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Rational design and synthesis of novel heparan sulfate mimetic compounds as antiadhesive agents

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Abstract—A biological evaluation of the antiadhesive activity of novel heparan sulfate glycosaminoglycans mimetic compounds (**KI**-compounds) is described. In an adhesion assay, **KI-111** [2-(4-fluoro-3-nitrobenzoyl)benzoic acetic anhydride] was found to exert potent inhibitory activities against the adhesion of human fibrosarcoma HT1080 cells and HeLa cells to fibronectin. Cell growth, migration, and invasion of HT1080 cells were also inhibited by **KI-111** at almost equal concentrations.

Heparan sulfate glycosaminoglycans (HSGAGs) are highly sulfated polysaccharides that are ubiquitous in nearly all animals. In vivo, HSGAGs exist at the cell surface and the extracellular matrix (ECM) as protein-conjugates referred to as heparan sulfate proteoglycans.[1] HSGAGs play regulatory roles in normal physiological processes (e.g., embryogenesis)[2] and pathological processes (e.g., tumorigenesis and metastasis).[3] Cell surface HSGAGs regulate signal transduction, such as that of the autocrine/paracrine loops of bFGF[4] and VEGF,[5] and also promote cell adhesion by acting as co-receptors for integrins.[6] Moreover, HSGAGs act as a physical barrier against cell invasion and as storage sheds for various proteins, such as growth factors and chemokines.[7] The *endo*-β-D-glucuronidase heparanase has been considered a major enzyme that degrades HSGAGs in mammalian tissues[8–11] and human tumors.[12]

In order to develop novel antitumor agents, as well as to dissect a variety of the pathological roles of HSGAGs, we designed novel HSGAG-mimetic compounds (**KI**-compounds) using database search techniques and molecular dynamics calculations. The design of a novel mimetic structure for HSGAGs was described previously.[13] The structure of a heparan sulfate (HS) disaccharide unit, HexUA–GlcNAc(6S) (where HexUA, GlcNAc, and 6S represent hexuronic acid, *N*-acetyl-D-glucosamine, and 6-*O*-sulfate, respectively), was used as a template. As a result of the database search and molecular dynamics calculations considering an ease of organic synthesis and Lipinski's 'rule of five',[14] 2-(3-nitrobenzoyl)benzoic acid was selected as a core structure. HSGAG-mimetic compounds (**KI**-compounds) were synthesized using 2-(4-fluorobenzoyl)benzoic acid (**KI-101**) as a starting material (Fig. 1A). The syntheses of **KI-102**–**KI-110** were previously described.[13] **KI-111** [2-(4-fluoro-3-nitrobenzoyl)benzoic acetic anhydride][15] was obtained by the nitration and acetylation of **KI-101**. **KI-111** was treated with thiophenol to give **KI-112** {2-[3-nitro-4-(phenylthio)benzoyl]benzoic acetic anhydride}[16] (Fig. 1A). Comparison of the energy minimized structures of HexUA–GlcNAc(6S) and **KI-111** showed that the direction of functional groups and the conformation of whole molecules were similar to each other (Fig. 1B).

As a result of cell-based assays against **KI**-compounds, **KI-105** has been found to exert potent inhibitory activities against migration and invasion of human fibrosarcoma HT1080 cells.[13] **KI-105** was also shown to increase the adherence of HT1080 cells and the amount of cell-surface HSGAGs and focal adhesions, and to possess a weak inhibitory activity against heparanase ($IC_{50} = 300\,\mu M$).[13] The inhibitory activities of **KI**-compounds were evaluated against the following enzymes;

