High-Throughput Turbidimetric Screening for Heparin-Neutralizing Agents and Low-Molecular-Weight Heparin Mimetics

Atsushi Sekiya, a Shinya Oishi, b Nobataka Fuji, b and Takaki Koide* a

a Department of Chemistry and Biochemistry, School of Advanced Science and Engineering, Waseda University; Tokyo 169–8555, Japan; and b Graduate School of Pharmaceutical Sciences, Kyoto University; Sakyo-ku, Kyoto 606–8501, Japan.

Received December 2, 2011; accepted December 26, 2011; published online January 5, 2012

Safer heparin-neutralizing agents are currently required to replace protamine, the use of which causes adverse effects such as anaphylaxia. Low-molecular-weight (LMW) heparin mimetics that potentiate anti-thrombin III (AT) action are also valuable as anti-thrombotics. This paper describes a high-throughput assay for both heparin-neutralizing agents and LMW heparin mimetics without the use of blood preparations. The assay is based on turbidimetric measurement of a solution of collagen, heparin, and a test compound. Native collagen molecules spontaneously form insoluble fibrils when transferred to a physiological buffer, and this process is inhibited by heparin. In the presence of a heparin-neutralizing agent or an LMW heparin mimetic, the inhibitory effect of heparin is canceled and turbidity increase is retrieved. We demonstrated that this assay is effective in detecting potential agents with high reliability (Z’ factor=0.9). The screening of a chemical library (34400 compounds) was further performed in a 384-well format, and led to the identification of a novel heparin-neutralizing agent. Since this assay protocol is feasible for an automated high-throughput screening (HTS) system, it could enhance the lead seeking process for drugs related to heparin/heparan sulfate (HS) functions.

Key words heparin; heparin mimetic; high-throughput screening; collagen

...
purchased from Sigma. Fondaparinux [Arixtra®, (methyl 0-2-deoxy-6-O-sulfo-2-(sulfamido)-α-D-glucopyranosyl (1→4)-O-β-D-glucopyra-nuronosyl-(1→4)-O-2-deoxy-3,6-di-O-sulfo-2-(sulfamido)-α-D-glucopyranosyl (1→4)-O-2-O-sulfo-α-L-idopyranuronosyl-(1→4)-2-deoxy-6-O-sulfo-2-(sulfamido)-α-D-glucopyranoside) decasodium salt] was purchased from Glaxo Smith Kline (London, U.K.). Polyethylenimine (PEI, MW approx. 750000) was purchased from Sigma. The screening library is a random collection of 34400 compounds, including drug-like compounds, known bioactive agents, and synthetic peptidomimetics from Kyoto University. The triple helix-forming collagen-like peptides, C4-c and C4-c scramble, are (Gly-Pro-Hyp)₇-Gly-Ile-Lys-Gly-His-Arg-Gly-Phe-Ser-Gly-Leu-Hyp-(Gly-Pro-Hyp)₇-Gly-amide and (Gly-Pro-Hyp)₇-Gly-Phe-His-Gly-Arg-Ser-Gly-Leu-Ile-Gly-Lys-Hyp-(Gly-Pro-Hyp)₇-Gly-amide (Hyp, 4-hydroxypro-
line), respectively.¹²)

Inhibition of Collagen Fibril Formation by Heparin
Mixtures containing 0.1 mg/mL type I collagen and variable concentrations of heparin in 20 mM sodium phosphate (pH 7.4), 100 mM NaCl, were prepared on ice. The temperatures of the mixtures were quickly raised to 37°C, and the degree of fibril formation was monitored by measuring the absorbance at 313 nm on a Vient XS multwell plate reader (DS Pharma Biomedical, Osaka, Japan) for 150 min.

% inhibition of collagen fibril formation was defined by the following equation:

\[
\% \text{ inhibition of collagen fibril formation} = \left(1 - \frac{\Delta A_{\text{heparin}(-)}}{\Delta A_{\text{heparin}(+)}}\right) \times 100
\]

where \(\Delta A_{\text{heparin}(-)}\) is the change in absorbance at 313 nm from time 0 to 150 min in the absence of heparin, and \(\Delta A_{\text{heparin}(+)}\) is that in the presence of each concentration of heparin.

Fibril Formation Assay
The protocol of the assay is illustrated in Fig. 1. The assay solution contains 0.1 mg/mL type I collagen, 5 μg/mL heparin, 20 μM test compound, 20 mM sodium phosphate (pH 7.4), 100 mM NaCl, and 1% dimethylsulfoxide (DMSO). The assay solution was prepared by mixing the incubation buffer (26.2 mM sodium phosphate, 132 mM NaCl, 6.6 μg/mL heparin), and 10-fold concentration solution of a test compound in 10% DMSO, and acid-soluble type I collagen (0.7 mg/mL) solution. The detailed procedure is as follows: Eleven microliters of 10× concentrated compound solution and 83 μL of the incubation buffer were mixed in a 384-well plate on ice. Fourteen microliters of collagen solution was dispensed in UV-star 384 plate (Greiner Bio-one, Frickenhausen, Germany). After 10 min, 86 μL of the pre-incubation mixture was added to collagen-containing 384-well plate on ice and thoroughly mixed by pipetting. The plate was

Fig. 1. Assay Protocol

![Assay Protocol](image1)

Fig. 1. Assay Protocol

(A) Collagen I molecules spontaneously form insoluble fibrils at physiological pH 7.4. (B) Heparin inhibits the collagen fibril formation. (C) Heparin-neutralizing agents restore the collagen fibril formation through the binding to heparin. (D) LMW heparin mimetics restore the collagen fibril formation by masking heparin-binding site in collagen.

