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Exploitation of Heparanase Inhibitors from Microbial Metabolites Using an Efficient Visual Screening System

KEISUKE ISHIDA[a,b], TAKAYUKI TERUYA[a,c], SIRO SIMIZU[a] and HIROYUKI OSADA[a,c,*]

[a] Antibiotics Laboratory, RIKEN Discovery Research Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
[b] Hanno Research Center, Taiho Pharmaceutical Co., Ltd., 1-27 Misugidai, Hanno, Saitama 357-8527, Japan
[c] Graduate School of Science and Engineering, Saitama University, 255 Shimo-okubo, Sakura-ku, Saitama, Saitama 338-8570, Japan

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In this paper we describe the establishment of an efficient visual method for screening heparanase inhibitors, and we present the results of screening 10,000 microbial culture broths. Heparanase-overexpressing stable clones of the human hepatocellular carcinoma HepG2 cells were established and used as an enzyme source. Digestion of heparan sulfate (HS) was detected using novel HS-containing tablets or SDS-polyacrylamide gel electrophoresis. This method was able to find suramin, a known heparanase inhibitor, from a library of typical enzyme inhibitors. By screening 10,000 culture broths of microorganisms (actinomycetes, fungi, and bacteria) an actinomycete strain, RK99-A234, was found to have heparanase inhibitory activity. RK-682 was identified in the fermentation broth as a heparanase inhibitor, $IC_{50}=17\ \mu M$.

Heparanase is an endo-β-D-glucuronidase and plays important roles in tumor metastasis[1~5]. When tumor cells spread out, they must penetrate the extracellular matrix (ECM) and the basement membrane (BM)[6]. At that time, tumor cells secrete matrix metalloproteases and heparanase to degrade major components of ECM and BM such as type IV collagen and heparan sulfate proteoglycans[5,7]. Moreover, heparanase releases several growth factors, such as bFGF and VEGF, which bind to heparan sulfate (HS) chains in ECM and BM to cause angiogenesis[8~10]. Therefore, heparanase is recognized as a successful target molecule for antitumor agents.

Although the importance of heparanase has been known for more than two decades[11~13], cloning of heparanase gene has not been achieved more recently because of the instability of the enzyme and the difficulties of detecting the enzyme activity[14~17]. Non-metastatic tumor cells acquired a highly metastatic phenotype by transfection of the heparanase gene. Heparanase consists of 543 amino acids and is classified into the new family 79 in Clan A of glycosidase classification. It is predicted that heparanase turns into an active form after the cleavage at the amino terminus and that it forms a heterodimer with a degradation fragment of 8 kDa[18].

An obstacle to high-throughput screening has hampered the search for heparanase inhibitors from a large number of natural sources or chemical libraries. Most heparanase inhibitors reported by now are derivatives of sulfated oligosaccharide similar to the substrate HS, and not low molecular weight compounds[19~22]. So we planned to establish an efficient assay method for heparanase inhibitors from microbial metabolites. First, we established heparanase-overexpressing cell lines, whose lysates were used as an enzyme source. Then a novel detection method for heparanase activity, namely, HS degradation activity, was established using an HS-containing polyacrylamide gel tablet for dealing with massive samples. After a screening from 10,000 microbial broths, RK-682[23] was identified as a potent heparanase inhibitor.

* Corresponding author: hisyo@riken.jp

Experimental

Cell Culture

The human hepatocellular carcinoma HepG2, HepG2-HP-# (# is the clone number as described bellow)[24], and HepG2-Neo cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and was incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Establishment of Heparanase-overexpressing Cell Lines

The cDNA of human heparanase was inserted into the expression vector, pcDNA3.1/MycHis(+)C (Invitrogen, Carlsbad, CA), and the vector was transfected into human hepatocellular carcinoma HepG2 cells, which did not express endogenous heparanase[25], using the FuGENE 6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN). The transfectants were selected in 400 μg/ml G418 (Geneticin, GIBCO Life Technologies, Grand Island, NY) and were named HepG2-HP-# (# is the clone number). The cell line transfected with the pcDNA3.1/MycHis(+)C vector was named HepG2-Neo.

