Biotechnol Lett (2009) 31:979–984
DOI 10.1007/s10529-009-9963-y

ORIGINAL RESEARCH PAPER

A new screening method based on yeast-expressed human dipeptidyl peptidase IV and discovery of novel inhibitors

Chen-Xi Hu · Hui Huang · Li Zhang · You Huang · Zhu-Fang Shen · Ke-Di Cheng · Guan-Hua Du · Ping Zhu

Received: 18 November 2008 / Revised: 8 February 2009 / Accepted: 8 February 2009 / Published online: 8 March 2009

Abstract Dipeptidyl peptidase (DPP) IV inhibitors provide a new strategy for the treatment of type 2 diabetes. Human DPP-IV gene was cloned from differentiated Caco-2 cells and expressed in *Pichia pastoris*. The recombinant enzyme was used in a new system for screening of DPP-IV inhibitors. By high throughput screening, a novel compound (W5188) was identified from 75,000 compounds with an IC_{50} of 6.5 μM. This method is highly reproducible and reliable for discovery of DPP-IV inhibitors as shown by Z′ value of 0.73 and S/N ratio of 6.89.

Keywords DPP-IV inhibitor · High throughput screening (HTS) · Diatetes · Recombinant human dipeptidyl peptidase IV (rhDPP-IV)

Introduction

Glucagon-like peptide-1 (GLP-1) is an incretin secreted by intestinal L-cells in response to food ingestion. Its biological effects include glucose-dependent enhancement of insulin secretion, inhibition of glucagon secretion, slowing of gastric emptying and reduction of food intake (Arulmozhi and Portha 2006). However, GLP-1 action has a short half life due to its degradation by dipeptidyl peptidase-IV (DPP-IV). DPP-IV is a homodimeric glycoprotein with an M_r of 110–150 kDa per subunit and is expressed in many tissues (Ludwig et al. 2003). Inhibition of DPP-IV prevents GLP-1 degradation in vivo, stimulates insulin secretion and improves

C.-X. Hu · K.-D. Cheng · P. Zhu (✉)
Department of Biosynthesis of Natural Products, Institute of Materia Medica, Peking Union Medical College & Chinese Academy of Medical Sciences, Beijing 100050, China
e-mail: Jack864900@sohu.com; zhuping@imm.ac.cn

C.-X. Hu
e-mail: Suhappy99@sohu.com

K.-D. Cheng
e-mail: chengkd@imm.ac.cn

H. Huang · Z.-F. Shen
Department of Pharmacology, Institute of Materia Medica, Peking Union Medical College & Chinese Academy of Medical Sciences, Beijing 100050, China

H. Huang
e-mail: Huanghui@imm.ac.cn

Z.-F. Shen
e-mail: shenzhf@imm.ac.cn

L. Zhang · G.-H. Du
National Centre for Drug Screening, Institute of Materia Medica, Peking Union Medical College & Chinese Academy of Medical Sciences, Beijing 100050, China

L. Zhang
e-mail: Zhangli@imm.ac.cn

G.-H. Du
e-mail: dugh@imm.ac.cn

Y. Huang
Institute and State Key Laboratory of Elemento-organic Chemistry, Nankai University, Tianjin 300071, China
e-mail: hyou@nankai.edu.cn

glucose tolerance (Mest and Mentlein 2005). Several DPP-IV inhibitors, such as Sitagliptin (Merck Co.) and Vildagliptin (Novartis Co.), are effective in the treatment of Type 2 diabetes.

Previous methods for screening DPP-IV inhibitors utilized DPP-IV isolated from pig liver or kidney, or Caco-2 cell lysates (Kim et al. 2005b). However, the identity of amino acid sequence of DPP-IV between human and pig or mouse is 85% (Misumi et al. 1992). Moreover, the 3D structure of native DPP-IV is a key factor in regulating the interaction with other components (Engel et al. 2003; Thoma et al. 2003). Therefore, the differences between DPP-IVs from human and other species might influence the specificity to their inhibitors. On the other hand, although human colon cancer cell line Caco-2 has been used to screen DPP-IV inhibitors, the components in crude cell lysates may greatly influence its specificity and it is not economical to culture Caco-2 cells for large-scale screening. In this report, we developed a new strategy by cloning and expressing human DPP-IV in *Pichia pastoris*. Partially purified recombinant human DPP-IV was then applied in a new screening protocol to search for inhibitors. By high throughput screening, a novel compound (W5188) was identified.

