Biochemical Pharmacology 78 (2009) 203–210

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Biochemistry, pharmacokinetics, and toxicology of a potent and selective DPP8/9 inhibitor

Jia-Jing Wu [a,1], Hung-Kuan Tang [a,1], Teng-Kuang Yeh [a], Chi-Min Chen [b], Hrong-Shing Shy [a], Yue-Ru Chu [a], Chia-Hui Chien [a], Ting-Yueh Tsai [a], Yu-Chen Huang [a], Yu-Lin Huang [a], Chih-Hsiang Huang [a], Huan-Yi Tseng [a], Weir-Torn Jiaang [a], Yu-Sheng Chao [a], Xin Chen [a,*]

[a] Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, No. 35 Keyan Rd., Zhu Nan Town, Miaoli County 350, Taiwan, ROC
[b] Animal Technology Institute Taiwan, Miaoli 350, Taiwan, ROC

ARTICLE INFO

Article history:
Received 3 February 2009
Accepted 31 March 2009

Keywords:
Dipeptidyl peptidase
DPP8
DPP9
Inhibitor
Pharmacokinetics
Toxicity

ABSTRACT

DPP-IV (EC 3.4.14.5) is a validated drug target for human type II diabetes. DPP-IV inhibitors without DPP8/9 inhibitory activity have been sought because a possible association has been reported between a "DPP8/9 inhibitor" and severe toxicity in animals. However, at present, it is not known whether the observed toxicity is associated with DPP8/9 inhibition, or an off-target effect induced by the compound. We investigated whether the inhibition of DPP8/9 is the cause of the severe toxicity in animals using a very potent and selective DPP8/9 inhibitor with different pharmacophore, 1G244. By Ki measurement, 1G244 is 15- and 8-fold more potent against DPP8 and DPP9, respectively, than the "DPP8/9 inhibitor". Strikingly, the "DPP8/9 inhibitor" does not penetrate the plasma membrane but remains outside the cells, whereas 1G244 readily enters the cells, even at low doses. By repeatedly exposing Sprague–Dawley rats to 1G244 by intravenous injection for a period of 14 days, we observed no significant toxicological symptoms associated with 1G244. Blood and serum chemistry parameters were all within the normal ranges for the treated animals. Because of the high potency, good membrane penetration and adequate tissue distribution of 1G244, the mild symptoms observed are probably associated with DPP8/9 inhibition.

1. Introduction

Dipeptidyl peptidase 8 (DPP8) and DPP9 belong to the prolyl peptidase family, which also includes DPP-IV (EC 3.4.14.5), fibroblast activation protein (FAP), prolyl oligopeptidase, and others [1]. Among these, DPP-IV is a validated drug target for human type II diabetes [2,3]. Chemical inhibitors of DPP-IV are effective in the treatment of type II diabetes, lowering blood sugar levels and improving beta cell function [4,5]. This inhibition is well tolerated and does not cause hypoglycemia or increase body weight in human [5,6]. Two inhibitors of DPP-IV, sitagliptin and vildagliptin, have been on the market since 2007. Two others, saxagliptin and alogliptin, have been submitted to the US Food and Drug Administration (FDA) as new drug applications.

Compared with well-studied DPP-IV, the functions of DPP8 and DPP9 are unclear. Knockout mice for DPP8 or DPP9 are not available. DPP8 and DPP9 are highly homologous proteins, with 62% sequence identity [7], and both are ubiquitously expressed [8,9]. They are dimeric soluble proteins that localize in the cytosol, with similar amino-dipeptidase activities [10,11]. Recently, it was demonstrated that the expression levels of DPP8 and DPP9 are upregulated in rats with experimentally induced asthma [12]. Despite tremendous efforts in the field, no potent and selective inhibitors of either DPP8 or DPP9 alone are available [13–16]. So far, the most potent and selective inhibitors of both DPP8 and DPP9 reported are 1G244 (compound 1), which we discovered previously, and the DPP8/9 inhibitor (compound 2) (Fig. 1) [13,17].

