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Effects of linagliptin on human immortalized podocytes: a cellular system to study dipeptidyl-peptidase 4 inhibition

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Running title:

DPP-4 and podocyte behaviour

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ABSTRACT

Background and Purpose

Dipeptidyl-peptidase 4 (DPP-4) is expressed by resident renal cells, including glomerular cells. Inhibitors of DPP-4 (gliptins) exert albuminuria lowering effects, but the role of renal DPP-4 as a pharmacological target has not been fully elucidated. To better understand the actions of gliptins, the effects of linagliptin on behaviour of immortalized human podocytes and mesangial cells have been evaluated here.

Experimental Approach

Expression of DPP-4 was measured at both mRNA and protein level. The effects of linagliptin on DPP-4 activity, cell growth, and cell cycle progression were measured. The contribution of the SDF-1-CXCR4/CXCR7 signalling pathways in mediating the effects of linagliptin were evaluated by studying the effects of AMD3100 (a CXCR4 antagonist and CXCR7 agonist) alone and in combination with linagliptin. The contribution of ERK1/2 activation was analysed by studying the effects of the MEK1/2 inhibitor AZD6244.

Key Results

Strong expression of DPP-4 was seen in podocytes and DPP-4 activity and podocyte growth were decreased by linagliptin (pIC₅₀ were 8.9 and 8.8, respectively). The effects of linagliptin on podocyte growth were mimicked by sitagliptin and related to the inhibition of cell proliferation, and were mimicked by AMD3100. Moreover, a synergistic interaction between these drugs was found. No interaction was seen between linagliptin and AZD6244.

Conclusion and Implications

Our cultures of immortalized human glomerular cells represent a reliable system for the investigation of the actions of gliptins. Moreover, DPP-4 is involved in the regulation of podocyte behaviour and its inhibition could underlie the effects of linagliptin on glomerular cells.

Keywords.

Gliptins; cellular systems; podocytes; stromal cell-derived factor-1.

Abbreviations

CXCR, chemokine, CXC motif, receptor; DPP-4, dipeptidyl-peptidase 4; ELISA, enzyme-linked immunosorbent assay; GLP-1, glucagon-like peptide-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide; RT-PCR, reverse transcriptase-polymerase chain reaction; SDF; stromal cell-derived factor.

Introduction

Dipeptidyl-peptidase (DPP)-4/CD26 inhibitors (gliptins) exert beneficial effects in diabetic patients and currently are registered in many countries as drugs for the treatment of type-2 diabetes mellitus (Beatta and Corsini, 2011; Davidson, 2013). Inhibition of the DPP-4-mediated incretin (e.g., glucagon-like peptide-1, GLP-1) inactivation is thought to mediate the therapeutic effects of these agents. Nevertheless, given the recognized involvement of DPP-4 in many intra- and inter-cellular signalling pathways (Gorrell *et al.*, 2001; Lambeir *et al.*, 2003; Mulvihill and Drucker, 2014), a more complex and pleiotropic mechanism of action, beyond the sole inhibition of incretin inactivation, could be suggested in order to explain the therapeutic effects of gliptins.

DPP-4 is a glycoprotein endowed with both enzymatic and non-enzymatic functions. It is a member of the serine peptidase/prolyl oligopeptidase which preferentially cleaves X-proline dipeptides from the *N*-terminus of polypeptides. Many substrates have been identified, including chemokines, growth factors, neuropeptides and hormones (Lambeir *et al.*, 2003; Mulvihill and Drucker, 2014). The non-enzymatic functions can result from the interaction between DPP-4 and cell-surface macromolecules, including adenosine deaminase (Kameoka *et al.*, 1993), chemokine CXC motif receptor (CXCR)4 (Herrera *et al.*, 2001), integrin β 1 (Gherzi *et al.*, 2006), among others.

