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Protein Chip-based Dipeptidyl Peptidase IV Assay System

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Abstract

Dipeptidyl peptidase IV (DPP-IV) is known to be a serine amino-peptidase that cleaves N-terminal X-Ala or X-Pro from target polypeptides. The enzyme plays a pivotal role in fatal diseases such as diabetes mellitus, obesity, tumor growths, and HIV infection. Inhibition of DPP-IV is currently being explored as a novel therapy for type II diabetes. Thus, we have developed a protein chip-based DPP-IV assay system for the screening of enzyme inhibitors. A biotin-labeled Gly-Pro sequence as a substrate was designed for immobilization on a ProteoChip™. DPP-IV was incubated with the substrate-immobilized ProteoChip. Also, Cy5-labeled streptavidin was applied to the chip, and the fluorescence intensities on the spots were detected using a laser scanner. DPP-IV activity on the chip was determined using data obtained from the fluorescence intensities. Galvus (vildagliptin) and virtually screened leads were tested using the DPP-IV assay chip for identification as novel diabetes inhibitors.

Keywords: DPP-IV, ProteoChip™, Protease assay, Diabetes inhibitor, Galvus

Introduction

DPP-IV is a serine protease that cleaves N-terminal dipeptide with a preference for Xaa-Pro dipeptides from oligopeptides[1]. This protease leads the biological activation or inactivation of regulatory peptides through specific enzyme processes. DPP-IV is expressed in many tissues and body fluids as either a membrane-bound or soluble enzyme[2]. Various substrates that are recognized by DPP-IV include neuropeptides, chemokines, and peptides of the glucagon family[3,4]. This mechanism of the enzyme is closely related with diabetes mellitus[5-9]. Suppression of DPP-IV increases the level of circulating Glucagon-like peptide-1 (GLP-1) and thus stimulates insulin secretion, which can improve hyperglycemia in type II diabetes[10-12]. A number of small molecule inhibitors of DPP-IV have been described[13-15]. GLP-1 is an incretin hormone secreted from the L cells of the small intestine as a response to food intake[16]. This hormone plays an important role in the inhibition of glucagon secretion, retardation of gastric emptying, induction of obesity, and stimulating the regeneration and differentiation of islet β cells[17,18]. However, GLP-1 is rapidly degraded by the action of DPP-IV *in vivo*, which degrades a dipeptide from the N-terminus to produce an inactive GLP amide[19,20].

In this paper, we show the development of a DPP-IV assay system using a protein chip for the analysis of DPP-IV in biological samples and enzyme inhibitor screening.

Results and Discussion

Development of DPP-IV Bioassay System based on ProteoChip

To perform an analysis of DPP-IV in biological samples and library screening of new anti-diabetic agents, we have developed a DPP-IV assay on a protein chip. Figure 1 shows a schematic diagram of the developmental process of DPP-IV using the ProteoChip system. The peptide containing a Gly-Pro sequence that is known to be a DPP-IV recognition site was designed as a specific substrate for the DPP-IV. A ProteoChip was used as a substrate attachment base plate. A monoclonal antibody (mAb) against $(His)_6$ was immobilized as a capture molecule on the ProteoChip for substrate binding. The designed peptide substrate labeled with biotin and His tag was then loaded onto an anti-His tag mAb-coated chip followed by application of DPP-IV and a DPP-IV inhibitor. Fluorescence dye (Cy5)-labeled streptavidin was applied to the chip, and the fluorescence intensities were detected for determining the DPP-IV activity *in vitro*. As shown in Figure 2A, it was noted that the DPP-IV assay chip revealed an enhancement of the substrate cleavage by DPP-IV with an increase in enzyme concentration. This result suggests that a successful DPP-IV assay is possible using protein chip technology. To confirm the applicability for the inhibitor screening using a DPP-IV assay chip, Galvus (vildagliptin), a known inhibitor of DPP-IV, was tested for validation of the DPP-IV assay chip on the enzyme inhibitor screening (Figure 2). Galvus showed a dose-dependent inhibition of on-chip DPP-