The potential functions of DPP8 and DPP9 were deduced in an inhibitor study with compound 2. The administration of compound 2 induces severe toxicity and various pathological symptoms, including alopecia, thrombocytopenia, anemia, enlarged spleen and death in rats, and bloody diarrhea in dogs [17]. Based on that study, great emphasis has been given in the search for DPP-IV

Abbreviations: DPP, dipeptidyl peptidase; DPP-IV, dipeptidyl peptidase IV; FAP, fibroblast activation protein; FDA, the US Food and Drug Administration; IV, intravenous; SD, Sprague–Dawley; GLP, Good Laboratory Practice; CC_{50}, cytotoxic concentration; AUC, the area under the curve.

* Corresponding author. Tel.: +886 37 246166x35718; fax: +886 37 586456.
E-mail address: xchen@nhri.org.tw (X. Chen).

[1] Equal contribution.

0006-2952/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2009.03.032

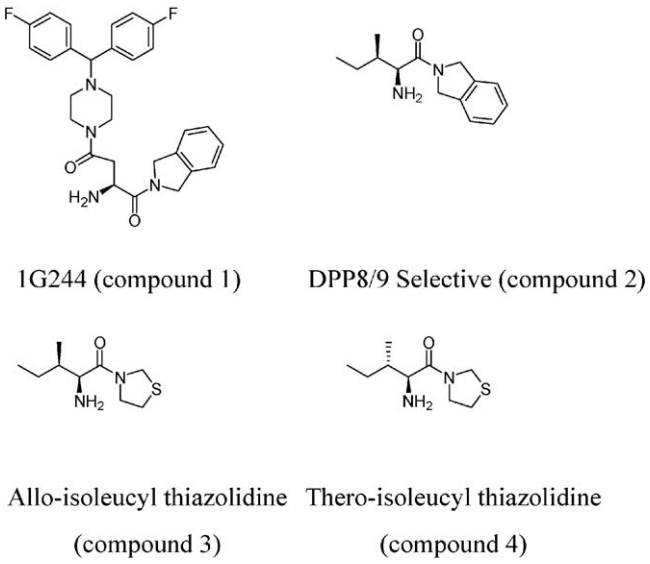

Fig. 1. Chemical structures of DPP8/9 inhibitors.

inhibitors to the "cleanness" of the compound, devoid of any DPP8/9 inhibitory activity. However, at present, it is not known whether the observed toxicity is associated with DPP8/9 inhibition, or whether it is an off-target effect induced by the compound. From the literature, sitagliptin and alogliptin have no DPP8/9 inhibitory activity, whereas vildagliptin has quite potent activities against both DPP8 and DPP9, especially DPP9 [17–19]. Vildagliptin has not yet been approved by the US FDA, though it was approved and on the market in Europe.

It is important to understand whether the inhibition of DPP8/9 is the cause of the severe toxicity observed in animals. A toxicological study with vildagliptin was carried out with high oral doses in a 13-week study to try to answer this question [20]. Vildagliptin is a potent DPP-IV inhibitor and a fairly weak DPP8/9 inhibitor, with Ki values of 810 and 95 nM, respectively [20]. Despite high concentrations of vildagliptin in the blood (above 2 μM), no toxicological consequences were observed with vildagliptin [20]. Because vildagliptin is a highly hydrophilic compound, questions have been raised about how well it penetrates the cell membrane to inhibit cytosolic DPP8/9 [21]. Therefore, whether DPP8/9 inhibition is the direct cause of toxicity in mammals remains to be resolved.

Here we report the characterization of the biochemical and pharmacokinetic properties of 1G244, and the investigation of its toxicological effects through repeated exposure of Sprague–Dawley (SD) rats to 1G244 by intravenous (IV) injection for a period of 14 days. The rats were evaluated with respect to mortality/moribundity, body weight, clinical signs, clinical pathology (hematology and serum chemistry), organ weights and gross necropsy.

