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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

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Abstract

Dipeptidyl peptidase-IV (DPP-IV) is involved in the inactivation of glucagon-like peptide-1 (GLP-1), a potent insulinotropic peptide. Thus, DPP-IV inhibition can be an effective approach to treat type 2 diabetes mellitus by potentiating insulin secretion. This study describes the biological effects of a new DPP-IV inhibitor, KR-62436 (6-{2-[2-(5-cyano-4,5-dihydropyrazol-1-yl)-2-oxoethylamino]ethylamino} nicotinonitrile) in vitro and in vivo. KR-62436 inhibited rat plasma DPP-IV, porcine kidney DPP-IV as well as human DPP-IV (Caco-2) with IC_{50} values of 0.78, 0.49, 0.14 μM, respectively. In addition, the compound (10 μM) almost completely inhibited DPP-IV-mediated degradation of GLP-1 in vitro. KR-62436 inhibited the enzyme in a competitive manner, and exhibited selectivity against several proteases including proline-specific proteases. In vivo efficacy of the compound was examined by using normal C57BL/6J mice and *ob*/*ob* mice, a type 2 diabetes animal model. Administration of KR-62436 to C57BL/6J mice either orally or subcutaneously resulted in the suppression of plasma DPP-IV activity, increase in intact GLP-1 and insulin levels in plasma. Furthermore, the plasma glucose concentrations during oral glucose tolerance test (OGTT) were reduced upon oral administration of KR-62436. This study demonstrates that KR-62436 could be a good lead compound for further development as a new anti-diabetic agent.

Keywords: Dipeptidyl peptidase-IV; Glucagon-like peptide-1; Insulin; Oral glucose tolerance test; *Ob*/*ob* mice

1. Introduction

Glucagon-like peptide-1 (GLP-1) is an incretin secreted from the L cells of the small intestine in response to food ingestion, and exhibits several biological effects including stimulation of insulin secretion, inhibition of glucagon secretion, slowing of gastric emptying and induction of satiety (Holst and Deacon, 2004). However, GLP-1 action has a very short half life of about 1 min due to its degradation by dipeptidyl peptidase-IV (DPP-IV) (Mentlein et al., 1993; Deacon et al., 1995). DPP-IV is a serine protease which removes the dipeptides from the N-terminus of substrate peptides by cleaving post proline or alanine residues. DPP-IV is expressed in many tissues and body fluids, and exists as either a membrane-bound or a soluble enzyme (Mentlein, 1999).

Since the discovery that GLP-1 secretion is impaired but retained its potency in type 2 diabetes (Toft-Nielsen et al., 2001; Gutniak et al., 1992; Nathan et al., 1992), several approaches have been employed to enhance GLP-1 action, thereby ameliorating type 2 diabetes (Drucker, 2001, 2003). Among them, DPP-IV inhibition has been proved to be an effective method for type 2 diabetes pharmacotherapy. Acute inhibition of DPP-IV activity by specific DPP-IV inhibitors has been shown to prevent GLP-1 degradation

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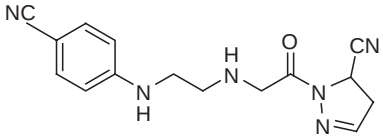

Fig. 1. Chemical structure of KR-62436.

and thus augment plasma insulin level in animal studies (Ahren et al., 2000; Villhauer et al., 2002; Pospisilik et al., 2002). Furthermore, the clinical efficacy of the inhibitors such as NVP-DPP728 and LAF-237 has been reported (Ahren et al., 2002; Ahren et al., 2004).

In the present study, we discovered an acylpyrazoline scaffold as a novel DPP-IV inhibitor by high throughput screening using compound collections from Korea Chemical Bank. Here, we report some biological effects of KR-62436, a representative compound, and propose KR-62436 as a good lead compound for further development. The structure of KR-62436 is shown in Fig. 1.