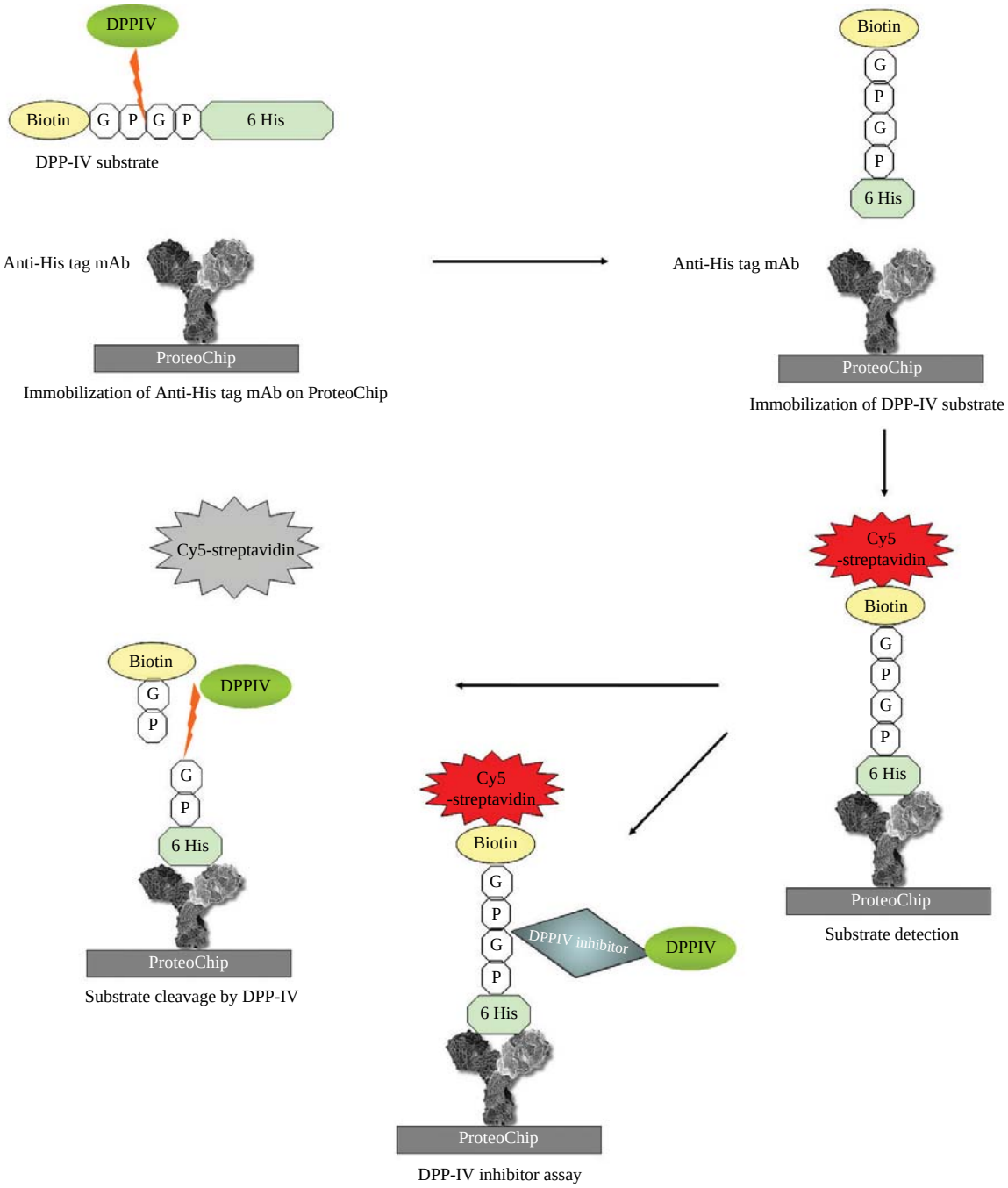

Figure 1. Schematic diagram of the DPP-IV assay on a ProteoChip.