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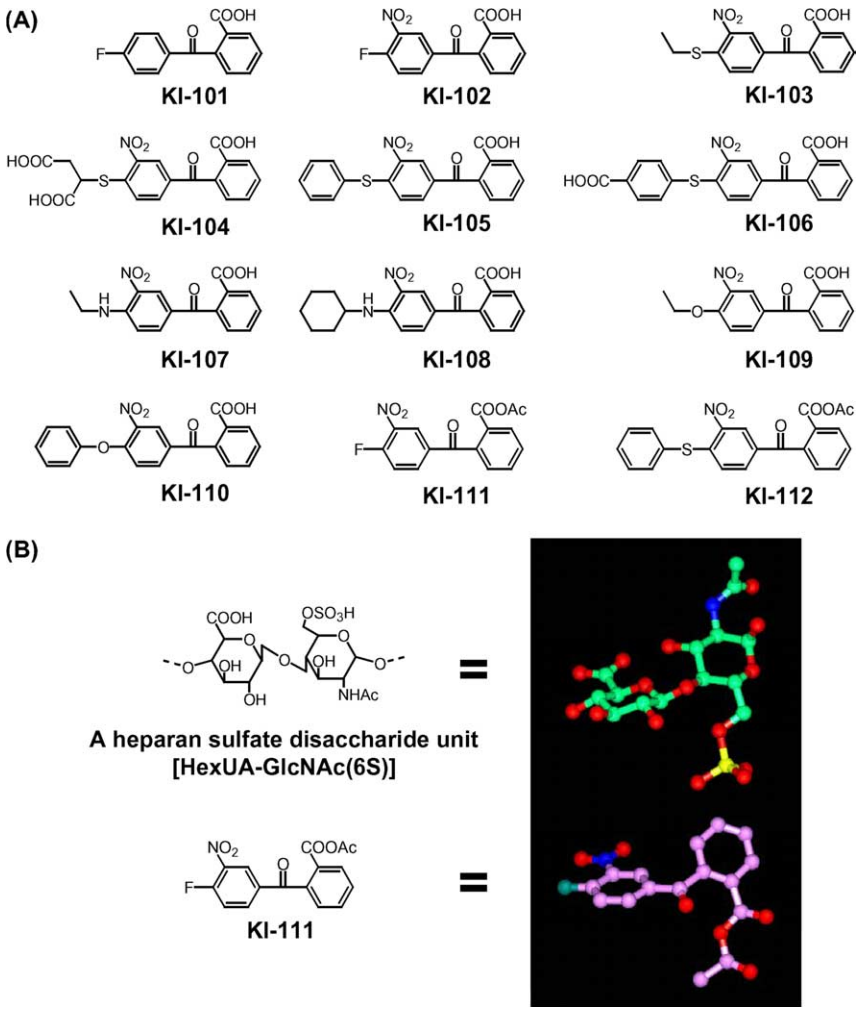

Figure 1. Novel HSGAG-mimetic compounds (**KI**-compounds). (A) Chemical structures of **KI**-compounds. (B) Energy-minimized structures of HexUA–GlcNAc(6S) and **KI-111**. The carbons of HexUA–GlcNAc(6S) and **KI-111** are represented in green and pink, respectively. Oxygen, nitrogen, sulfur, and fluorine are represented in red, blue, yellow, and blue-green, respectively. The hydrogen atoms are not shown.

coagulant factors (factor Xa, thrombin, trypsin), matrix metalloproteinases (MMPs) (MMP-2, MMP-9, the collagenase from *Clostridium histolyticum*), urokinase-type plasminogen activator, and heparanase. None of these enzymes were inhibited by **KI**-compounds at 100 μM. In this study, we further carried out a biological evaluation of the antiadhesive activity of **KI**-compounds.

The inhibitory activities against the adhesion of human fibrosarcoma HT1080 cells and HeLa cells to fibronectin were assessed using Biocoat fibronectin coat plates (Fig. 2). The RGD peptide (GRGDNP: Gly-Arg-Gly-Asp-Asn-Pro) used as a positive control inhibited the adhesion of HeLa cells, but not that of HT1080 cells. The RGD peptide has been known to interrupt the binding between integrins (e.g., α4β1 and α5β1) and the central cell-binding domain of fibronectin that contains the amino acid sequence Arg-Gly-Asp (RGD).[17–19] HS, paclitaxel, and the RGD control peptide (GRADSP: Gly-Arg-Ala-Asp-Ser-Pro; an inactive control for the RGD peptide) did not inhibit the adhesion of either type of cells to fibronectin. No **KI**-compounds except **KI-111** showed remarkable inhibitory activities against the adhesion of either type of cells to fibronectin. Only **KI-111** inhibited the adhesion of both cell types. The percentages of inhibition by 100 μM **KI-111** were 89% for HT1080 cells and 85% for HeLa cells. The target site of **KI-111** is thought to be different from that of the RGD peptide, based on the finding that **KI-111** inhibited the adhesion of both types of tumor cells.