DPP-4 is widely distributed and high levels have been found in many tissues (Gorrell *et al.*, 2001; Lambeir *et al.*, 2003). The expression/activity of DPP-4 by glomerular cells was originally reported by Fukasawa *et al.*, (1981) and subsequently confirmed by other groups (Hartel *et al.*, 1988; Kettmann *et al.*, 1992; Stefanovic *et al.*, 1993; Mentzel *et al.*, 1996). Nevertheless, the pathophysiological and pharmacological roles of glomerular DPP-4 remain elusive, although convergent lines of evidence suggest its potential relevance. Indeed, increased DPP-4 expression has been measured in human glomerular endothelial cells exposed to high glucose concentrations (Pala *et al.*, 2003), whole kidneys (Kirino *et al.*, 2009) and glomeruli of rats receiving a high fat diet and streptozotocin (Yang *et al.*, 2007; Shi *et al.*, 2016), *db/db* mice (Sharkovska *et al.*, 2014) and insulin-dependent diabetic patients (Sharkovska *et al.*, 2014; Maeda *et al.*, 2015). Moreover, albuminuria-lowering effects have been described in both preclinical and clinical studies on different gliptins, including sitagliptin, vildagliptin and linagliptin (Hattori, 2010; Mega *et al.*, 2011; Liu *et al.*, 2012;

Groop *et al.*, 2013; Nistala *et al.*, 2014; Eun Lee *et al.*, 2016). Whether these effects result from an action on the enzyme expressed by resident renal cells is not known. However, a contribution of the glomerular DPP-4 as a therapeutic target of these drugs cannot be excluded.

Members of the stromal cell-derived factor (SDF)-1 family are plausible mediators of the effects of gliptins on glomerular cells. Indeed, SDF-1 peptides are released by resident renal cells (e.g., podocytes) and play essential roles during glomerular development, in the maintenance of glomerular integrity and may also sustain regenerative processes (Mazzeo *et al.*, 2008; Stokman *et al.*, 2010; Chen *et al.*, 2014). In addition, they are rapidly processed by DPP-4 (Lambeir *et al.*, 2003; De La Luz Sierra *et al.*, 2004). To date, however, the role of SDF-1 peptides in mediating the renal effects of gliptins has not been clearly established. Interestingly, Takashima *et al.*, (2016) have shown that SDF-1 can mediate the renal effects of DPP-4 inhibition in animal models of diabetes mellitus. Thereby, elucidation of the mechanism underlying the renal effects of gliptins, as well as the role of the SDF-1 signalling pathway needs appropriate analytical studies.

In general, the findings of analytical investigations, especially those on subtle pharmacological actions, are made more informative by limiting the number of variables that might influence the measured responses. Compared with more complex experimental systems – such as *in vivo* models – cellular systems meet this premise and could be helpful by providing data to (dis)prove and generate novel hypothesis. Therefore, in the present series of investigations, designed to provide an insight into the role of the glomerular DPP-4 as a target for gliptins, the effect of linagliptin on immortalized human podocytes and mesangial cells was investigated.

Methods

Cell cultures

In this study, lines of immortalized human podocytes and mesangial cells were used. Immortalized cells were obtained from primary podocytes and mesangial cells by infection with a hybrid Adeno5/SV40 virus. Cells were characterized as previously described (Conaldi *et al.*, 1998; Doublier *et al.*, 2001; Miglio *et al.*, 2011 and 2012). Under standard conditions, cells were cultured in Dulbecco's modified Eagle's medium, supplemented with foetal bovine

serum (10%), penicillin G (100 U mL⁻¹), streptomycin (100 µg mL⁻¹), and L-glutamine (2 mM). Cell culture medium was replaced every 2 days, and cultures were maintained at 37 °C, 95% air-5% CO₂ in a humidified incubator.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses

Total RNA was extracted from cell cultures using the EuroGold Trifast Kit, according to the manufacturer's instructions. First-strand cDNA was synthesized from 10 ng of total RNA using the RevertAid First Strand cDNA Synthesis Kit. PCRs were performed in 25 µL-reaction mixtures containing 2 µL of cDNA, 2.5 µL of 10× reaction buffer, dNTPs (0.2 mM), MgCl₂ (2.5 mM), EuroTaq ThermoStable DNA polymerase (2.5 U), and a specific primer pair (0.5 µM; Table 1S). RT-PCR amplicons were resolved in agarose gels by electrophoresis and visualized with ethidium bromide.