Western Blotting

Cells (1×10^6) were washed and collected with 1 ml of cold PBS. After centrifugation at 4,000 rpm for 5 minutes, the supernatant was removed and 100 μl of lysis buffer (10 mM HEPES, 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2% Nonidet P-40, 0.1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.2) was added to the cells. The cells were lysed at 4°C with sonication. After centrifugation at 15,000 rpm for 15 minutes, 90 μl of supernatant was added to the 30 μl of loading buffer (42 mM Tris-HCl, pH 6.8, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol, and 0.002% bromophenol blue). The mixture was heated at 95°C for 3 minutes and then subjected to SDS-PAGE. The proteins were transferred to PVDF membranes and immunoblotted with specific antibodies, after which they were detected using SuperSignal West Pico Chemiluminescence Substrate (Pierce, Rockford, IL).

HS Degradation Assay

HS sodium salt from bovine kidney, chondroitin sulfate (CS) sodium salt from sturgeon notochord, and keratan sulfate (KS) sodium salt from bovine cornea were purchased from Seikagaku Corp. (Tokyo, Japan). Heparin (HR) sodium salt from porcine intestinal mucosa was purchased from Sigma Chemical Co. (St. Louis, MO). Alcian blue 8GX was purchased from Wako Pure Chemical Industries (Osaka, Japan). HepG2-HP-1 cells were collected with cold PBS (pH 6.2) and were subjected to three cycles of freezing and thawing. After centrifugation, the supernatant was collected as cell lysate. The mixture of 90 μl of cell lysate (2.0 mg/ml of protein) and 10 μl of HS solution (10 mg/ml in PBS, pH 6.2) with or without compound solution was incubated for 24 hours at 37°C. After adding 20 μl of HS sampling solution (glycerol 8 ml, 0.5% bromophenol blue 5 ml, H_2O 19 ml), 10 μl of the reaction mixture was subjected to SDS-PAGE (20%). After an electrophoresed gel was soaked in H_2O for 2 hours to remove SDS, it was stained with 0.5% Alcian blue for 1 hour and de-stained with AcOH - EtOH - H_2O (1 : 2 : 7) for 12 hours[26]. The volumes of bands were measured using an MD Scanning Imager equipped with MD ImageQuant Software Version 3.22 (Molecular Dynamics Inc., Sunnyvale, CA) for quantification. In the case of HR, CS, or KS degradation assay, all procedures were same as described above except for the use of HR, CS, or KS instead of HS.

HS Containing Polyacrylamide Gel Tablet Assay

To 0.9 ml of acrylamide solution (4 M acrylamide and 0.05 M *N*,*N*′-methylenebisacrylamide) was added 3.6 ml of H_2O, HS solution (10 mg/ml in PBS, pH 6), 100 μl of ammonium peroxodisulfate (10%), and 10 μl of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine. 100 μl of the solution was immediately transferred to wells of a 96-well plate (Sumilon, 96 well flat bottom, Sumitomo Bakelite Co., Ltd., Tokyo, Japan). The solution gelled in a few minutes. The gels were transferred to polycarbonate tubes (1.5 ml) and were washed with distilled water. HepG2-HP-1 lysate (1 ml) and a sample solution (1 μl) were added to the tubes and incubated at 37°C for 7 days. After a wash with distilled water, the gels were stained with 1 ml of the Alcian blue solution (0.5% in AcOH - EtOH - H_2O, 1 : 2 : 7) for 1 hour and de-stained with AcOH - EtOH - H_2O (1 : 2 : 7) until the control gel that did not contain HS was completely de-stained. Quantification of the gels was carried out using the MD Scanning Imager equipped with MD ImageQuant Software Version 3.22 (Molecular Dynamics Inc., Sunnyvale, CA).