The influence of three adhesion molecules in the ECM, fibronectin, laminin, and collagen IV, as well as the influence of plastic was assessed using dishes pre-coated with these molecules. HT1080 cells were incubated with **KI-111** at several concentrations for 1 h and adherent cells were counted. The results are shown in Fig. 3A. **KI-111** inhibited the adhesion of HT1080 cells to fibronectin and plastic more strongly than the adhesions to laminin or collagen IV. The IC_{50} values against fibronectin, plastic, laminin, and collagen IV were 7, 5, 17, and 43 μM, respectively (Table 1). Though large differences of IC_{50} values were not observed, **KI-111** inhibited the adhesion between HT1080 cells and fibronectin most effectively.

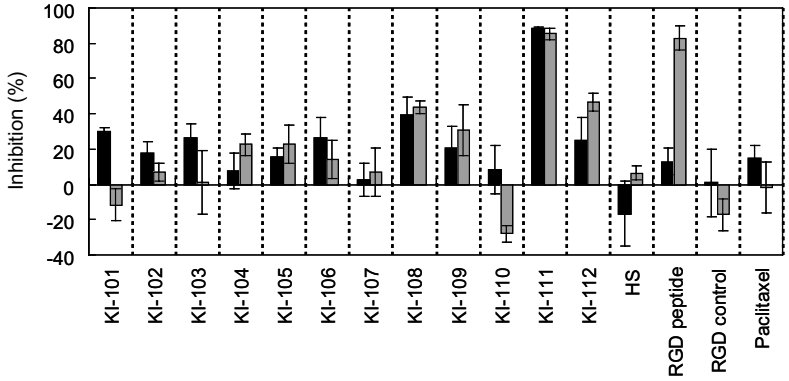

Figure 2. Antagonistic effects of **KI**-compounds in an adhesion assay. HT1080 or HeLa cells (6×10^4 cells) were incubated with **KI**-compounds (100 μM), HS (100 μM in disaccharide unit), RGD peptide (50 μM), RGD peptide control (50 μM), or paclitaxel (1 μM) for 1 h at 37 °C on fibronectin pre-coated 96-well plates. After washing the cells with PBS, adhering cells were stained with crystal violet and were counted. The results shown are the mean ± SD of three experiments. Black and hatched bars indicate the results using HT1080 and HeLa cells, respectively.