Enzymatic assays

Activity of DPP-4 was measured in extracts prepared from confluent cell cultures and in fresh/conditioned cell culture media. Cell extracts were prepared as described by Thomas *et al.*, (2008) with minor modifications. In brief, cells were washed twice with Mg²⁺-free phosphate-buffered saline (PBS) and lysed at 4 °C in a buffered solution (10 mM Tris-HCl, 150 mM NaCl, 0.04 U mL⁻¹ aprotinin, 0.5% Nonidet P40, pH 8.0). Samples were collected then centrifuged at 16000 g for 30 min. Resulting supernatants were stored at -80 °C. Assays were performed by mixing 20 µL of either vehicle alone or linagliptin with 50 µL of the DPP-4 substrate, H-Ala-Pro-7-amido-4-trifluoromethylcoumarin (final concentration in the assay buffer 100 µM), and 30 µL of cell extract/culture media (100-fold diluted in the assay buffer: 100 mM Tris-HCl, 100 mM NaCl, pH 7.8). Plates were maintained at room temperature for 1 h and fluorescence was measured at 5 min intervals at excitation/emission wavelengths of 405/535 nm by using a VICTOR X4 plate reader (PerkinElmer, Waltham, MA, USA). The activity of DPP-4 measured in different samples was normalized to protein content of the samples.

Western blot analyses

Western blot analyses of cell extracts were performed as previously described (Miglio *et al.*, 2011 and 2012). DPP-4 was detected following incubation with a goat anti-DPP-4 polyclonal antibody (0.2 µg mL⁻¹). Expression of p21 and phosphorylated ERK1/2 were detected with mouse anti-p21 or anti-pERK1/2 monoclonal antibodies (at dilution factors of 1:200 and

1:2000, respectively). Cyclin D1 and p27 expression was detected with rabbit anti-cyclin D1 and anti-p27 polyclonal antibodies (at dilution factors of 1:200 and 1:100 respectively). To confirm equal protein loading, membranes were stripped, and incubated with an anti- β -actin monoclonal antibody. Finally, membranes were overlaid with Western Lightning Chemiluminescence Reagent Plus, luminescence detected using Hyperfilm ECL film. Protein bands were quantified by densitometry using Image J 1.49 (National Institutes of Health, Bethesda, MD, USA).

Measurement of cell growth and cell cycle analyses

Cells were plated (2×10^3 cells well⁻¹) in 24-well culture plates and exposed to vehicle alone (control), linagliptin, sitagliptin, AZD6244, and/or AMD3100. Cell growth was evaluated in sub-confluent cultures by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay; results were confirmed by determining cell density, as previously described (Miglio *et al.*, 2011 and 2012).

The percentage of cells in different phases of the cell cycle was determined as previously described (Miglio *et al.*, 2005). In brief, at the end of each treatment, cells were washed with PBS, harvested, then centrifuged. Pellets were resuspended in 1 mL of ice-cold 70% ethanol and maintained at -20 °C for at least 24 h. Afterward, cells were washed twice with PBS, and treated (1 h, 37 °C) with RNase (0.5 mg mL^{-1} , final concentration). Finally, propidium iodide (PI; $50 \text{ }\mu\text{g mL}^{-1}$) was added. Fluorescence of individual nuclei was measured on a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA). Cell cycle analysis was performed using Flowing Software version 2.5 (Centre for Biotechnology, Turku, Finland).

Measurement of SDF-1 α concentration

Cell culture supernatants were collected and the level of SDF-1 α was quantified with an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturers instructions.

Compliance with design and statistical analysis requirements

Data were collected, blinded and analysed by an observer. Results were expressed as mean \pm SEM of at least five experiments done in triplicate. The -Log value of the molar concentration of an agent that decreases the baseline response measured in control samples by 50% (pIC₅₀) was calculated with Origin 6.0 software (Microcal Software, Northampton, MA, USA) by using a Hill regression model for analysis of the concentration-response data. The Bliss

independence model (Bliss, 1939) was adopted to analyse the effects exerted by a mixture of two agents, *A* and *B*, both exerting overtly similar effects: agent *A* at concentration *a* exerts the effect Y_a , and agent *B* at concentration *b* exerts the effect Y_b . If *A* and *B* act independently, the combined effect, $Y_{ab,P}$, can be predicted using the additivity of probability theory as:

$$Y_{ab,P} = Y_a + Y_b - Y_a \times Y_b \quad (1)$$

The observed combined effects, $Y_{ab,O}$, are then compared with $Y_{ab,P}$ to establish the scenario as: $Y_{ab,O} > Y_{ab,P}$, synergy; $Y_{ab,O} = Y_{ab,P}$, independence; $Y_{ab,O} < Y_{ab,P}$, antagonism.

Statistical significance was evaluated by either Student's *t* test or ANOVA and Bonferroni *post hoc* test (Prism 5, GraphPad Software, La Jolla, CA, USA). Differences were judged statistically significant when $P < 0.05$.