2. Materials and methods

2.1. Materials

All chemicals were purchased from commercial suppliers: Ala-Pro-7-amino-4-trifluoromethylcoumarin (AFC) (Enzyme system products); *Z*-Gly-Pro-methylcoumaryl-7-amide (MCA) and Cbz-Glu-Tyr (Fluka Co.); DPP-IV (porcine kidney), elastase, trypsin, *N*α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA), aminopeptidase N, leucine-*p*-nitroanilide, carboxypeptidase P, and ninhydrin (Sigma-Aldrich Co.). Prolyl oligopeptidase was prepared from the brain cortex of Sprague–Dawley rats, and the supernatant obtained from the centrifugation of the homogenates was used as the enzyme source. DPP-II was prepared from the crude lysates of COS-7 cells transfected with pcDNA3.1-DPP-II cDNA. To determine DPP-IV activity from lysates of Caco-2 cells (human colonic adenocarcinoma cells), the cells were cultured and induced differentiation as previously described (Hara et al., 1993), and lysed by lysis buffer (0.15 M NaCl, 1% Triton X 100, 10 mM Tris–HCl, pH 7.4, 10% glycerol) and the supernatant after centrifugation at 12,000 rpm for 10 min was used as the enzyme source. Experimental protocols concerning the use of laboratory animals conformed to the European Community guidelines for the use of experimental animals.

2.2. DPP-IV enzyme assay

DPP-IV activity was measured using Ala-Pro-AFC as a substrate. Briefly, 20 μl of aliquots of enzyme sources (rat plasma, porcine kidney, Caco-2 lysates) were added to 96-well plates, followed by the addition of 160 μl of reaction buffer (50 mM Tris, pH 7.5) containing KR-62436. The compound was dissolved in DMSO and the concentration of stock solution was 2 mM. The reaction was initiated by addition of 20 μl Ala-Pro-AFC (final 40 μM), and incubated for 1 h at room temperature. The fluorescence was measured using fluorometer (Synergy HT, BioTek; excitation 360 nm, emission 485 nm).

To further confirm the inhibitory effect of KR-62436 on DPP-IV, the protection of GLP-1 degradation by DPP-IV action was measured in vitro by using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI–TOF MS) as described previously (Pauly et al., 1996). The reaction mixture contained 15 μM GLP-1 amide (aa 7-36), porcine kidney DPP-IV (1 μU) and 10 μM KR-62436 in a buffer containing 0.1 mM Tricine (pH 7.5). After 24 h incubation at room temperature, the reaction mixture was applied to MALDI–TOF MS using α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix solution. The amount of intact GLP-1 and inactive GLP-1 was quantitated.

2.3. DPP-IV inhibition kinetics

Initial rates at five different concentrations of KR-62436 (0.25, 0.5, 1, 2, 4 μM) were measured. For each concentration, measurements were performed at seven different concentrations (12.5, 25, 50, 100, 200, 400, 800 μM) of Ala-Pro-AFC in a buffer containing 50 mM Tris/HCl, pH 7.5. The inhibition pattern was evaluated, and K_i was determined using a direct curve fitting program (Grafit Software).

2.4. Isoenzyme selectivity

The selectivity of KR-62436 was determined against several enzymes including elastase, trypsin, DPP-II, and post proline-cleaving enzymes. For DPP-II enzyme assay, the enzyme (10 μg/well) was incubated in a buffer containing 50 mM sodium acetate buffer (pH 5.0) and 40 μM Ala-Pro-AFC with or without KR-62436 for 1 h at room temperature. The fluorescence of AFC was determined (excitation 360 nm; emission 485 nm). For trypsin enzyme assay, the enzyme (2 μg/well) was incubated in a buffer containing 50 mM Tris buffer (pH 8.0) and 2 mM BAPNA with or without KR-62436 for 1 h at room temperature. The absorbance at 405 nm was measured for the detection of *p*-nitroanilide released from enzyme reaction. For elastase enzyme assay, the enzyme (0.8 units/ml) was incubated in a buffer containing 90 mM Tris buffer (pH 8.0), 10 mM $CaCl_2$ and succinyl-ala-ala-ala-*p*-nitroanilide (1 mM) with or without KR-62436 for 1 h at room temperature. The absorbance at 405 nm was measured. For aminopeptidase N enzyme assay, the enzyme (0.2 μg/well) was incubated in a buffer containing 50 mM Tris buffer (pH 8.0) and L-leucine-*p*-nitroanilide (0.5 mM) with or without KR-62436 for 30 min at room