Isolation and Structure Determination of RK-682

An actinomycete strain, RK99-A234, isolated in Okinawa, Japan, was cultured in 1.5 liters of production medium (soluble starch 2.0%, glucose 1.0%, soy bean meal 1.5%, malt extract 0.5%, $MgSO_4\cdot7H_2O$ 0.05%, KH_2PO_4 0.05%, V8 juice 10%, and potato dextrose 2.2%) for 72 hours at 28°C. The mycelia and supernatant were extracted

with acetone - methanol (1 : 1). The extract was evaporated and partitioned between chloroform and water. The chloroform extract was concentrated *in vacuo* and applied to a silica gel column (Silica gel 60, Merck KGaA, Darmstadt, Germany) with chloroform - methanol (1 : 1). The active fractions were collected and purified by a Sephadex LH-20 column (Sigma Chemical Co., St. Louis, MO) with chloroform - methanol (1 : 1). The active fractions were collected and further purified by a preparative medium pressure liquid chromatography (YAMAZEN Corp., Osaka, Japan) on silica gel (Silica gel 60, Merck KGaA, Darmstadt, Germany) with chloroform - methanol (95 : 5) to yield 57 mg of RK-682. NMR experiments were carried out on a Varian Unity Plus 500 spectrometer (Varian, Inc., Palo Alto, CA). Mass spectra were recorded on a JEOL JMS-SX102 spectrometer (JEOL, Tokyo, Japan).

Results

Establishment of Heparanase-expressing Stable Clones

The cloning of the heparanase gene has enabled us to assess the enzyme activity more clearly. As a result, we have established heparanase-expressing stable clones by transfecting the heparanase gene to HepG2 cells, which was found to express the heparanase mRNA at an undetectable level. Because it was difficult to obtain anti-heparanase antibodies, we planed to detect the heparanase protein by using the MycHis tag. Since heparanase is processed at the *N*-terminal side, the MycHis tag was attached at the *C*-terminal side of the heparanase protein. The cell lines harboring pcDNA3.1-*hpa*-MycHis(+)C (Fig. 1, A) or vector alone were labeled HepG2-HP-# (# indicates clone number) or HepG2-Neo, respectively. The result of western blotting using anti-c-*myc* antibody (Fig. 1, B) showed that an 80-kDa band was detected in the cell lines of HepG2-HP-1, 2, 3, 4, and 6, but not in HepG2-Neo, HepG2-HP-5, or HepG2-HP-7. This band was regarded as heparanase protein having the MycHis tag at the *C*-terminal, in view of its molecular weight: heparanase (65 kDa) plus MycHis-tag (15 kDa). A 62-kDa band was assigned as endogenous c-myc protein.

After examination of some methods (such as cellulose-acetate chromatography), a combination of SDS-PAGE (20%) and Alcian blue staining was found to easily detect HS degradation fragments. Next we examined the conditions necessary for making cell lysate. We found that the enzyme activity of heparanase was extremely sensitive to sonication and detergent treatments. The lysate prepared by repeatedly freezing and thawing particularly maintained the enzyme activity. Consequences of the transfection of the heparanase gene are shown in Fig. 2, A. HS degradation activity was detected only in the case of HepG2-HP-1, and not parent HepG2 or HepG2-Neo. The HepG2-HP-7 cells showing a trace 80-kDa band (Fig. 1, B) did not show HS degradation activity even though the heparanase gene was transfected. These results also support the assumption of the 80-kDa band as heparanase-MycHis. The activity was correlated with the amount of HepG2-HP-1 cells. The substrate specificity of HepG2-HP-1 lysate is shown in Fig. 2, B. The band of HS became pale by the treatment of HepG2-HP-1 lysate. The band of heparin (HR) and chondroitin sulfate (CS) was not changed. The band of keratan sulfate (KS) became slightly pale by the treatment. These results indicate that this lysate possesses HS selectivity. The effect of pH in lysate preparation was also assessed. HS degradation activity was kept up in the range of pH 5～6, but the activity disappeared at pH below 4 or over 7. The following experiments were executed using the lysate prepared by repeatedly freezing and thawing at pH 6 and containing no detergents.

Fig. 1. Diagram of pcDNA3.1-*hpa*-MycHis(+)C construct (A) and western blot analysis with anti-c-*myc* antibody (B).