Materials and methods

Cloning, expression and purification of recombinant human DPP-IV (rhDPP-IV)

Human colon cancer cell line Caco-2 (from the Peking Union Medical College, Beijing, China) were cultured in MEM medium containing 1% (v/v) non-essential amino acids (Gibco) and 10% (v/v) FBS at 37°C and in a humidified 5% CO_2 environment for 12 days to induce differentiation of the cells (Baricault et al. 1995). DPP-IV cDNA was obtained from total RNA of differentiated Caco-2 cells with ThermoScript RT-PCR Kit (Invitrogen Co.). PCR primers were: FW1 (5′-G**GAATTC**ATGAAGACAC CGTGGAAG-3′) with an *Eco*RI site (bold letters); RV1(5′-G**GCGGCCGC**TCA*GTGGTGGTGGTGGT GGTG*AGGTAAAGAGAAACATTG-3′) with a *Not*I site (bold letters) and 6 × his tag (italics). Then the cDNA was subcloned into pPIC9 K plasmid (Invitrogen Co.) resulting in the recombinant plasmid pPIC9 K-rdpp (Fig. 1).

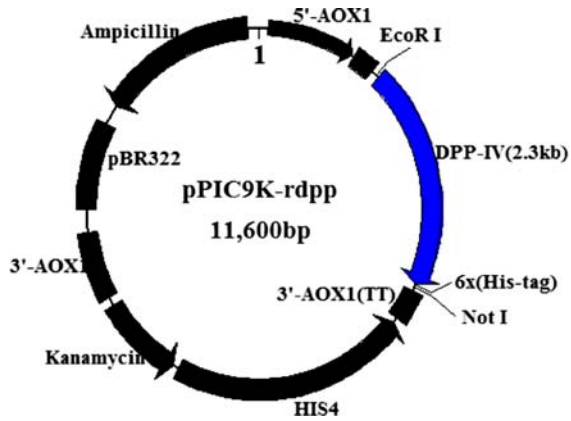


Fig. 1 Schematic diagram of recombinant plasmid pPIC9K-rdpp construction. The recombinant plasmid contains 5′-AOX1 promoter region, DPP-IV coding region, 6×his-tag, 3′-AOX1 transcription termination region (TT), kanamycin and ampicillin resistant gene, etc

The recombinant plasmid pPIC9 K-rdpp was transformed into *Pichia pastoris* GS115 (Invitrogen Co.) by electroporation. The original pPIC9 K plasmid was also transformed resulting in mock transformant as control. Twenty positive transformants with phenotype Mut^+ were screened for the expression of rhDPP-IV. Transformants were grown at 30°C in YPD liquid medium (1% yeast extract, 2% peptone and 2% glucose) for 24 h, then in YPM liquid medium (1% yeast extract, 2% peptone and 2% methanol) in baffled shake-flasks at 30°C and 280 rpm for 5 days. Methanol at 2% (v/v) was added to YPM medium every day. The culture filtrate was centrifuged at 15,000*g* for 20–30 min. The supernatant was freeze-dried and dialyzed in 50 mM Tris/HCl (pH 8.0). After $(NH_4)_2SO_4$ precipitation, the 40–80% fraction was purified on gel chromatography (Sephacryl S-200 HR column) eluting with 50 mM Tris/HCl (pH 8.0) containing 100 mM NaCl. The fractions of first protein peak were collected and concentrated as rhDPP-IV stock solution.

The rhDPP-IV was verified by 9% (v/v) SDS-PAGE and Western blot analysis (the first antibody was anti-his-tag monoclonal antibody (Novagen Co.).

Determination of rhDPP-IV enzymatic activity

DPP-IV standard protein (isolated from pig kidney) and its substrate Gly-Pro-*p*-nitroanilide hydrochloride were from Sigma. Each well contained 150 µl

substrate (0.26 mM, in 100 mM Tris/HCl, pH 8.0, buffer) and 50 µl rhDPP-IV stock solution (50 mU ml^{-}) in a 96-well plate. The plate was incubated at 37°C for 60 min. The rhDPP-IV activity was defined as the increase in absorbancy between 0 and 60 min (at 405 nm).