IV activity. These data demonstrate that a DPP-IV assay chip is a useful tool for the library screening of an enzyme inhibitor.

Inhibitor Screening using DPP-IV Assay Chip

To screen a novel DPP-IV inhibitor from phytochemical libraries, a structure-based virtual screening was first carried out. The compound inhibitors screened using an *in-silico* method tested the inhibitory effects on DPP-IV activity using the DPP-IV assay chip at a given compound concentration (100 nM) (Figure 3A). The chip-based method allowed us to screen four potent inhibitors of DPP-IV activity in the nanomolar range. Four different compounds appeared to be effec-

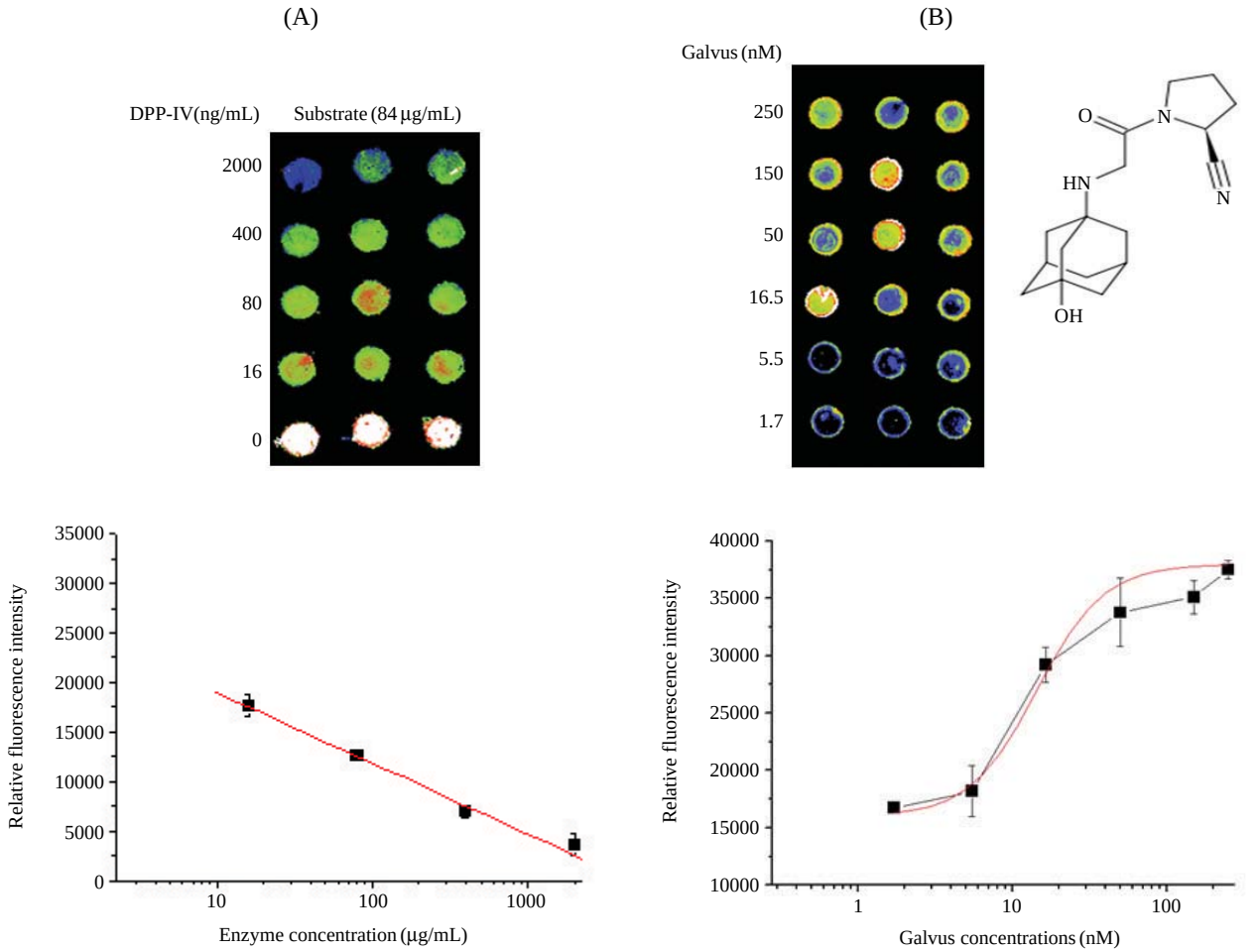

Figure 2. The DPP-IV activity assay using a ProteoChip. (A) Dose-dependent cleavage of the substrate by DPP-IV. (B) Inhibitory effect of Galvus on the DPP-IV activity on the enzyme assay chip.

tive in inhibiting DPP-IV activity compared with Galvus, a positive control. The inhibitory efficacy of A11, one of the potent inhibitors, was higher than that of Galvus (Figure 3B). Further works on the biological functions of these compounds still remain.

Conclusions

We have developed a new DPP-IV assay system using protein chip technology and demonstrated its application for a new inhibitor screening. A monoclonal antibody (mAb) against $(His)_6$ was immobilized on the ProteoChip for substrate binding. A biotin-labeled substrate tagged with $(His)_6$ was designed for immobilization on a ProteoChip™. DPP-IV was incubated with the substrate-immobilized ProteoChip. Fluorescence dye (Cy5)-labeled streptavidin was applied to the chip, and the fluorescence intensities were detected for determining the DPP-IV activity *in vitro*. The DPP-IV assay chip was used for screening of potent inhibitors of DPP-IV activity from the phytochemical libraries. A11, a potent inhibitor, suppressed DPP-IV activity on the chip.

Materials and Methods

Materials

The DPP-IV substrate using biotin-GPGPHHHHHH peptide was designed and synthesized by Peptron, Inc. (Taejeon, Korea).

Anti-His tag mAb (Koma-biotech, Seoul, Korea), DPP-IV in active form (Calbiochem, Darmstadt, Germany), ProteoChip (Proteogen, Chuncheon, Korea), Phytochemical libraries (InterBioScreen, Russia), Galvus (Norvatis, Korea), and Cy5-straptavidin (GE healthcare, USA) were purchased.