Materials

Linagliptin and sitagliptin were kindly provided by Boehringer Ingelheim GmbH (Biberach, Germany). AZD6244 was generously supplied by Dr M. Gallicchio (Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Turin, Italy). Dulbecco's modified Eagle's medium, foetal bovine serum, penicillin G, streptomycin, and L-glutamine were obtained from Lonza (Basel, Switzerland). EuroGold Trifast Kit, and EuroTaq ThermoStable DNA polymerase were from EuroClone (Milan, Italy). RevertAid First Strand cDNA Synthesis Kit was from Thermo Scientific (Waltham, MA, USA). PCR primers were from Sigma Life Science (Milan, Italy). H-Ala-Pro-7-amido-4-trifluoromethylcoumarin was obtained from Bachem (Bubendorf, Switzerland). ELISA kit to quantify SDF-1 α was from Peprotech House (London, UK). Anti-DPP-4 antibody was obtained from RD System (Minneapolis, MN, USA). Antibodies against phosphorylated ERK1/2, anti-mouse, and anti-rabbit horseradish peroxidase-linked-antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti- β -actin antibody was obtained from Sigma-Aldrich (Milan, Italy). Antibodies against cyclin D1, p21, p27, and goat horseradish peroxidase-linked antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Western Lightning Chemiluminescence Reagent Plus was obtained from PerkinElmer Life Science (Norwalk, CT, USA). Hyperfilm ECL film was obtained from Amersham Biosciences (Piscataway, NJ, USA). All other reagents and chemicals were obtained from Sigma-Aldrich (Milan, Italy).

Results

Expression of DPP-4 by immortalized human glomerular cells

The expression of DPP-4 was studied in immortalized human podocytes and mesangial cells by RT-PCR. A single product of the predicted size was detected in all reactions. Moreover, as shown in Figure 1A, the gene encoding for DPP-4 was expressed by podocytes, and at a markedly lower level by mesangial cells. To extend these findings, DPP-4 enzymatic activity was measured in both cell extracts and cell culture media. As shown in Figure 1B, compared with podocytes, a significantly lower activity was determined in extracts from mesangial cells, thus confirming the PCR findings. Enzymatic activity in podocyte extracts was even higher than those measured in culture media, indicating that DPP-4 is mainly retained by cells. To confirm these findings, DPP-4 expression was assessed at protein level by Western blot analyses. A single band at the expected molecular weight was found in podocyte extracts (Figure 1C), although no band corresponding to DPP-4 was detected consistently in mesangial cell extracts (data not shown), thus confirming previous data. Finally, to further confirm the identity of the enzymatic activity, the effects of increasing linagliptin concentrations (0.01–100 nM) on the enzymatic activity were evaluated. Enzymatic activity measured in podocyte extracts was inhibited by linagliptin in a concentration-dependent manner and abolished at 30–100 nM (Figure 1D). Notably, the $pIC_{50} = 8.9$ was consistent with the expected value (Thomas *et al.*, 2008). Enzymatic activity measured in mesangial cell extracts and culture media was also abolished by 100 nM linagliptin (data not shown). Therefore, by expressing high DPP-4 levels, our cultures of immortalized human podocytes fulfil an essential prerequisite to be evaluated as a system to study the effects of DPP-4 inhibitors on this cell type.

Effects of glucose on DPP-4 activity in immortalized mesangial cells

Contrary to podocytes, DPP-4 is expressed at very low levels by our immortalized human mesangial cells. These cells were cultured under the same conditions as podocytes, except for glucose concentrations in the culture media, that were 1.0 g L^{-1} and 4.5 g L^{-1} , respectively. Given that glucose concentration has been reported to influence DPP-4 expression/activity (Pala *et al.*, 2003), its effect on the enzymatic activity in mesangial cells was studied. Mesangial cells were exposed to higher glucose concentration (4.5 g L^{-1}) up to 48 h and enzymatic activity was assessed in cell extracts. As shown in Figure 2, compared with the

basal level (0 h), no significant change in the enzymatic activity was measured in cells exposed to the higher glucose concentration for 24 or 48 h (longer time significantly affected cell viability; data not shown).

Thus, low DPP-4 levels are expressed by our immortalized human mesangial cells, irrespective of the glucose concentration in the culture media. Such cells could however be helpful to evaluate DPP-4-independent effects of gliptins.