Activities of standard DPP-IV dilutions with a series of concentrations were also measured and plotted as a standard curve (activity/concentration). The rhDPP-IV concentration was determined from the standard curve.

Validation of screening method in 96-well plate

(*S*)-1-Cyclohexyl-2-oxo-2-(thiazolidin-3-yl)-ethylamine, INDP-2 (a homologue of Sitagliptin, see Table 1) was used as reference compound. Its synthetic route was provided by Dr. Jinyou Xu, Merck Co., USA. Each well contained 125 µl substrate (0.26 mM), 50 µl rhDPP-IV stock solution (50 mU ml^{-1}), and 25 µl INDP-2 solution. INDP-2 was from 8 nM to 8.4 µM. A concentration-activity curve was obtained for the calculation of its IC_{50}.

Calculation of Z′ factor and S/N ratio

The reaction rate of DPP-IV at 405 nm was linear at 37°C with up to 0.3 mM product for up to 120 min (Nagatsu et al. 1976). To assess the robustness of this screening method, the parameter of Z′ factor was used:

$$Z' = 1 - \left[(3\sigma_c + 3\sigma_{c-})/(\mu_{c+} - \mu_{c-})\right]$$

wherein, σ is the standard deviation of the data, μ is the mean of a sample and the subscripts "c+" and "c−" mean positive and negative controls respectively (Zhang et al. 1999). Data of 80 positive control wells (INDP-2, 100 µg ml^{-1}) and 80 negative control wells (100 mM Tris/HCl buffer without test compound) were used to calculate the Z′ factor.

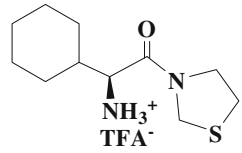
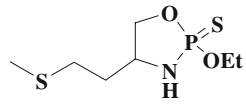
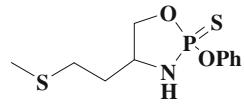
The signal/noise ratio is defined as: S/N = (mean value of negative controls − mean value of backgrounds)/mean value of backgrounds. All the data were expressed as mean ± SD and statistical analysis was performed by Microsoft Excel software.

Results

Production and validation of rhDPP-IV

Human *DPP-IV* cloned from Caco-2 cells was 2.3 kb and with 100% homogeneity to the reported sequence (GenBank BC065265). Compared with the culture filtrate of mock transformant, rhDPP-IV was identified as a 110-120 kDa protein by SDS-PAGE and Western blot (Fig. 2). According to concentration-activity curve of pig DPP-IV, the rhDPP-IV stock

Table 1 Inhibitory effects of compounds against rhDPP-IV

Compound	Structure	IC_{50} (µM)
INDP-2[a] (reference compound)		0.46
W5188		6.5
W5186		43.5

Note: [a] INDP-2 is a known inhibitor of DPP-IV. The listed IC_{50} value of INDP-2 was consistent with published data (Xu et al. 2006)

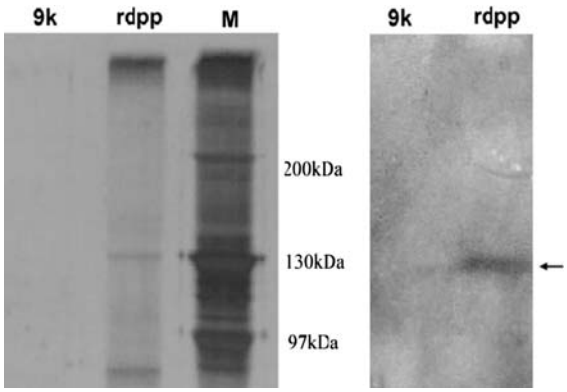


Fig. 2 Silver-stained SDS-PAGE (left) and Western blot (right) of culture filtrates of transformants. 9k: mock transformant. rdpp: rhDPP-IV transformant. M: MW marker. The band was identified around 110–120 kDa (arrow)

solution was made at 50 mU ml^{-1}. The yield of rhDPP-IV was calculated to be about 1.2 mg l^{-1} of growth medium.

Establishment of screening system

After studying the influence of substrate concentration and enzyme amounts on enzymatic activity (Figs. 3 and 4), we establish the screening system in 96-well plates and 384-well plates.