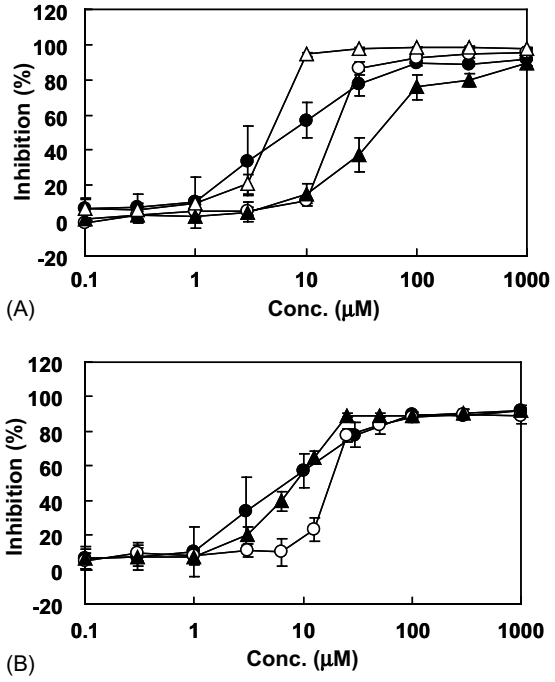

Figure 3. Dose–response curves of adhesion inhibition by **KI-111**. (A) Influence of the use of different adhesion molecules on the adhesion of HT1080 cells. HT1080 cells (5×10^5 cells) were incubated with **KI-111** for 1 h at 37 °C on human fibronectin-coated, laminin-coated, collagen IV-coated, or noncoated 35 mm dishes. The results shown are the mean ± SD of three experiments. Closed-circles, open-circles, closed-triangles, and open-triangles indicate the results of adhesion against fibronectin, laminin, collagen IV, and plastic, respectively. (B) Influence of the use of different tumor cells on the adhesion to fibronectin. HT1080, HeLa, or A431 cells (5×10^5 cells) were incubated with **KI-111** for 1 h at 37 °C on human fibronectin-coated 35 mm dishes. The results shown are the mean ± SD of three experiments. Closed-circles, open-circles, and closed-triangles indicate the results using HT1080, HeLa, and A431 cells, respectively.

The influence of different tumor cells was assessed using HT1080 cells, HeLa cells, and human epidermoid carcinoma A431 cells. All cell lines were incubated with **KI-111** on fibronectin pre-coated dishes for 1 h. Large differences were not observed (Fig. 3B). The IC_{50} values of **KI-111** against HT1080, HeLa, and A431 cells were 7, 16, and 8 μM, respectively (Table 1). **KI-111** was thought to be a broad adhesion inhibitor for various types of tumor cells.

Table 1. The IC_{50} values of **KI-111** against adhesion, migration, invasion, and growth

	IC_{50} (μM)
Adhesion (fibronectin/HT1080 cells)	7
Adhesion (laminin/HT1080 cells)	17
Adhesion (collagen IV/HT1080 cells)	43
Adhesion (plastic/HT1080 cells)	5
Adhesion (fibronectin/HeLa cells)	16
Adhesion (fibronectin/A431 cells)	8
Migration (HT1080 cells)	2
Invasion (HT1080 cells)	2
Growth (HT1080 cells)	10

There are several important functions of tumor cells in tumor progression. In addition to adhesion, tumor cell growth, migration, and invasion are very representative characteristics of tumor cells. Tumor cell growth was assessed using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium] assay[20] (Nacalai Tesque, Kyoto, Japan), and in vitro invasion/migration was assayed using a BD BioCoat Matrigel invasion chamber and cell culture insert (8 μM pore size; Becton Dickinson Labware, Bedford, MA).[21] The results are shown in Figure 4. **KI-111** inhibited not only adhesion but also growth and invasion/migration. The IC_{50} values of **KI-111** against adhesion, growth, migration, and invasion were 5, 10, 2, and 2 μM, respectively (Table 1).

The manner in which **KI-111** peeled HT1080 cells from the substrata was analyzed by immunofluorescence staining. HT1080 cells were incubated with vehicle or **KI-111** (5 or 50 μM) for 1 h, then stained with Alexa Fluor 568 phalloidin, monoclonal antibody against human vinculin, and Hoechst 33258 to visualize F-actin, focal adhesions, and nuclei (Fig. 5A). The amount of F-actin was gradually decreased as the concentration of

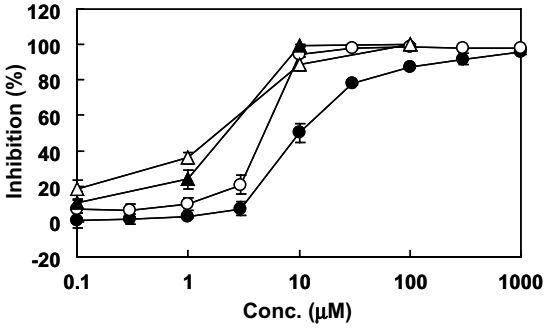

Figure 4. Inhibitory activities of **KI-111** against cell-based assays. The results shown are the mean ± SD of three experiments. Closed-circles, open-circles, closed-triangles, and open-triangles indicate growth, adhesion, migration, and invasion of HT1080 cells, respectively.

KI-111 increased (Fig. 5A-a,e,i). The number of focal adhesions also decreased (Fig. 5A-b,f,j). Nuclei were not altered by the **KI-111** treatment (Fig. 5A-c,g,k). The merged image of 5 μM **KI-111** treatment (Fig. 5A-h) showed that HT1080 cells began to peel off from the area where focal adhesions disappeared (yellow arrow).