Effects of DPP-4 inhibitors on the cell growth of immortalized human glomerular cells

Inhibitors of DPP-4 have been reported to decrease proliferation of different cell types, including T cells (Schön *et al.*, 1985; Reinhold *et al.*, 1997) and smooth muscle cells (Ervinna *et al.*, 2013; Wronkowitz *et al.*, 2014). To investigate whether linagliptin modulates glomerular cell behaviour, its effect on cell growth in culture was studied. Podocytes and mesangial cells were exposed to linagliptin (30 nM; 1–5 days) and cell growth was measured by MTT assay. As shown in Figure 3A, podocyte growth was decreased by linagliptin in a time-dependent manner. Significant effects were measured at 3–5 days (differences between control and linagliptin-treated cells decreased at longer treatment times due to cell confluency; data not shown). By contrast, mesangial cell growth was unaffected by linagliptin. MTT data were confirmed by determining cell number in each well (Figure 1AS and 1BS), indicating that the observed changes in the rate of cell growth actually reflect differences in terms of cell density.

As the effects of linagliptin were observed on podocytes, but not on mesangial cells, they may depend on DPP-4 inhibition. To confirm this hypothesis, podocytes were exposed to increasing concentrations of either linagliptin (0.01–100 nM) or sitagliptin (0.01 nM–1000 nM), and cell growth was measured at 5 days. Cell growth was decreased by both drugs in a concentration-dependent manner (Figure 3B). The pIC₅₀ values (8.8 and 7.6, calculated with respect to their maximal effects: $-27.4 \pm 3.4\%$ and $-28.5 \pm 2.4\%$ vs. vehicle alone, respectively) were comparable to the expected values for the inhibition of DPP-4 activity (Kim *et al.*, 2005; Thomas *et al.*, 2008), indicating a probable link between the two effects. Therefore, as already observed in other cell types, significant changes in the rate of podocyte growth results from DPP-4 inhibition.

Effects of linagliptin on cell cycle progression and apoptosis

A decreased rate of cell growth can result from inhibition of cell proliferation, toxicity, or both. To better understand the effects of linagliptin on podocyte growth, cells were exposed to

linagliptin (1 nM or 100 nM) for 5 days, before nuclei were stained with PI to determine the percentage of cells in different phases of the cell cycle. As shown in Table 1, cell cycle progression was altered by linagliptin, and significant differences, with respect to the control cells, were determined for linagliptin 100 nM. In particular, changes in the percentage of cells in the G₀/G₁ (increase) and S (decrease) phases were determined. No significant difference was determined in the percentage of cells sub-G₁ (apoptotic) phase. To further understand these effects, the expression of cyclin D1, p27, and p21, which are involved in the regulation of cell cycle progression (Lasagni *et al.*, 2013), was evaluated in podocyte extracts by Western blot analyses. Compared with control cells, a lower level of cyclin D1 was observed in linagliptin treated (100 nM, 5 days) cells (Figure 4), whereas the constitutive low expression of p27 was not changed by the drug treatment (Figure 4); p21 was not detected (data not shown).

Therefore, in our immortalized human podocytes a slowed-down cell cycle progression results from DPP-4 inhibition.

Expression of SDF-1, CXCR4 and CXCR7 by immortalized human podocytes

The effects of gliptins on cell growth could result from a perturbation of signalling pathways involving molecules that are DPP-4 substrates and that also play a role in the control of cell proliferation. By fulfilling these prerequisites (see Introduction), the SDF-1 signalling pathway could mediate the effects of linagliptin on podocyte growth. To assess this hypothesis, the expression of the genes encoding for SDF-1 and its cognate receptors (CXCR4 and CXCR7) by our immortalized podocytes was studied by RT-PCR. As shown in Figure 5A, *SDF-1*, *CXCR4*, and *CXCR7* were constitutively expressed by our cells. Moreover, strengthening these data, the local production of SDF-1 α (as a representative member of the SDF-1 chemokine family) was evaluated by measuring the peptide levels in the extracellular milieu by ELISA. Compared with the basal value (2.72 ± 0.18 ng mL⁻¹), SDF-1 α concentration significantly increased ($P < 0.05$) throughout the culture growth, and was 7.76 ± 0.17 ng mL⁻¹ after 5 days of culture (Figure 5B).

Therefore, by expressing all components of the SDF-1-CXCR4/CXCR7 pathways, our cultures of human glomerular cells could be helpful to study the role of these pathways in mediating the effects on these cell types resulting from DPP-4 inhibition.