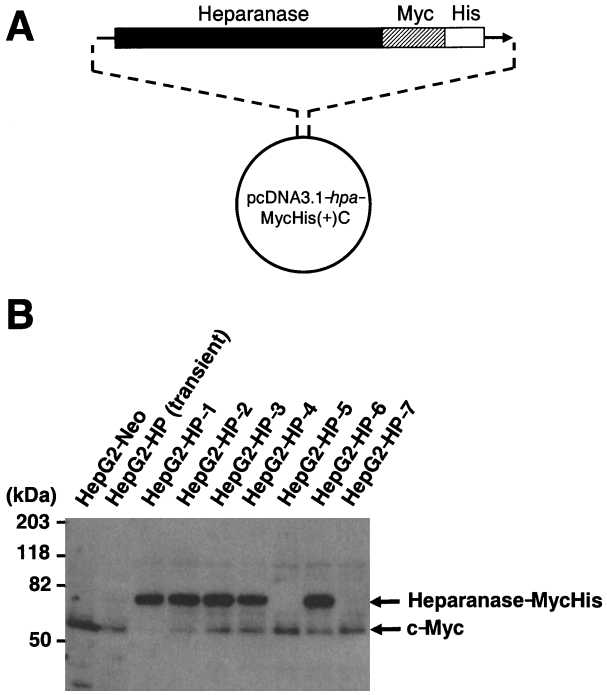
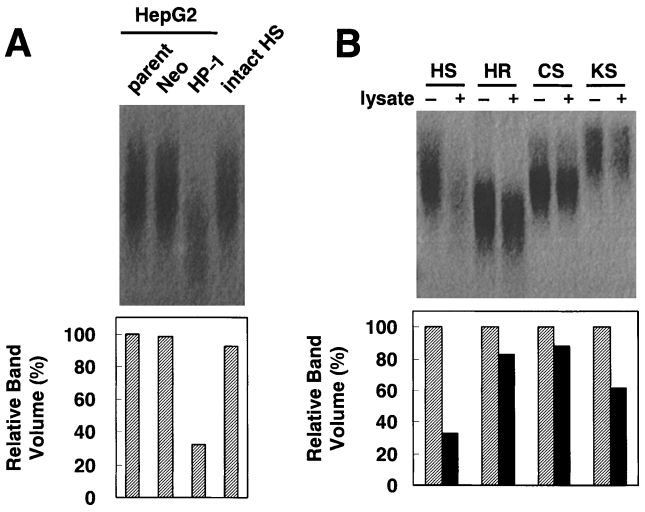
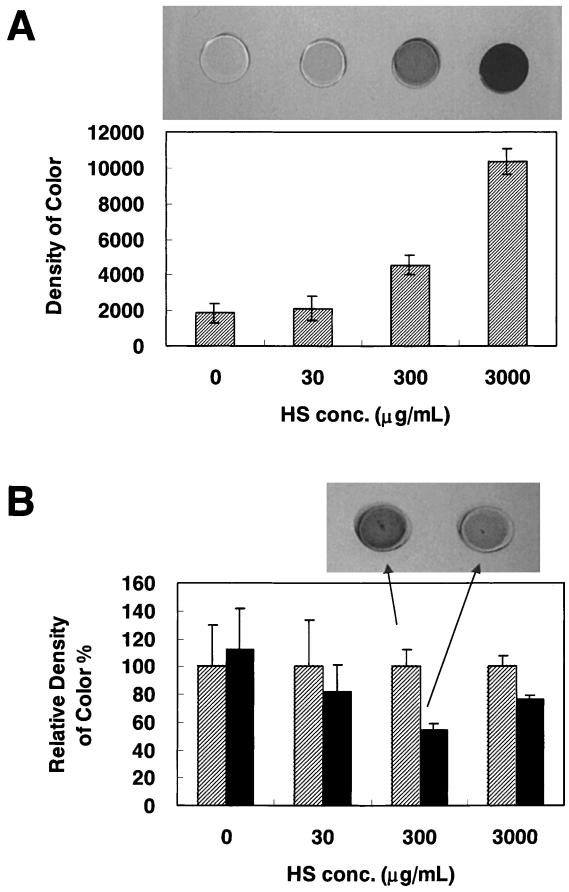


Fig. 2. Consequence of the transfection of heparanase gene (A) and substrate specificity of the HepG2-HP-1 lysate (B).