Each test well in 96-well plate (total volume of 200 µl) contained: 50 µl rhDPP-IV (50 mU ml^{-1}); 125 µl Gly-Pro-p-NA hydrochloride (0.26 mM); 25 µl test compound (0.1 mM ~ 0.1 nM). Reagents

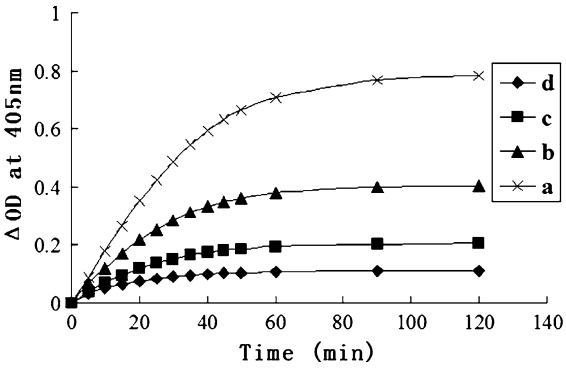


Fig. 3 Influence of the substrate concentration on rhDPP-IV activity. Each well in a 96-well plate contains 150 µl substrate solution and 50 µl rhDPP-IV stock solution (50 mU ml^{-1}). The substrate concentrations were: (**a**) 0.26 mM (symbol ×), (**b**) 0.13 mM (symbol ▲), (**c**) 0.065 mM (symbol ■), and (**d**) 0.0325 mM (symbol ◆)

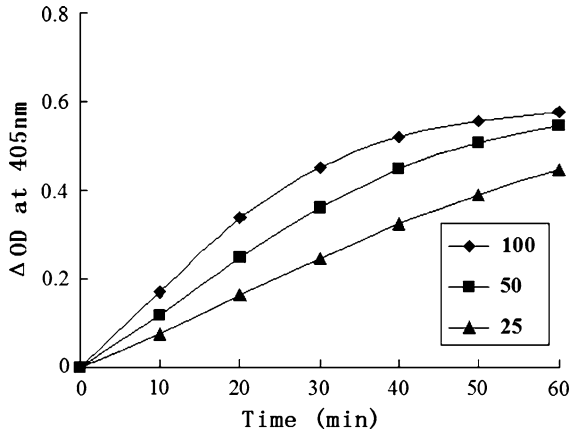


Fig. 4 Influence of the rhDPP-IV concentration on rhDPP-IV activity. The total volume was 200 µl and the substrate concentration was 0.26 mM. The rhDPP-IV amount is 25 µl (symbol ▲), 50 µl (symbol ■) and 100 µl (symbol ◆) (corresponding to 6.25, 12.5 and 25 mU ml^{-1}, respectively)

used in 384-well plate (total volume of 40 µl) were decreased proportionally.

Evaluation of the assay reproducibility

Z′ factor and S/N ratio were used to evaluate the reproducibility. The mean values of △ A over 60 min of positive control and negative control were 0 ± 0.01 and 0.383 ± 0.025 (Fig. 5). Therefore, the Z′ factor for this assay was calculated to be 0.73. Z′ > 0.5 indicates that the assay is applicable, and the closer the Z′ factor is to 1, the higher its reliability is (Zhang et al. 1999). The S/N ratio of HTS screening was calculated as 6.89. In general, a screening method is reliable when the S/N ratio is more than 5. Therefore, Z′ factor and S/N ratio both showed the stability and reliability of our method.

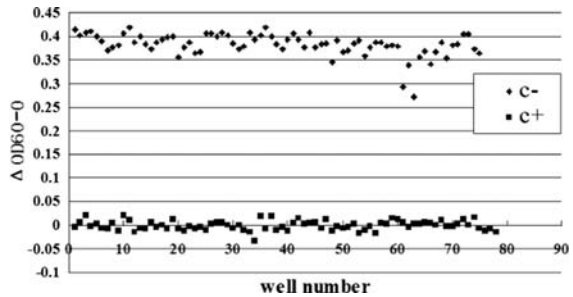


Fig. 5 Z′ factor assay for 384-well method. 80 positive control wells (c+, INDP-2 compound solution (100 µg ml^{-1}), symbol ■) and 80 negative control wells (c−, 100 mM Tris/HCl buffer with no test compound, symbol ◆) were tested