![Principle of the Assay](image2)

Fig. 2. Principle of the Assay

(A) Soluble collagen molecule form insoluble fibrils at physiological pH 7.4. (B) Heparin inhibits the collagen fibril formation. (C) Heparin-neutralizing agents restore the collagen fibril formation through the binding to heparin. (D) LMW heparin mimetics restore the collagen fibril formation by masking heparin-binding site in collagen.
left at room temperature for 10 min. To start the collagen fibril formation, the temperature was quickly raised to 37°C, and the degree of fibril formation was monitored by measuring the absorbance at 313 nm.

‘% Fibril formation’ was defined by the following equation:

\[
\% \text{ fibril formation} = \left[ \left( \Delta A - \mu_- \right) / (\mu_+ - \mu_-) \right] \times 100
\]

(2),

where \( \Delta A \) is the change in absorbance at 313 nm from time 0 to 150 min, \( \mu_+ \) is the average \( \Delta A \) of wells containing collagen and heparin, and \( \mu_- \) is the average change of absorbance of the collagen only in control wells on the same plate.

\( Z' \) values were calculated using the method of Zhang and coworkers\(^1\) as follows:

\[
Z' \text{ factor} = 1 - \left[ 3(\delta_+ + \delta_-) / (\mu_+ - \mu_-) \right]
\]

(3),

where \( \delta_+ \) and \( \delta_- \) are the standard deviations of wells containing collagen and heparin and of wells of collagen only, respectively.

**Statistical Analysis**

Statistical analyses were performed using Origin ver. 7.5 data analysis software (Origin Lab, Northampton, MA, U.S.A.). IC\(_{50}\) values were estimated from midpoint of the % inhibition of collagen fibril formation or the %fibril formation curves.

**Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Analysis**

Mass spectrometric analysis of compound 1 was performed by MALDI-TOF on a Bruker Autoflex II mass spectrometer. \( \alpha \)-Cyano-4-hydroxycinnamic acid was used as the matrix.

**Results**

**Principle and Validation of the Assay**

The assay is based on the phenomenon that native type I collagen molecules solubilized at acidic pH (pH 3) form insoluble fibrils by spontaneous self-assembly when transferred to a buffer of physiological pH (pH 7.4) at 37°C. This process is readily monitored by the turbidity of the solution (Fig. 2A). In the presence of heparin, the self-assembly is disturbed, probably because of the steric hindrance of heparin bound to collagen (Fig. 2B). When adding a heparin-neutralizing agent, the effect of heparin on the collagen fibril formation is canceled and the turbidity of the solution is restored (Fig. 2C).

The profiles of the time-dependent increase in the presence (+) and absence (−) of 5 μg/mL heparin are shown in Fig. 3A. The data were validated using \( Z' \) factor (Eq. 3, Fig. 3A). The \( Z' \) factor was estimated to be 0.90, which is sufficiently high to be applied to an HTS.\(^1\)

Heparin inhibited collagen fibril
formation in a dose-dependent manner (IC$_{50}$ = 1.0 μg/mL) (Fig. 3B). On the basis of this result, we fixed the assay concentration of heparin to be 5 μg/mL. We next examined whether activities of some known heparin inhibitors could be detected by this method. In the presence of 20 μM protamine, surfen, or C4-c peptide, the inhibitory effect of heparin on collagen fibril formation was canceled and the turbidity of the solution was increased to a level similar to that of the collagen-only solution (Fig. 3C). Here, C4-c is a recently identified heparin-binding collagen-like triple helical peptide containing the heparin-binding motif, Lys-Gly-His-Arg-Gly-Phe, and C4-c scramble (C4-c scr) is a negative control with a randomized motif sequence (see Materials) (Fig. 3C). This result indicated that the assay is reliable and powerful in selecting heparin-neutralizing agents. Normally, the heparin-neutralizing activity of the compounds can be evaluated by measuring % fibril formation at 150 min (Fig. 3D). The values of % fibril formation were calculated using Eq. 2. In addition, dextran sulfate (Dex), a functional analog of heparin, can also be used in the same system. Addition of Dex instead of heparin retarded the turbidity increase, and the effect of Dex was canceled in the presence of protamine (data not shown).