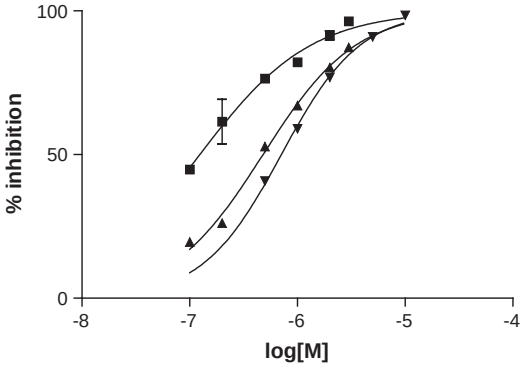

Fig. 2. Concentration dependent inhibition of DPP-IV by KR-62436. Different enzyme preparations (rat plasma; ▼, porcine kidney; ▲, Caco-2 cell; ■) were incubated with different concentrations of KR-62436. The enzyme activity was measured by the fluorescence of AFC released by enzyme reaction, and the results were expressed as percent inhibition of control activity (mean±S.E.M.) (N=3 of separate experiments).

temperature. The absorbance at 405 nm was measured. For prolyl oligopeptidase enzyme assay, the enzyme (10 μg/well) was incubated in a buffer containing 25 mM potassium phosphate buffer (pH 7.0) and *Z*-Gly-Pro-MCA (200 μM) for 1 h at room temperature. The fluorescence of MCA produced by enzyme reaction was measured (excitation 360 nm; emission 485 nm). For