(A) Heparan sulfate (HS) was treated with the lysate of the indicated cells at 37°C for 24 hours. (B) Glycosaminoglycans were incubated with or without the lysate at 37°C for 24 hours. HS: heparan sulfate, HR: heparin, CS: chondroitin sulfate, KS: keratan sulfate.

Fig. 3. Photographs and color density of HS-containing polyacrylamide gel tablets.



(A) Correlation between HS concentration and color density of the tablets. (B) Color change of the tablets caused by the treatment with HepG2-HP-1 lysate. Hatched bars and black bars indicate relative density of color of the tablets treated with PBS and HepG2-HP-1 lysate, respectively.

Establishment of an Efficient Visual Screening Method

Chromogenic substrates are commercially available for some exoglycosidases, but in the case of heparanase, such chromogenic substrates are not commercially available. It is assumed to be very difficult to develop chromogenic substrates for heparanase, since heparanase is an endoglycosidase. Heparanase assay methods previously reported are also not suitable for dealing with massive samples, since those methods use radioisotope-labeled HS as a substrate and gel-filtration for detection. Consequently, it was necessary to establish a convenient and efficient heparanase assay method.

The separated steps of reaction and detection cause vexatious complications for biological assay. It is thought that if capsules containing substrates were made and the progress of the reaction could be visually detected, these vexatious complications would be resolved. So we have planned to create a novel substrate that is visually detectable and easily transferred.

After considering several methods, we found that the amount of HS in polyacrylamide gel could be quantified by Alcian blue staining. Tablets of polyacrylamide, each containing a different amount of HS were stained with Alcian blue and the density of the colors was quantified. The color density of tablets was correlated with the concentrations of HS (Fig. 3, A). Then the tablets were incubated in the lysate of HepG2-HP-1 cells and stained with Alcian blue. In the case of HS concentration of 300 μg/ml, the relative density of color was reduced more thoroughly than with other concentrations (Fig. 3, B). This result indicates that heparanase in the lysate of HepG2-HP-1 can degrade HS immobilized in a polyacrylamide tablet, and the degraded fragments of HS are excluded from the tablet. From these results, we assumed that it is possible

to use this HS-containing polyacrylamide tablet for heparanase assay. The concentration of HS in each tablet was set to 300 μg/ml for screening.

Search for Heparanase Inhibitors from Microbial Culture Broths

Before the screening from microbial culture broths, we assessed some typical inhibitors. No inhibitory activity was observed in any representative kinase inhibitors (PD 98059 and staurosporine), protein phosphatase inhibitors (okadaic acid and cyclosporine A), mitochondrial function inhibitors (antimycin and oligomycin), histone deacetylase inhibitor (trichostatin A), tubulin assemble inhibitor (vinblastine), tubulin disassemble inhibitor (paclitaxel), topoisomerases inhibitors (adriamycin, etoposide, and camptothecin), RNA synthesis inhibitor (actinomycin D), protein synthesis inhibitor (cycloheximide), Hsp90 inhibitors (geldanamycin and radicicol), proteasome inhibitor (MG-132), or PKC activator (TPA). Only suramin, a pleiotropic function inhibitor, completely inhibited the HS degradation activity at 100 μM. Suramin is also reported to have an inhibitory effect on heparanase[27]. This result proves the potential of this method to allow heparanase inhibitors to be searched from a library.

Among 10,000 culture broths of microorganisms, an actinomycete strain of RK99-A234 was found to have heparanase inhibitory activity. After purification of the active substance using consecutive column chromatography, the structure was identified by means of NMR and MS. The result of negative ion FAB-MS showed a strong peak at *m/z* 367 $(M-H)^-$. A molecular structure was speculated by the presence of 1D- (^1H and ^{13}C) and 2D-NMR (DQF-COSY, HMQC, and HMBC). Finally the inhibitor was identified to be RK-682, which was previously identified as a protein tyrosine phosphatases and dual-specificity phosphatases inhibitor (Fig. 4, A). The IC_{50} value for heparanase was about 17 μM (Fig. 4, B) and it was rather higher than that for VHR (Table 1). After rational drug design using RK-682 as a lead compound, we could develop a heparanase selective inhibitor, 4-benzyl-RK-682 (4-Bn-RK-682).

