted in triplicate, and the means and standard derivations were calculated for a minimum of three independent experiments.

Visualization of Cytoskeletal Rearrangement

Jurkat or HepG2 cells were plated onto glass coverslips in the presence of adhesamine (6 μM). They were fixed after 5 or 3 hr, respectively, with 4% paraformaldehyde for 20 min. The cell membrane was permeabilized by a 5-min treatment with 0.1% Triton X-100 (in PBS). Samples were washed twice with PBS, and actin cytoskeleton was visualized with rhodamine-conjugated phalloidin according to the manufacturer's instruction (Invitrogen). The cell images were captured with a Yokogawa CSU22 confocal fluorescence microscope with 568-nm laser excitation.

Immunofluorescent Staining of Vinculin

Jurkat cells were plated onto glass coverslips in the presence of adhesamine (6 μM) and incubated for 5 hr. They were fixed with 4% paraformaldehyde for 30 min, treated with 0.1% Triton X-100 in PBS, and incubated with blocking solution (PBS containing 3% FBS) for 60 min. Blocking solution was removed, and cells were incubated overnight with anti-vinculin monoclonal antibody. Cells were washed and then were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG in blocking solution. After extensive washing, the cell images were captured with a Yokogawa CSU22 confocal fluorescence microscope with 488-nm laser excitation.

Cytoskeletal Disruption

Jurkat cells (5×10^4) were cultured in 100 μl of RPMI and were incubated with cytochalasin B (0–100 μM) or nocodazole (0–0.5 μM) for 37°C. The treated cells were then assayed for their responses to adhesamine (6 μM). Their responses to fibronectin- or poly-*L*-lysine-coated plates were also evaluated. The coated plates were prepared by pretreating wells of 96-well plates with fibronectin or poly-*L*-lysine (1 ng/well).

Western Blotting

Jurkat cells were maintained in an RPMI medium containing 10% FBS and were resuspended in a serum-free RPMI medium. Five to six million Jurkat cells were dispensed to a well of a 6-well plate in a final volume of 2 ml and then were incubated at 37°C for 0–5 hr in the presence of adhesamine or poly-*L*-lysine. Nonadherent cells were removed and pelleted, and the remaining adherent cells were lysed in a 125 mM Tris-HCl buffer (pH 6.8) containing 4% SDS, 10% glycerol, 0.006% bromophenol blue, and 1.8% β-mercaptoethanol. The adherent cell homogenate was combined with pelleted nonadherent cells from the same well, and the combined sample was boiled for 5 min. The samples were separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membranes were blocked in 1% BSA and incubated with primary antibody, and then with horseradish peroxidase–conjugated secondary antibody. The blots were developed using the enhanced chemiluminescence technique.

Microinjection

The solutions of adhesamine (**1**), Type I collagen, poly-*L*-lysine hydrochloride, and fibronectin were prepared by diluting with PBS containing 1% (v/v) DMSO. Ten microliters of each solution (1 mg/ml) was added to glass-bottom culture dishes (35 mm uncoated dish). Two milliliters of cell suspension in a culture medium was added to each well at a density of 2×10^5 cells/ml. After 14 hr of incubation at 37°C, Alexa Fluor 594 (Molecular Probes, USA) at a concentration of 5 mg/ml was injected into Jurkat cells by using a CI-2000 automated cell injection system (Fujitsu, Japan). Success rates of the microinjection were evaluated by direct observation of the fluorescent cells through a fluorescent-microscopic window of the injection system. The data shown are means ± SD for a minimum of three experiments. Twenty cells were injected in each experiment.

SUPPLEMENTAL DATA

Supplemental data include seven figures, Supplemental Experimental Procedures, and one movie and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00206-3.

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