New discovery of DPP-IV inhibitors screening

In high throughput screening, all addition were performed robotically. The test compound was at 100 μg ml^{-1}. Plates were incubated at 37°C for 60 min. Each plate contains one positive control well (INDP-2, 100 μg ml^{-1}) and one negative control well (100 mM Tris/HCl buffer). The inhibition percentage was calculated according to the formula below. Only compounds with inhibition percentage more than 50% were selected as candidates.

$$\text{Inhibition}(\%) = [(\Delta A_{405} \text{ of negative control} - \Delta A_{405} \text{ of test compound})/\Delta A_{405} \text{ of negative control}] \times 100\%.$$

A total of 75,000 compounds in our chemical library were screened with HTS. Two compounds (W5186 and W5188, see Table 1) were confirmed as active inhibitors. Compound W5188 was more effective than W5186 and was selected for further study. W5188 inhibited DPP-IV in concentration dependent manner (Fig. 6). The IC_{50} values of W5188 and INDP-2 were 6.5 and 0.46 μM, respectively (Table 1).

Discussion

Recently, several in vitro methods based on DPP-IV from pig, rat or mouse tissues or cultured human cells (e.g. from Caco-2 cells) have been used to identify DPP-IV inhibitors (Kim et al. 2005a, b). However, inhibitors of non-human DPP-IV might lack specificity when used against human targets. On the other hand, protocols using raw human cell lysate can have reduced sensitivity and S/N ratio due to disturbance of other unknown substances in the extract. In our new strategy, we solved these problems by expressing human DPP-IV gene in *P. pastoris* and purifying secreted rhDPP-IV protein from the media. The rhDPP-IV leads to higher S/N ratio of 6.89. Furthermore, large-scale screening demands a sufficient supply of DPP-IV protein. Our protocol is therefore more economical, sensitive and convenient than the protocols based on DPP-IV from non-human tissues (Fukasawa et al. 1978, 1981; Kyouden et al. 1992) or even crude human cell lysates.

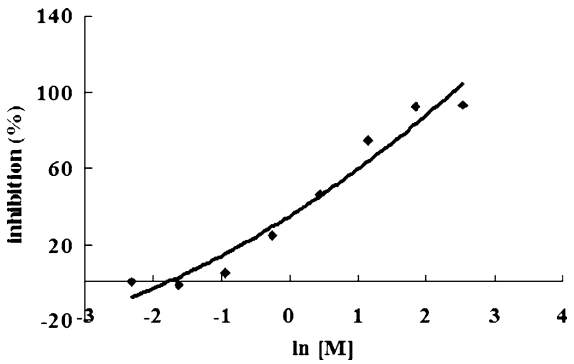


Fig. 6 Dose-dependent inhibitory effects of W5188. Symbol ◆ represents the inhibition percentage ($n = 3$ of separate experiments). Each well contains 25 μl substrate (0.26 mM), 10 μl rhDPP-IV stock solution (50 mU ml^{-1}) and 5 μl W5188 compound at 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195 and 0.098 μg ml^{-1}

So far, there are three classes of DPP-IV inhibitors: reversible product analogue inhibitors (e.g. pyrrolidines and thiazolidines), covalently-modified product analogues (e.g. cyanopyrrolidines) and reversible non-peptidic heterocyclic inhibitors (e.g. xanthines and aminomethylpyrimidines). Some of them have a five- or six-member ring derived from proline. Though compound W5188 (see Table 1) had a five-member ring structure, our findings suggested a novel class of rhDPP-IV inhibitors (such as W5188) which have a completely different heteroatom (P, O and N) in the ring structure as compared with other inhibitors. This new class of inhibitors has not been reported previously and is presumed to be a covalently modified product analogue. Moreover, the selectivity of DPP-IV inhibitors against other isoenzymes appears to be essential for the development of safe agents. So the structure-activity relationship research of its derivatives and study of their selectivity to DPP-IV isoenzymes are now under progress.