Discussion

Heparanase is involved in the important steps of tumor metastasis, invasion, and angiogenesis[3,4]. Heparanase facilitates an invasion of tumor cells by degradation of a barrier of HS, and causes an angiogenesis by releasing growth factors, such as bFGF and VEGF, sequestered in HS[3,8~10]. Heparanase has attracted a great deal of interest as a promising target for antitumor agents[1,2].

Reports on the search for heparanase inhibitors have been limited by the instability of the protein and the

Fig. 4. The chemical structures of RK-682 and 4-Bn-RK-682 (A) and the heparanase inhibitory activity of RK-682 (B).

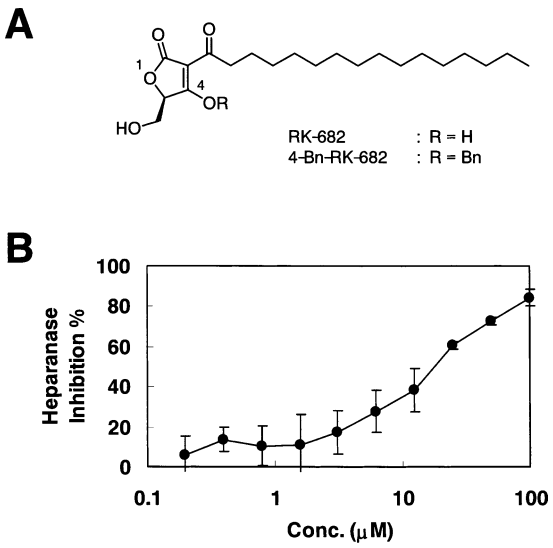


Table 1. Heparanase and VHR inhibitory activities of RK-682 and 4-Benzyl-RK-682.

Compounds	IC_{50} (μM)		Selective Index[a]
	Heparanase	VHR	
RK-682	17	6	0.4
4-Benzyl-RK-682	17	>100	>6

[a] Selective Index = (IC_{50} of VHR) / (IC_{50} of heparanase).

difficulty of assays. To overcome this problem, we established heparanase-expressing stable clones by transfection to HepG2 cells. We also innovated on a visual and facile screening method using a novel HS-containing polyacrylamide tablet for dealing with massive samples. Among 10,000 screened broths, only an actinomycete showed heparanase inhibitory activity. After activity-guided purification and structure determination by means of NMR and MS, the known compound RK-682[23] was identified as the active substance.

The biological activities of RK-682 have already been reported; as a phospholipase A2 inhibitor isolated from *Streptomyces* sp. AL-462 (IC_{50}=16 μM)[28], and as an HIV-1 protease inhibitor from *Actinomycete* strain DSM7375 (IC_{50}=84 μM)[29,30]. We have also isolated RK-682 from *Streptomyces* sp. RK88-682 as an inhibitor of protein phosphatases (IC_{50} of CD45 and VHR=54 and 2.0 μM, respectively)[23].

In this paper, we identified RK-682 as a heparanase inhibitor (IC_{50} 17 μM). This result indicates that RK-682 is more potent than suramin, (IC_{50} 46 μM), a known heparanase inhibitor[27]. However as described above, RK-682 is known to have other biological activities. SODEOKA *et al.* reported[31] that methylation of the enol group at the C4 position of RK-682 almost abolished the VHR activity. The IC_{50} values for the VHR activity of RK-682 and 4-Me-RK-682 are 12 and >250 μM, respectively. We synthesized 4-Bn-RK-682, which had heparanase selective inhibitory activity. The IC_{50} values for heparanase and VHR were 17 μM and >100 μM, respectively (Table 1)[32].

Taken together, we innovated on a visual and facile bioassay method to discover heparanase inhibitors, and the usefulness of the bioassay was proven because RK-682 and its 4-Benzyl-derivative were identified as heparanase inhibitors.

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