Effects of AMD3100 on the growth of immortalized human podocytes

To assess whether pharmacological modulation of the SDF-1-CXCR4/CXCR7 pathways mediates the effects of linagliptin in our system, a pharmacological analysis was performed. In particular, the effects of AMD3100/plerixafor, which acts as a CXCR4 competitive antagonist (Zhang *et al.*, 2002), and as a CXCR7 agonist (Kalatskaya *et al.*, 2009; Gravel *et al.*, 2010), on podocyte growth were studied. Moreover, to evaluate whether an interaction between AMD3100 and linagliptin could be established, the effects exerted by mixtures of these agents were measured. Podocytes were exposed to increasing concentrations of AMD3100 (0.01–1 μ M), either in the absence or presence of linagliptin (1 nM), and cell growth was measured at 5 days by MTT assay. Podocyte growth was decreased by AMD3100 in a concentration-dependent manner ($pIC_{50} = 6.4$; Figure 6). Moreover, the value of the slope parameter (3.1 ± 1.6) was consistent with a heterogeneous effector function. Of note, when the effects of mixtures of AMD3100 (0.01–1 μ M) + linagliptin (1 nM) were assessed and compared with the hypothetical independence (no interaction), potentiation was observed, indicating the establishment of a synergistic interaction between the two agents (the pIC_{50} was 7.1 ± 0.1 and the slope parameter 2.8 ± 1.2). MTT data were confirmed by determining cell number in each well (Figure 2S).

Thereby, a pharmacological modulation of the SDF-1-CXCR4/CXCR7 signalling pathways contributes to the regulation of podocyte growth in our experiments.

Effects of linagliptin and AMD3100 on ERK1/2 activation in immortalized human podocytes

To further evaluate the contribution of the SDF-1-CXCR4/CXCR7 pathways in mediating the effects of linagliptin on podocytes, activation of ERK1/2 – intracellular signalling molecules activated in response to CXCR4 and CXCR7 stimulation (Wang *et al.*, 2008; Gravel *et al.*, 2010) – was investigated. Podocytes were exposed to linagliptin (1 nM), AMD3100 (0.3 μ M), or linagliptin + AMD3100, and activation of ERK1/2 (pERK1/2) at 18 h was assessed by Western blot (Figure 7). Compared with control cells, a marked increase in pERK1/2 level was measured in linagliptin and/or AMD3100-treated cells, thus supporting the hypothesis that SDF-1-CXCR4/CXCR7 pathways are perturbed by DPP-4 inhibition.

Effects of AZD6244 on the growth of immortalized human podocytes

If the effects of linagliptin on cell growth depend on ERK1/2 activation, they should be sensitive to MEK inhibition. To assess this, the effects of a potent MEK1/2 inhibitor – AZD6244 (Huynh *et al.*, 2007) – on podocyte growth, were studied. In particular, podocytes

were exposed to increasing concentrations of this drug (0.1–10 μM) either alone or in combination with linagliptin (1–100 nM), and cell growth was measured after 5 days by MTT assay. Podocyte growth was decreased by both AZD6244 alone and AZD6244 + linagliptin (Figure 8 and Table 2). In addition, when the observed and predicted effects were compared, no interaction was observed. MTT results were confirmed by determining cell density in each well (Figure 3S).

Therefore, ERK1/2 activation is not involved in DPP-4 mediated inhibition of podocyte growth in our hands.

Discussion and conclusion

DPP-4 inhibition underlies the beneficial effects of gliptins, especially on blood glucose levels (Beatta and Corsini, 2011; Davidson JA, 2013). The wide distribution of the glycoprotein, together with the demonstrated broad spectra of its biological roles (Gorrell *et al.*, 2001; Lambeir *et al.*, 2003; Mulvihill and Drucker, 2014), suggests multiple additional consequences of DPP-4 inhibition, some of which may help explain interesting clinical data. Nevertheless, the actions of DPP-4 inhibitors are difficult to appreciate in some cases. A notable example is the renal actions of gliptins. Beneficial, partially glycaemia-independent renal effects of these drugs have been reported (Hattori S., 2010; Mega *et al.*, 2011; Liu *et al.*, 2012; Groop *et al.*, 2013; Nistala *et al.*, 2014; Eun Lee *et al.*, 2016). However, their intrarenal actions are poorly understood. Whether inhibition of the renal DPP-4 contributes to the beneficial effects of gliptins is an intriguing hypothesis and merits further investigation.