Acknowledgements We thank Dr. Jinyou Xu in Merck Co. (USA) for providing the synthetic route of INDP-2 and thank Dr. Haihong Huang and Dr. Ailin Liu in our Institute for chemical synthesis of the positive compound and technical assistance of compound library, respectively. Also, we thank Dr. Chenhui Zou in Boston, USA for his technical assistance. This work was partially supported by the Hi-Tech Research and Development (863) Program of China (2002AA27343B).

References

Arulmozhi DK, Portha B (2006) GLP-1 based therapy for type 2 diabetes. Eur J Pharm Sci 28(1–2):96–108

Baricault L, Fransen JA, Garcia M, Sapin C, Codogno P, Ginsel LA, Trugnan G (1995) Rapid sequestration of DPP IV/CD26 and other cell surface proteins in an autophagic-like compartment in Caco-2 cells treated with forskolin. J Cell Sci 108(5):2109–2121

Engel M, Hoffmann T, Wagner L, Wermann M, Heiser U, Kiefersauer U (2003) The crystal structure of dipeptidyl peptidase IV (CD26) reveals its functional regulation and enzymatic mechanism. Proc Natl Acad Sci USA 100(9):5063–5068

Fukasawa KM, Fukasawa K, Harada M (1978) Dipeptidyl aminopeptidase IV, a glycoprotein from pig kidney. Biochim Biophys Acta 535:161–166

Fukasawa KM, Fukasawa K, Hiraoka BY, Harada M (1981) Comparison of dipeptidyl peptidase IV prepared from pig liver and kidney. Biochim Biophys Acta 657:179–189

Kim D, Wang L, Beconi M, Eiermann GJ, Fisher MH, He H (2005a) (2R)-4-oxo-4-[3-(trifluoromethyl)-5, 6-dihydro[1, 2, 4]triazolo[4, 3-a]pyrazin-7(8H)-yl]-1-(2, 4, 5-tri-fluorophenyl)butan-2-amine: a potent, orally active dipeptidyl peptidase IV inhibitor for the treatment of type 2 diabetes. J Med Chem 48:141–151

Kim KR, Rhee SD, Kim HY, Jung WH, Yang SD, Kim SS, Ahn JH (2005b) KR-62436, 6-{2-[2-(5-Cyano-4, 5-dihydropyrazol-1-yl)-2-oxoethylamino]ethylamino}nicotinonitrile, is a novel dipeptidyl peptidase-IV (DPP-IV) inhibitor with anti-hyperglycemic activity. Eur J Pharmacol 518(11):63–70

Kyouden T, Himeno M, Ishikawa T, Ohsumi Y, Kato K (1992) Purification and characterization of dipeptidyl peptidase IV in rat liver lysosomal membranes. J Biochem 111: 770–777

Ludwig K, Yan S, Fan H, Reutter W, Bottcher C (2003) The 3D structure of rat DPPIV/CD26 as obtained by cryo-TEM and single particle analysis. Biochem Biophys Res Commun 304(1):73–77

Mest HJ, Mentlein R (2005) Dipeptidyl peptidase inhibitors as new drugs for the treatment of type 2 diabetes. Diabetologia 48:616–620

Misumi Y, Hayashi Y, Arakawa F, Ikehara Y (1992) Molecular cloning and sequence analysis of human dipeptidyl peptidase IV, a serine proteinase on the cell surface. Biochim Biophys Acta 1131(3):333–336

Nagatsu T, Hino M, Fuyamada H, Hayakawa T, Sakakibara S, Nakagawa Y (1976) New chromogenic substrates for X-prolyl dipeptidyl-aminopeptidase. Anal Biochem 74: 466–476

Thoma R, Loffler B, Stihle M, Huber W, Ruf A, Hennig MF (2003) Structural basis of proline-specific exopeptidase activity as observed in human dipeptidyl peptidase IV. Structure 11:947–959

Xu J, Wei L, Mathvink RJ, Edmondson SD, Eiermann GJ, He H, Leone JF, Leiting B, Lyons KA, Marsilio F, Patel RA, Patel SB, Petrov A, Scapin G, Wu JK, Thornberry NA, Weber AE (2006) Discovery of potent, selective, and orally bioavailable oxadiazole-based dipeptidyl peptidase IV inhibitors. Bioorg Med Chem Lett 16(20):5373–5377

Zhang J, Chung TDY, Oldenburg KR (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 4(2):67–73