The effect of **KI-111** on the phenotype of HT1080 cells was different from that of representative adhesion inhibitors; e.g., cytochalasin B and okadaic acid (Fig. 5B). F-actin formed large and small dots after treatment with cytochalasin B, an inhibitor of the actin polymerization[22] (Fig. 5B-a). On the other hand, intracellular small dots and pericellular filaments of F-actin were observed by treatment with okadaic acid, an inhibitor of the protein serine/threonine phosphatase type 2 (PP-2A)[23] (Fig. 5B-b). These results indicate that the antiadhesive mechanism of **KI-111** is neither actin de-polymerization nor PP-2A inhibition.

HSGAGs have been known to have regulatory roles in many biological processes of cancer, that is, tumorigenesis, tumor progression, and metastasis. Nonsaccharide-based HSGAG regulators with low molecular

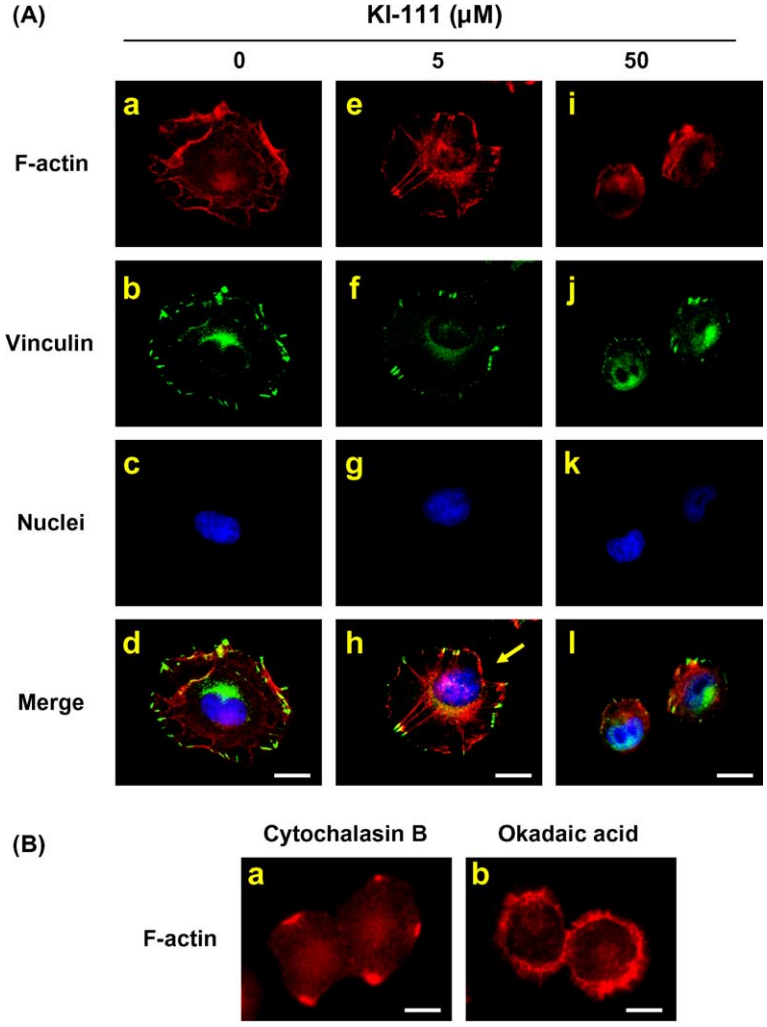

Figure 5. Effects of **KI-111** on the phenotype of HT1080 cells. (A) HT1080 cells were treated with vehicle (a, b, c, d), 5 μM of **KI-111** (e, f, g, h), or 50 μM of **KI-111** (i, j, k, l) for 1 h. F-actin (red), vinculin (green), and nuclei (blue) were stained with Alexa Fluor 568 phalloidin, antivinculin antibody, and Hoechst 33258, respectively. White bars indicate 10 μm. (B) HT1080 cells were treated with 2 μM of cytochalasin B (a) or 50 nM of okadaic acid (b) for 3 h. F-actin (red) was stained with Alexa Fluor 568 phalloidin. White bars indicate 10 μm.