Here, we have evaluated the effects of linagliptin on cultures of immortalized human podocytes and mesangial cells. Our cellular systems have been previously characterized. For example, our lines of human podocytes phenotypically reassemble primary normal cells; they appear as arborized epithelial cells with a large cytoplasmic-to-nuclear area ratio and express podocyte-specific markers (e.g., synaptopodin and nephrin; Conaldi *et al.*, 1998; Doublier *et al.*, 2001; Miceli *et al.*, 2010; Miglio *et al.*, 2011 and 2012), and grow *in vitro* under typical culture conditions. Moreover, they have been employed to study the effects of agents acting on angiotensin II receptors (Miceli *et al.*, 2010; Rosa *et al.*, 2012) and peroxisome proliferator-activating receptors (Miceli *et al.*, 2010; Miglio *et al.*, 2011 and 2012) among others. In these circumstances, they have proved reliable model systems for analytical pharmacology studies.

Robust expression of active DPP-4 has been observed in our cultures of human podocytes. In addition, a decrease in the rate of podocyte growth has been observed when the effects of either linagliptin or sitagliptin were evaluated. These effects were concentration-dependent and apparent at drug concentrations which are able to inhibit DPP-4 activity both *in vitro* and *in vivo* and which are achieved after oral administration of therapeutic doses in healthy individuals and diabetic patients (Kim *et al.*, 2005; Thomas *et al.*, 2008; Baetta and Corsini, 2011). Moreover, they have been associated with decreased cell proliferation without toxicity. The significance of these results should however be carefully weighted. In mature glomeruli, podocytes behave as post-mitotic cells (Lasagni *et al.*, 2013), while immortalized podocytes proliferate. Hence, behaviour of normal cells in mature glomeruli is not identical to that in our model. Despite this, the ability of our cells to proliferate offers a convenient parameter (measurement of cell growth) to study the actions of gliptins. Notably, DPP-4 inhibitors have been reported to decrease the rate of growth of different cell types, including mitogen-treated T cells (Schön *et al.*, 1985; Reinhold *et al.*, 1997) and smooth muscle cells (Ervinna *et al.*, 2013; Wronkowitz *et al.*, 2014). The mechanism underlying these effects remains uncertain in most cases, however, the perturbation of intra/intercellular signalling following DPP-4 inhibition is one possibility. Consistent with this argument, the effects on the rate of growth of our immortalized podocytes exerted by two structurally different gliptins suggest a response resulting from DPP-4 inhibition. However, acquisition of a ‘quiescent phenotype’ could be as retained a potentially favourable response, with regard the maintenance of the glomerular integrity, but this speculation deserves further evaluation.

Due to the existence of a relationship between DPP-4 activity and podocyte proliferation, we investigated the role of signalling pathways involving DPP-4 as mediators of linagliptin effects. The GLP-1-GLP1 receptor axis was excluded since GLP-1 is not produced by renal cells and moreover, the GLP-1 receptor is not found in our cultures of glomerular cells (data not shown), confirming previous data which exclude a prominent action of incretin hormones on podocytes in mature glomeruli (Fujita *et al.*, 2013; 2014; Pyke *et al.*, 2014). By contrast, a role for the SDF-1-CXCR4/CXCR7 signalling pathways is supported by convergent lines of evidence: *i*) a strong expression of CXCR4 and CXCR7 by our podocytes; *ii*) the constitutive production of SDF-1 α ; *iii*) activation of the cognate intracellular signalling molecules (ERK1/2) after a relatively short-term exposure to linagliptin; *iv*) the effects exerted by AMD3100 alone and; *v*) the synergistic interaction between AMD3100 and linagliptin. Our data corroborate previous findings on the expression of DPP-4 by podocytes, the role played by DPP-4 in SDF-1 proteolytic processing (SDF-1 being one of the best DPP-