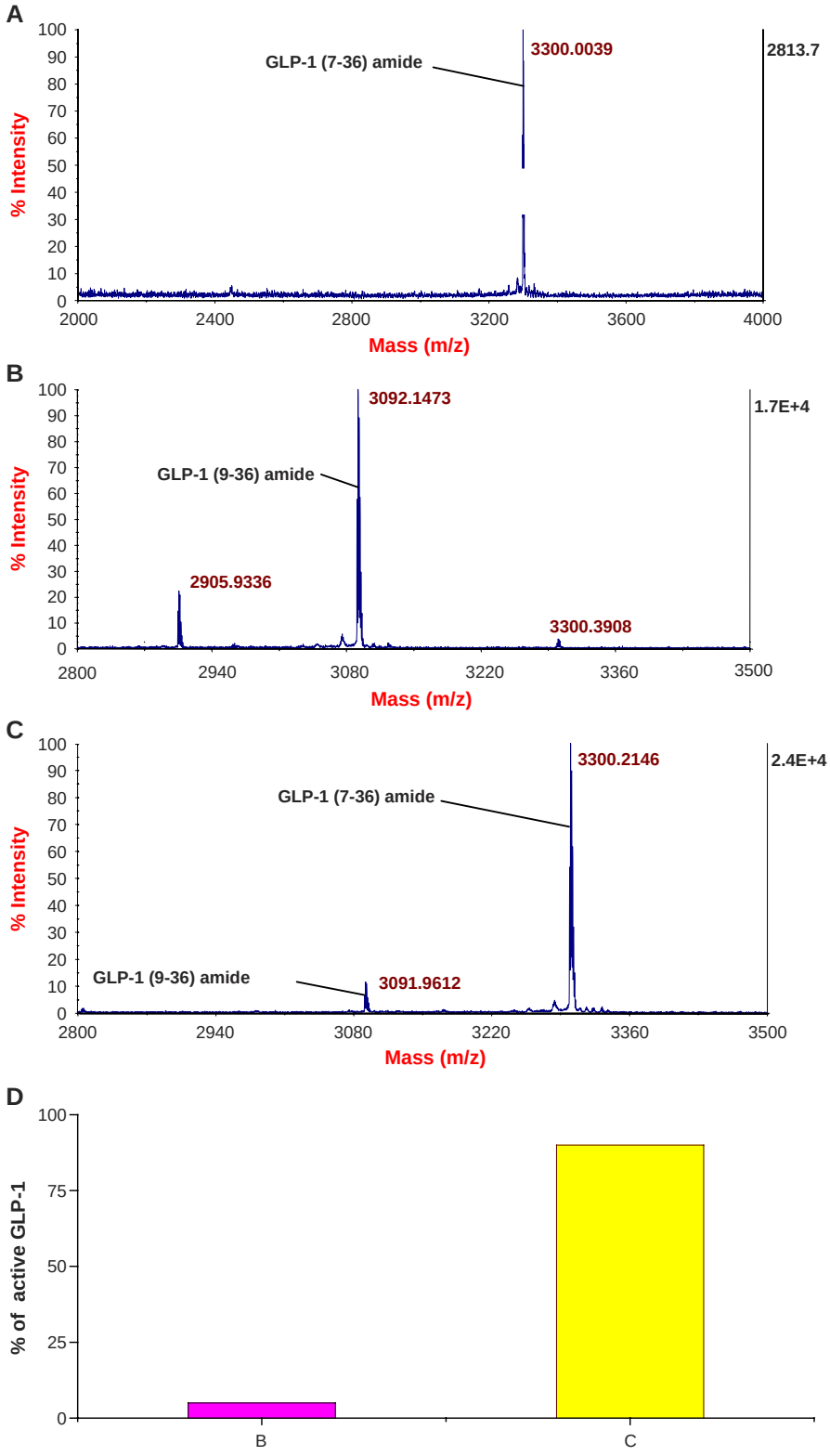

Fig. 3. Effect of KR-62436 on the GLP-1 degradation by DPP-IV in vitro. GLP-1 (15 μM) was incubated with porcine kidney DPP-IV (1 μU) in the presence or absence of 10 μM KR-62436. The amount of intact GLP-1 was quantitated after Maldi–Tof mass spectrometry. A. GLP-1 alone, B. GLP-1 plus DPP-IV, C. GLP-1 plus DPP-IV in the presence of 10 μM KR-62436, D. Percent of active GLP-1 in total GLP-1 was calculated in the cases of B and C.

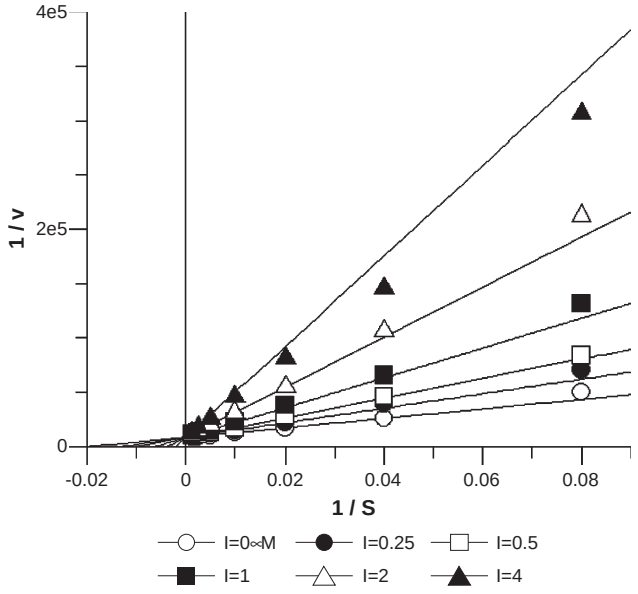

Fig. 4. Inhibition kinetics of DPP-IV by KR-62436. Different concentrations of KR-62436 (0, 0.25, 0.5, 1, 2, 4 μM) were incubated in the presence of various concentrations of Ala-Pro-AFC. Initial rates of the reaction were measured, and the results were expressed as a Lineweaver–Burk plot. Data are represented as mean of three separate experiments.

carboxypeptidase P enzyme assay, the enzyme (0.02 units/200 μl) was incubated in a buffer containing 50 mM sodium acetate buffer (pH 3.7) and 0.5 mM Cbz-glu-Tyr (0.5 mM) with or without KR-62436 for 20 min at room temperature. The amount of tyrosine released from enzyme reaction was measured by ninhydrin reaction as previously described (Yaron and Mlynar, 1968). The absorbance at 570 nm was measured.

2.5. In vivo DPP-IV inhibition using C57BL/6J mice

C57BL/6J mice (9 weeks, male) were given various doses of KR-62436 or vehicle either subcutaneously or orally (10 ml/kg). At various time points, blood (40 μl) was withdrawn from opthalamic venous plexus without anesthesia and added into heparinized capillary tube, and the DPP-IV activity was measured as described above. Either 0.5% carboxymethylcellulose (CMC) (*p.o.*) or 10% PEG in saline (*s.c.*) was used for vehicle. To determine the effect of KR-62436 on plasma intact GLP-1 levels, KR-62436 was administered orally 30 min before oral glucose challenge (2 g/kg), and plasma was withdrawn at 5 min after oral glucose challenge. Plasma GLP-1 levels were measured with Glucagon-Like Peptide (Active) enzyme-linked immunosorbent assay (ELISA) (Linco Research, St. Charles, MO). Plasma insulin concentrations were determined with an insulin ELISA kit (Shibayagi Co, Shibukawa, Japan) at 10 min after oral glucose challenge.