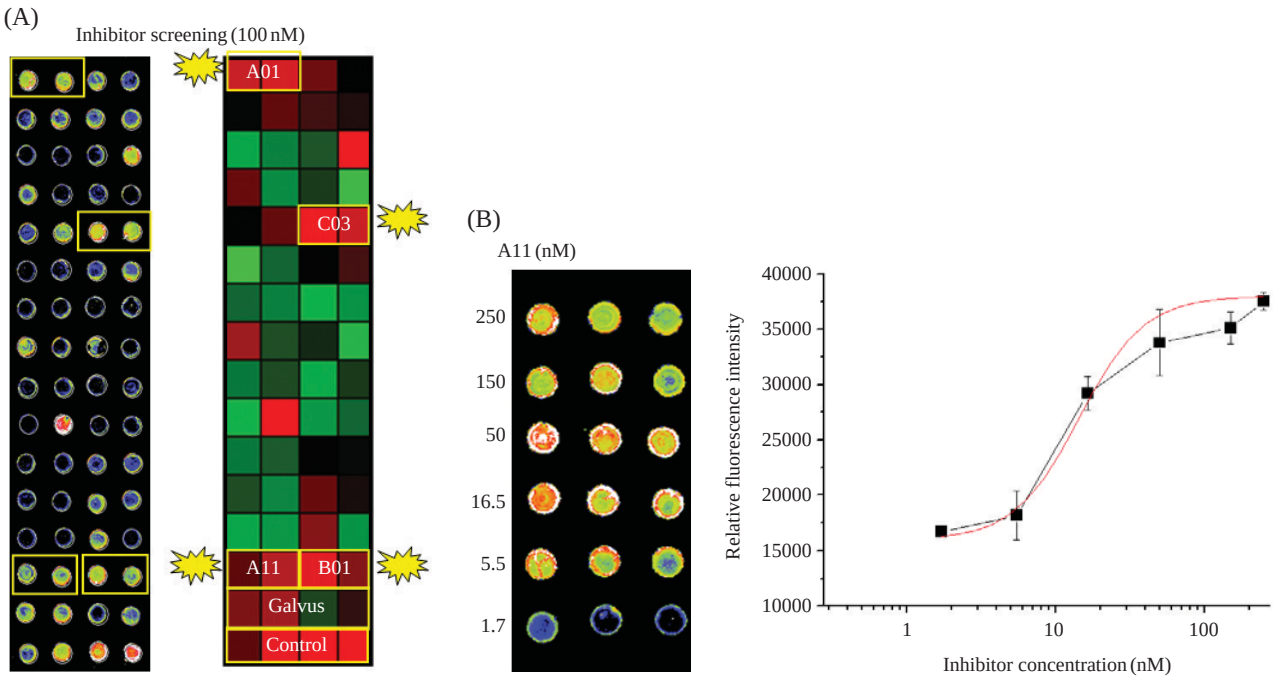

Figure 3. Library screening of the DPP-IV inhibitor using the enzyme chip. (A) The primary screening of the DPP-IV inhibitor from phytochemical compound libraries that were screened using *in-silico* methods. (B) An inhibitor (A11) that was shown to inhibit the effect of DPP-IV screened from the phytochemical libraries suppressed the enzyme activity on the DPP-IV chip in a dose-dependent manner.

Protein Chip-based DPP-IV Assay

ProteoChip (Proteogen Inc., Korea) was used as miniaturized 96-well chips, which were applicable in 1 μL of each well. Anti-His tag mAb (100 μg/mL, 30% glycerol) was immobilized overnight on a ProteoChip as a substrate capture protein at 4˚C. After rinsing with PBST, the biotin-GPGPHHHHHH (10-250 μg/mL, 30 % glycerol) substrate was immobilized on the ProteoChip for 3h at 4˚C. After rinsing with PBST, the chip was blocked using 5% BSA for 1 h at room temperature, and the chip was then rinsed with PBST and dried under N_2 gas. DPP-IV enzyme (0-2,000 ng/mL) containing an assay buffer consisted of 50 mM Tris-HCl pH 7.8 containing 0.1 mg/mL BSA, 100 mM NaCl, and 10% Glycerol. The reaction mixture was applied on the chip and incubated for 1 h at 30˚C. Cy5-straptavidin (1 : 100) was applied to the rinsed chip by PBST for 1 h at 30˚C and then rinsed with PBST and DW. The chip was then dried in a stream of N_2 gas.

Inhibitor Screening using DPP-IV Assay Chip

In order to utilize the DPP-IV inhibitor screening, a substrate concentration of 100 μg/mL was spotted onto to the anti-$(His)_6$ antibody immobilized ProteoChip. Selected compounds from the virtual screening were dissolved in 100% DMSO, and diluted to the desired concentration in reaction buffer. DPP-IV (40 ng/mL) and libraries (2,500 nM, 500 nM, 100 nM) containing the reaction buffer mixture were applied to the screening of the DPP-IV assay explained above. Galvus (vildagliptin) was used as a control for the DPP-IV inhibitor. Determinations of the IC_{50} values of the compounds were calculated using a non-linear best fit regression of the DPP-IV inhibition of different compound concentrations (250 nM, 150 nM, 50 nM, 16.5 nM, 5.5 nM, and 1.7 nM).

Protein Chip Image Analysis

In order to detect fluorescence signals, the chip was scanned using a Genetix aQuire™ scanner (Genetix, UK) and saved as a TIFF file. The scanned images were analyzed using GenePix Pro 6.0 (Axon Instruments, CA, USA). The IC_{50} values were calculated using Origin6.1 (Originlab, MA, USA).

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