weight have not been reported, whereas there have been many reports on sulfated oligosaccharide derivatives acting as HSGAG mimics.[24–28] We have previously reported on novel HSGAG-mimetic compounds (**KI**-compounds) that act as antitumor agents.[13] In this study we focused on the antiadhesive activity of these compounds. Our adhesion assay showed that a novel HSGAG-mimetic compound (**KI-111**) had potent inhibitory activity against tumor cells. **KI-111** inhibited the adhesion of three types of tumor cell lines (e.g., HT1080, HeLa, and A431 cells) to fibronectin, and also inhibited the adhesion of HT1080 cells to laminin, collagen IV, and plastic. In addition to adhesion, some other cell-based processes (growth, migration, and invasion) of HT1080 cells were inhibited by **KI-111**. The mechanism of **KI-111** is unique and different from that of the representative adhesion inhibitors, such as the RGD peptide (an inhibitor of the binding between integrins and the central cell-binding domain of fibronectin), cytochalasin B (an actin polymerization inhibitor), and okadaic acid (a PP-2A inhibitor).

Acknowledgements

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15. Synthesis of **KI-111**: A suspension of 11.0 g of 2-(4-fluorobenzoyl)benzoic acid in 30 mL of fuming HNO_3 and 30 mL of acetic acid and 30 mL of acetic anhydride was stirred at 4 °C for 1 h and at room temperature for 2 h. The reaction mixture was poured into ice-water and stirred at room temperature for 12 h. The resultant white precipitate was filtrated, washed with water, and then purified by recrystallization from acetone. Yield, 46%; mp 143–145 °C; IR (neat) ν_{max} 1793, 1773, 1540, 1347, 1198, 1185, 1094, 1036, 1000, 963 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) 2.00 (3H, s), 7.43 (1H, dd, J 9.0, 10.0 Hz), 7.55 (1H, ddd, J 1.0, 7.5, 8.0 Hz), 7.59 (1H, ddd, J 1.0, 1.0, 8.0 Hz), 7.65 (1H, ddd, J 1.0, 7.0, 7.5 Hz), 7.77 (1H, ddd, J 1.0, 1.0, 7.0 Hz), 7.90 (1H, ddd, J 2.0, 4.5, 9.0 Hz), 8.25 (1H, dd, J 2.0, 6.5 Hz); ^{13}C NMR (125 MHz, acetone-d_6) 22.1, 104.4, 120.6 (d, J_{CF} 21 Hz), 124.4, 124.6, 126.7, 127.6, 132.6, 134.2 (d, J_{CF} 10 Hz), 136.4 (d, J_{CF} 4 Hz), 136.7, 148.3, 156.9 (d, J_{CF} 263 Hz), 168.2, 169.2, 206.7; MS (FAB, Neg) m/z 331 (M^-); HRMS (FAB, Neg) $C_{16}H_{10}NO_6F$: calcd 331.0492, found m/z 331.0513 (M^-).
16. Synthesis of **KI-112**: The mixture of 3.2 g of **KI-111** and 1.1 g of thiophenol and 1.2 g of DIPEA in 10 mL of acetone was stirred at room temperature for 1 h, and was extracted with chloroform. The chloroform extract was evaporated and purified by recrystallization from acetone–hexane. Yield, 76%; mp 69–71 °C; IR (neat) ν_{max} 1789, 1775, 1520, 1212, 1063, 1050, 938 cm^{-1}; ^1H NMR (270 MHz, DMSO-d_6) 2.14 (3H, s), 6.89 (1H, d, J 8.9 Hz), 7.54–7.85 (9H, m), 7.95 (1H, d, J 7.3 Hz), 8.37 (1H, d, J 1.9 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) 21.6, 103.5, 122.7, 122.8, 125.9, 126.3, 128.7, 129.9, 130.0, 130.1, 130.3, 130.4, 131.1, 134.4, 135.1, 136.0, 141.7, 144.4, 146.6, 167.1, 167.7, 206.5; MS (FAB, Neg) m/z 421 (M^-); HRMS (FAB, Neg) $C_{22}H_{15}NO_6S$: calcd 421.0620, found m/z 421.0644 (M^-).
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