2.6. In vivo OGTT using ob/ob mice

Ob/*ob* mice were bred at in-house facilities. The mice (8–9 weeks, male) were fasted overnight, and plasma glucose and body weights were measured and matched between groups. Either KR-62436 (10, 20, 40, 80 mg/kg) or vehicle (0.5% CMC) in a volume of 10 ml/kg was given orally 30 min before glucose (2 g/kg) administration (*p.o.*). Blood samples (40 μl each time from the same animal) were taken at 0, 15, 30, 60, 120 min and plasma glucose concentration was measured by colorimetric assay using the Selectra 2 (Vital Scientific N.V., Spankeren, The Netherlands).

2.7. Statistical analysis

Data are expressed as means ± S.E.M., and statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Dunnett's test.

3. Results

3.1. DPP-IV enzyme assay

The inhibitory activity of KR-62436 on DPP-IV was determined by using rat plasma, porcine kidney and Caco-2 cell lysates as enzyme sources. As shown in Fig. 2, KR-62436 inhibited DPP-IV activity in a concentration dependent manner. The IC_{50} values were estimated as 0.78 μM (rat plasma), 0.49 μM (porcine kidney) and 0.14 μM (Caco-2 lysates). By comparison, previously reported DPP-IV inhibitors such as NVP-DPP728, LAF-237 and P-32/98 inhibited human Caco-2 DPP-IV activities with IC_{50} values of 29 nM, 117 nM and 1.3 μM, respectively, under our experimental conditions. In addition, KR-62436 could protect GLP-1 almost completely from the degradation mediated by DPP-IV in vitro (Fig. 3). About 90% of intact GLP-1 was recovered in the presence of KR-62436 (10 μM).

3.2. DPP-IV inhibition kinetics

To examine whether KR-62436 inhibits DPP-IV through interaction with the enzyme's active site, we tested enzyme kinetics of KR-62436 for DPP-IV inhibition with Ala-Pro-AFC as the substrate and porcine kidney as the enzyme source. KR-62436 inhibited DPP-IV activity in a competitive manner as determined by Lineweaver–Burk plot (Fig. 4). K_i value was estimated to be 0.47 μM.

3.3. Isoenzyme selectivity

To examine the selectivity profile of KR-62436 against related proteases in vitro, we evaluated the effect of KR-62436 on various proteases including proline-specific proteases. As shown in Table 1, KR-62436 exhibited clear selectivity against DPP-II, elastase, trypsin, prolyl oligopeptidase, aminopeptidase N, car-

Table 1
Selectivity of KR-62436 against several related proteases

Isozyme	IC_{50} (μM)
DPP-II	56.1
Trypsin	>100
Elastase	>100
Aminopeptidase N	>100
Prolyl oligopeptidase	59.1
Prolidase	>100
Carboxypeptidase P	>100
DPP-IV (porcine kidney)	0.49

boxypeptidase P and prolidase, where at least the selectivity was more than 100 folds.

3.4. In vivo DPP-IV inhibition using C57BL/6J mice

The in vivo inhibitory activity of KR-62436 was measured using C57BL/6J mice. Subcutaneous administration of KR-62436 dose-dependently resulted in the reduction of plasma DPP-IV activity (Fig. 5A). The ED_{50} was estimated to be 4.1 mg/kg. KR-62436 was also orally active with ED_{50} of 5.9 mg/kg (Fig. 5B). By comparison, ED_{50} values of NVP-DPP728, LAF-237 and P-32/98 by oral administration were estimated as 2.4, 0.1 and 8.5 mg/kg, respectively. Duration of action of KR-62436 was determined by oral administration of the compound, and the remaining DPP-IV activity was measured at various times. The effect of the compound remained active till 8 h after administration (Results not shown). Consistent with the inhibitory effect on DPP-IV, KR-62436 treatment resulted in the increase of plasma intact GLP-1 and insulin levels during OGTT in a dose-dependent manner (Fig. 6A and B), consequently decreasing plasma glucose levels.

3.5. In vivo OGTT using ob/ob mice

To examine whether the DPP-IV inhibition by KR-62436 results in the lowering of plasma glucose concentrations in type 2 diabetes, we tested the effect of KR-62436 on plasma glucose levels in OGTT using *ob*/*ob* mice, the type 2 diabetes animal model. As shown in Fig. 7, oral administration of KR-62436 reduced plasma glucose levels in a dose-dependent manner during OGTT with an ED_{50} of 15 mg/kg. This effect was accompanied by DPP-IV inhibition, suggesting that KR-62436 enhanced in vivo glucose clearance by elevation of intact GLP-1 and insulin levels in plasma.