**Feasibility in the Screening of LMW Heparin Mimetics**

LMW heparin mimetics are also therapeutically valuable drugs related to the blood coagulation system. If a heparin mimetic does not disturb the collagen self-assembly by itself, and if it competes with heparin at the same binding sites in collagen, the activity could be detected by the similar collagen fibril formation assay (Fig. 2D). In order to investigate the feasibility of the assay system to select LMW heparin mimetics, we checked the effect of fondaparinux, a clinically used LMW heparin analogue, on the fibril formation of type I collagen. As shown in Fig. 4A, fondaparinux itself did not significantly affect the collagen fibril formation. In the presence of heparin, 50 μg/mL fondaparinux effectively restored the collagen fibril formation that had been inhibited by heparin (Fig. 4B). This result indicated that LMW heparin mimetics could also be selected by the screening based on the same collagen fibril formation assay as for obtaining heparin-neutralizing agents.

**Results of an HTS**

An HTS on 384-multiwell plates based on the above-mentioned protocol was performed against a chemical library that contains a total of 34400 small-molecule compounds including peptidomimetics, known bioactive agents, and commercially available drug-like compounds. As shown in Fig. 5A, 12 compounds showed values of % fibril formation greater than 50%. Samples marked by asterisks were non-specific detections. (B) Lag-time analysis of the 12 samples. Only compound 1 showed a normal lag time. The remaining 11 samples (Fig. 5A asterisks) showed rapid increase of turbidity without lag times (dashed lines).
March 2012 375

compounds were further determined to be false positives because they showed turbidity increase in the absence of collagen and heparin. The false positives were colored compounds or precipitate-forming ones. (data not shown)

Characterization of Hit Compound 1 From the screening, one possible hit compound 1 was obtained (Fig. 5). The registered chemical structure of 1 is shown in Fig. 6A.14 However, the MALDI-TOF mass analysis detected signals over MS 5000 with regular mass intervals of 87, indicating that the sample was a mixture of polymers. Figure 6C shows the mass spectrum of 1 in the range of MS 1000—2000. This result suggested that the active compounds were PEI-like derivatives generated by ring-opening poly-condensation of the original epoxy-methanamine compound (Fig. 6B).

We thus compared the heparin-neutralizing activity of 1 with that of PEI, which is a polyamine known to interact with heparin/HS proteoglycans,15,16 by the collagen fibril formation assay (Fig. 7). Polymer compound 1 showed dose-dependent restoration of collagen fibril formation with an IC50 value of 1.2 μg/mL, and the heparin-neutralizing activity was comparable to that of PEI (IC50 = 0.7 μg/mL).

Discussion

On the basis of the phenomenon that collagen-binding macromolecules disturb collagen fibril formation in vitro, we previously developed an HTS system for inhibitors of collagen-binding proteins including heat shock protein 47, glycoprotein VI, and von Willebrand factor.11 In this study, we expanded the feasibility of the system to screening for heparin-neutralizing agents and LMW heparin mimetics.

Therapeutically, heparin-related drugs are valuable in treatments of disorders of the blood coagulation system. Protamine has long been used to suppress excessive anti-coagulation activity of heparin.17 However, administration of protamine...
sometimes has severe adverse effects. In addition, protamine does not neutralize the activity of LMW heparins. Safer protamine surrogates are thus required. Factor Xa inhibitors have been developed as anti-coagulant drugs that can replace warfarin. Fondaparinux, a synthetic pentasaccharide heparin mimic, potentiates AT-mediated inhibition of factor Xa activity that leads to blood coagulation. The screening system developed in this study is applicable to obtaining both heparin-neutralizing agents, such as protamine, and LMW heparin mimetics, such as fondaparinux. The advantages of the system are: i) no use of fresh blood preparations, ii) no use of expensive enzymes such as blood coagulation factors, iii) no bound/free separation, iv) label-free, v) high-throughput, vi) high Z' factor, and vii) feasible for various types of test compounds, such as small molecules, peptides, and polymeric materials. These features provide the opportunity for its use as an automated HTS.

An actual screening result was demonstrated using a chemical library containing 34400 compounds (Fig. 5A). Twelve compounds (0.035%) showed signals above 50% fibril formation, indicating very low background noise of the screening. Among the 12 candidates, 11 compounds were false positives showing heparin-independent turbidity increase. It should be noted that the false positives can be easily eliminated by their lack of a lag time at the start of the turbidity increase (Fig. 5B). The hit compound 1 was a mixture of polymers with PEI-like structure (Fig. 6). PEI is reported to have GAG-binding property, and utilized as an HS proteoglycan-mediated DNA transfection agent and a GAG stain for histology. The heparin-inhibiting property of compound 1 was similar to that of PEI (Fig. 7). Taking these findings together, compound 1 was concluded to be a novel heparin-neutralizing agent.

In conclusion, the easy-to-use turbidimetric assay based on the collagen fibril formation in vitro was applicable to an HTS for both heparin-neutralizing agents and LMW heparin mimetics. Such an efficient system would be valuable because heparin-related agents have recently been elucidated to be also effective in terms of anti-tumor, anti-angiogenesis, and anti-inflammation activities, in addition to their classical anti-thrombotic effects. The screening system could enhance the lead seeking process for drugs related to heparin functions. In addition, the system could also be applicable to the screening of inhibitors for other collagen-binding proteoglycans such as syndecan, perlecan, lumican, and decorin.

Acknowledgement This work was supported by a Grant-in-Aid for Scientific Research (B) (No. 19390032) from the Japan Society for the Promotion of Science.

References