2. Materials and methods

2.1. Materials

Strep-Tactin® resin was purchased from EMD Chemicals Inc. (Darmstadt, Germany). The enzyme substrates Ala–Pro–pNA and Gly–Pro–pNA were purchased from Bachem (Torrance, CA, USA). 1G244 was synthesized by Ryss Inc. (Taipei, Taiwan), as described previously [13]. Compound 2 was synthesized according to Lankas et al. [17]. Principles of laboratory animal care' (NIH publication no. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) were followed.

2.2. Expression and purification of human recombinant DPP-IV, DPP8, DPP9, DPP2 and FAP proteins

DPP8, DPP9, DPP-IV and FAP were expressed and purified as described previously [11,22–26]. Recombinant DPP2 protein was purified as described previously, with some modifications [27,28]. The purity of the protein was confirmed by SDS-PAGE with Coomassie blue staining. The protein concentrations were determined with the Bradford method using bovine serum albumin as the standard.

2.3. IC_{50} determination

The assay was carried out essentially as described previously [13,29–32]. More specifically, enzyme activities were assayed in a total volume of 100 μL for 30 min at 37 °C at an emission wavelength of 405 nM with a Power Wave X spectrometer (Bio-Tek Instrument, Inc., Winooski, VT, USA). The IC_{50} values for DPP8, DPP9 and FAP were determined in phosphate-buffered saline (PBS, pH 8.0), in the presence of 2.5 mM Gly–Pro–pNA, 1.5 mM Gly–Pro–pNA and 1.5 mM Ala–Pro–pNA, respectively. The IC_{50} for DPP-IV was determined in 2 mM Tris–HCl (pH 8.0) in the presence of 500 μM Gly–Pro–pNA. The IC_{50} for DPP2 was determined in PBS (pH 5.5), in the presence of 1.5 mM Gly–Pro–pNA. The inhibitor concentrations ranged from 100 to 0.003 μM. IC_{50} values were computed with commercially available curve-fitting programs such as SigmaPlot.

2.4. Inhibition constant (Ki) measurement

The inhibitory activity of 1G244 against DPP8 or DPP9 was determined from its ability to inhibit the hydrolysis of Ala–Pro–pNA. Ki was measured as described previously, with a Beckman DU 800 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA) [28,33]. The assay was performed in PBS in a total volume of 100 μL with 10 nM DPP8 or 10 nM DPP9. For the slow-tight binding assay of DPP8 with 1G244, the concentration of the substrate was 125 μM, and the concentrations of 1G244 ranged from 0 to 12.5 μM. For the competitive binding assays, the substrate and inhibitor concentrations were 62.5–500 μM and 0–50 μM for DPP8 with compound 2; 93.75–750 μM and 0–30 nM for DPP9 with 1G244; and 93.75–750 μM and 0–200 nM for DPP9 with compound 2, respectively. The hydrolysis of the Ala–Pro–pNA substrate was monitored continuously by measuring the light emission at 405 nM for 3–5 min. The light intensity was corrected using a standard curve. The data were fitted to slow-binding inhibition and competitive inhibition as described previously [28,33].

2.5. Mammalian cell culture and inhibitor uptake

The mammalian cells were cultured with a standard protocol suggested by the American Type Culture Collection (ATCC). To measure the inhibitor uptake into the mammalian cells, human embryonic kidney (HEK) 293T cells were cultured in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) or serum-free DMEM (Invitrogen, Carlsbad, CA, USA). The inhibitors were dissolved in dimethyl sulfoxide (DMSO), added to the culture medium, and incubated with the cells for 6 h. The final DMSO concentration was less than 1%. After treatment, the cells were collected and washed five times with PBS. The cells were lysed in lysis buffer containing 142.5 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes (pH 7.4), 1 mM EGTA and 0.2% NP40 on ice for 15 min. The cell lysates were clarified and collected, and the total protein concentrations were quantified with the Bio-Rad Protein Assay Kit (Bio-Rad laboratories, Hercules, CA, USA). Equal amounts of protein