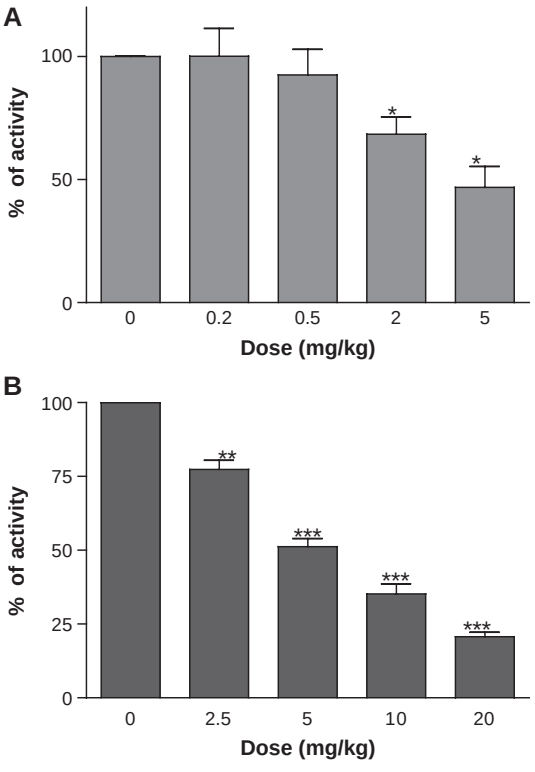

Fig. 5. In vivo inhibitory effect of KR-62436 on DPP-IV activity. Indicated doses of KR-62436 were given to C57BL/6J mice either subcutaneously (A) or orally (B). Plasma DPP-IV activity was measured at 30 min (A) or 1 h (B) after the administration of KR-62436. Data are represented as mean±S.E.M. ($n=8$). *P value <0.05 vs. control, **P value <0.01 vs. control, ***P value <0.005 vs. control.

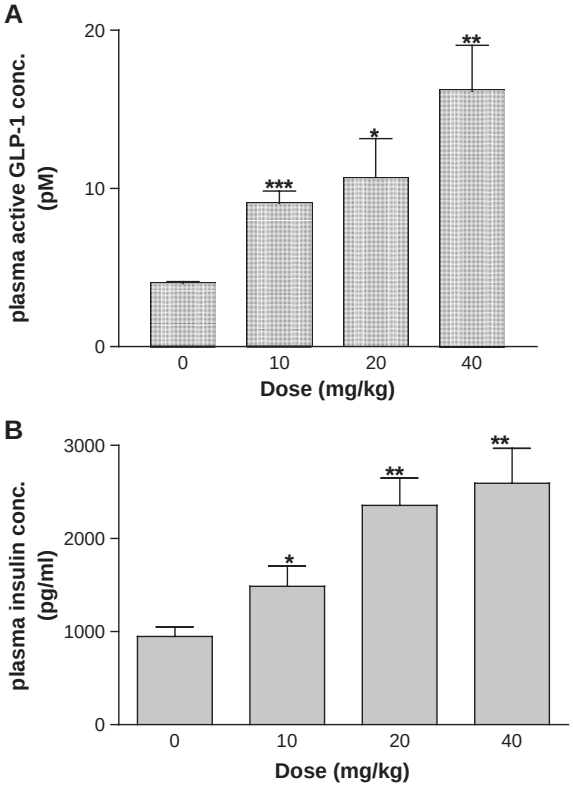

Fig. 6. Effect of KR-62436 on plasma GLP-1 and insulin levels by oral treatment. Indicated doses of KR-62436 were given to C57BL/6J mice 30 min before oral glucose challenge (2 g/kg). Plasma was withdrawn at 5 and 10 min for the measurements of GLP-1 and insulin levels. Intact GLP-1 and insulin levels were determined as described in Materials and methods. A. Plasma active GLP-1 concentration. B. Plasma insulin concentration. Data are represented as mean±S.E.M. ($n=8$). *P value <0.05 vs. control, **P value <0.01 vs. control, ***P value <0.005 vs. control.

4. Discussion

Dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5), a serine aminopeptidase, acts by cleaving penultimate proline or alanine residues, resulting in the regulation of various peptides activity (Mentlein, 1999). Among several peptide substrates of DPP-IV, GLP-1 is one of the well-characterized physiological and pharmacological substrates of the enzyme. GLP-1 action includes stimulation of insulin secretion, inhibition of glucagon secretion, promotion of satiety, inhibition of food intake, and tropic effect on pancreatic β cells, where all these effects contribute to glucose homeostasis (Holst and Deacon, 2004). However, active form of GLP-1 is rapidly degraded to its inactive form by DPP-IV.

The importance of DPP-IV inhibition in glucose homeostasis via elevation of intact GLP-1, thus maintaining glucose homeostasis is proved by various studies. DPP-IV knockout mice showed enhanced insulin secretion and blood glucose clearance where DPP-IV inhibitor was not effective. This effect was accompanied by an increase of endogenous intact GLP-1 level (Marguet et al., 2000). Furthermore, a DPP-IV deficient rat strain, Fisher 344/DuCrj rat, exhibited improved glucose tolerance when compared with DPP-IV positive wild type (Nagakura et al., 2001). Although the absolute specificity of small molecule DPP-IV inhibitors was not clear, several DPP-IV inhibitors have shown to increase insulin secretion and reduce blood glucose after glucose challenge (Ahren et al., 2000; Villhauer et al., 2002). Thus, DPP-IV inhibition represents an attractive strategy to develop anti-diabetic agents.

With the aim to develop novel DPP-IV inhibitors as anti-diabetic agents, we discovered acylpyrazoline deriva-

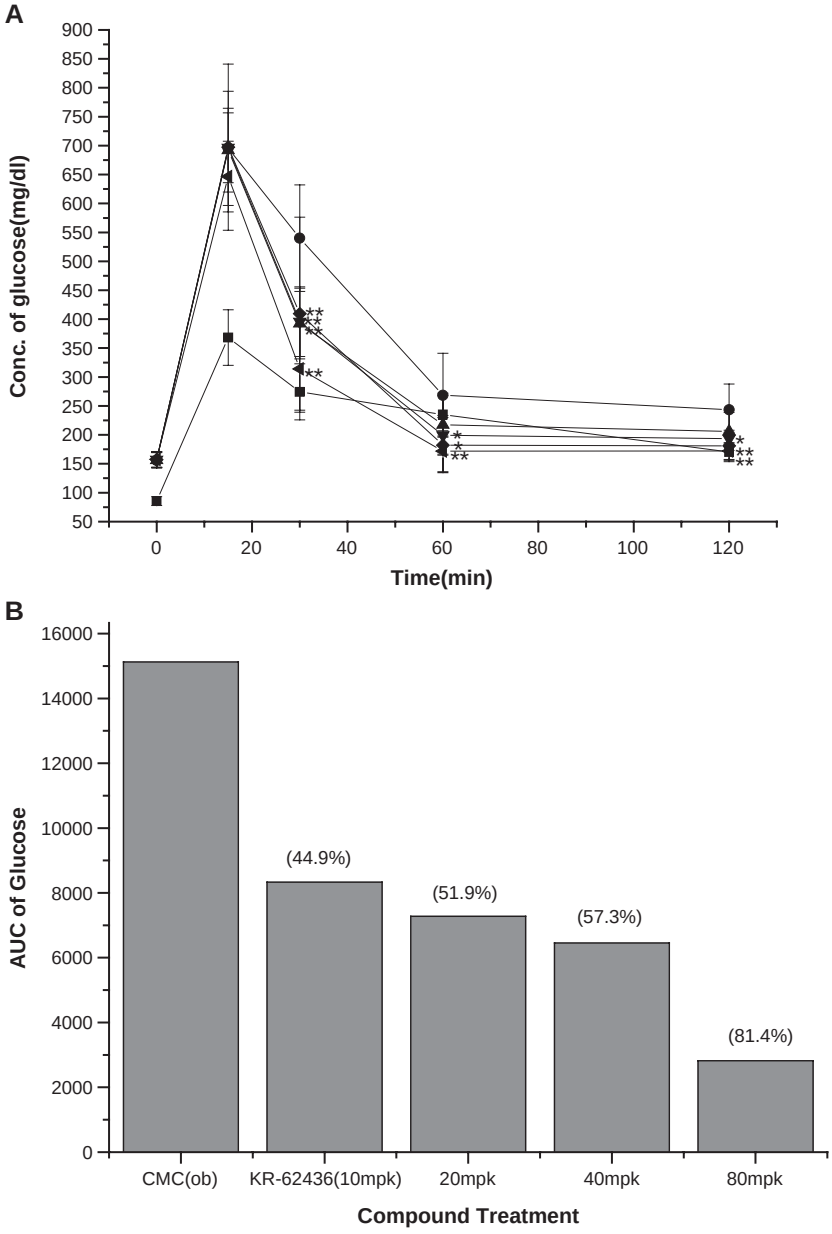

Fig. 7. Oral glucose tolerance test of KR-62436 using *ob*/*ob* mice. Various doses of KR-62436 were orally administered to *ob*/*ob* mice, and glucose (2 g/kg, *p.o.*) was given 30 min later (0 min). Plasma glucose concentration was measured at the indicated time (A). In A, (■) C57BL/6J (lean) mice vehicle, (●) *ob*/*ob* mice vehicle, (▲) *ob*/*ob* mice KR-62436 10 mg/kg, (▼) *ob*/*ob* mice KR-62436 20 mg/kg, (♦) *ob*/*ob* mice KR-62436 40 mg/kg, (◀) *ob*/*ob* mice KR-62436 80 mg/kg. Data are represented as mean ± S.E.M. ($n = 8$). *P value <0.05 vs. control, **P value <0.01 vs. control. B. AUC of glucose concentration curve (A) was calculated and displayed. In B, percent inhibition values for each treatment (in parentheses) were generated from the AUC data normalized to the vehicle-treated lean C57BL/6J mice.

tives by random screening of chemical library (about 70,000 compounds). KR-62436, a representative of these series, inhibited DPP-IV activity from different enzyme sources including human. The inhibition mediated by KR-62436 appears to be competitive, indicating that KR-62436 may bind to the active site of the enzyme. Recently, crystal structure of soluble form of human DPP-IV in complex with the inhibitor valine pyrrolidide was reported (Rasmussen et al., 2003). Determination of cocrystal structure of human DPP-IV with KR-62436 is under progress. To further confirm the inhibitory effect of KR-62436 on DPP-IV, we tested the effect of compound on GLP-1 degradation by incubation of intact GLP-1 peptide (aa 7-36) with porcine kidney DPP-IV in the presence or absence of the compound. Maldi–Tof analysis of the incubation mixture revealed that KR-62436 did protect exogenous GLP-1 degradation from DPP-IV-mediated degradation to 90% of control level.

Since DPP-IV modulates the biological activity of several peptide hormones, chemokines and neuropeptides (De Meester et al., 1999), chronic inhibition of DPP-IV may produce unwanted side effects. However, the side effects of DPP-IV inhibition therapy are likely to be mild, and indeed known DPP-IV inhibitors have not been reported to cause serious side effects except that NVP-DPP728 produced transient prunitis and nasopharyngitis (Ahren et al., 2002). Furthermore, the side effects induced by NVP-DPP728 appear to be compound specific rather than class specific based on the currently available information (Deacon et al., 2004). On the other hand, the selectivity of DPP-IV inhibitors against other proteases appears to be essential for the development of safe anti-diabetic agents. KR-62436 exhibited fair selectivity against several proteases including post proline-cleaving enzymes (prolyl oligopeptidase, DPP-II). Thus, KR-62436 appears to be a specific DPP-IV inhibitor.

In vivo efficacy of KR-62436 was evaluated by using C57BL/6J mice and *ob*/*ob* mice. We found that KR-62436 inhibited plasma DPP-IV activity in a dose-dependent manner by either subcutaneous or oral administration, along with increased GLP-1 and insulin, and decreased glucose levels. The duration of action of KR-62436 lasted till 8 hours after oral administration. Furthermore, we also found that oral administration of KR-62436 exhibited improved glucose tolerance after oral glucose challenge, consistent with its good pharmacokinetic properties in the rat (F=87%, CL=33.7 ml/min/kg, T_{max} 0.7 h, $t_{1/2}$ 1.2 h). Thus, it is likely that KR-62436 potentiated the active GLP-1 level by DPP-IV inhibition, consequently enhancing insulin secretion and reducing plasma glucose levels in *ob*/*ob* mice.

In conclusion, present study shows that KR-62436 acts as a specific and competitive DPP-IV inhibitor in vivo as well as in vitro. The results suggest the usefulness of KR-62436 for further development as a therapeutic agent for impaired glucose tolerance and type 2 diabetes.

Acknowledgments

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