4 substrates) and the expression of SDF-1 peptides and their receptors by podocytes (see (Lambeir *et al.*, 2003; De La Luz Sierra *et al.*, 2004; Mazzingi *et al.*, 2008; Stokman *et al.*, 2010; Chen *et al.*, 2014). Collectively, these findings allow us to propose a linkage between renal effects of gliptins resulting from the inhibition of the glomerular DPP-4 and perturbation of the SDF-1-CXCR4/CXCR7 pathways. The complexity of the SDF-1 signalling pathways makes this linkage difficult to be study. Indeed, for example SDF-1 α (1-68) is known to act as a full CXCR4 and CXCR7 agonist (Crump *et al.*, 1997; Gravel *et al.*, 2010; Southern *et al.*, 2013), whereas SDF-1 α (3-68) is a CXCR4 antagonist (Crump *et al.*, 1997), and has been proposed to act as a CXCR7 agonist (Gravel *et al.*, 2010). Moreover, CXCR7 activation has been reported to interfere with the CXCR4-mediated responses in some cellular systems (Levoye *et al.*, 2010; Uto-Konomi *et al.*, 2013). Nevertheless, data resulting from our pharmacological analysis allows us to propose the following mechanistic model: *i*) mixtures of CXCR4- and CXCR7-ligands (SDF-1 peptides) are produced by podocytes, *ii*) some of the SDF-1 peptides act at their cognate receptors, thus establishing autocrine/paracrine circuits which govern cell behaviour (Figure 9A) and; *iii*) changes in the peptide mixture composition and/or in the protein-protein interaction (e.g., DPP-4-CXCR4 complexes; Herrera *et al.*, 2001) could results from DPP-4 inhibition and be directly upstream of the effects of gliptins on podocyte behaviour (Figure 9B). The changes resulting from DPP-4 inhibition on either the composition of the SDF-1 peptide mixture or the function of DPP-4-CXCR4 complexes cannot easily detected by conventional molecular assays, however, it likely takes place in our systems. The effects of linagliptin and sitagliptin on cell growth were mimicked by AMD3100, which alters CXCR4 and CXCR7 signalling in a DPP-4-independent manner. Therefore, under normal conditions proliferation of our podocytes is sustained by a stimulus mediated by CXCR4 activation. However, in the presence of DPP-4 inhibitors, AMD3100, or both, CXCR7 seems to play an important opposing role. Although further studies are needed to confirm these findings, a novel mechanism of action of gliptins could be inferred from them: namely the modulation of intrarenal autocrine/paracrine signals resulting from DPP-4 inhibition and involving SDF-1-CXCR4/CXCR7 signalling.

Whether the perturbation of SDF-1-CXCR4/CXCR7 signalling contributes to the clinical effects of gliptins requires further investigation. Several intra- and extra-renal cell types are targeted by SDF-1 and it acts together with other signalling molecules (including DPP-4 substrates). For example, the expression at mRNA level of SDF-1, CXCR4 and CXCR7 was observed not only in podocytes, but also in mesangial cells (see Figure 4S). Therefore, a cascade of autocrine and paracrine actions could be triggered by the inhibition of

the intra-glomerular DPP-4-mediated SDF-1 processing. Until now, conflicting results have been reported on the role of the SDF-1-CXCR4/CXCR7 pathways in the kidney. Indeed, some studies have suggested a beneficial function for SDF-1 (Mazzeo *et al.*, 2008; Stokman *et al.*, 2010; Chen *et al.*, 2014; Nistala *et al.*, 2014). By contrast, it has been shown that a CXCR4-mediated podocyte proliferation could contribute to the development of certain glomerular diseases (Ding *et al.*, 2006; Rizzo *et al.*, 2013). As discussed, a perturbation of the SDF-1 signalling has been postulated to mediate the potentially favourable effects of gliptins on podocyte behaviour observed in our experiments. These findings contribute to the compelling evidence on the role of the pharmacological modulation of the intrarenal SDF-1 signalling pathways. Recently, an *in vivo* study on the renal effects on linagliptin has been published (Takashima *et al.*, 2016). By using different animal models of diabetes, it was demonstrated that the beneficial effects resulting from DPP-4 inhibition are mediated by the SDF-1 signalling, although the exact mechanism remains unclear. Therefore, consistent with our conclusion, the pharmacological modulation of the intrarenal SDF-1 signalling pathways is may be a promising mechanism requiring additional studies to understand the therapeutic effects of gliptins. Since the possess almost all essential features, our cellular systems could prove useful in this endeavour.

In conclusion, our data indicate that DPP-4 is involved in the regulation of podocyte behaviour. Inhibition of DPP-4 activity could promote potentially beneficial changes with respect to the maintenance of the glomerular integrity. These findings give rise to a novel hypothesis and could contribute to a better understanding of the renal actions of linagliptin.

Author contributions

G.M. devised the experiments; G.M, G.V. and E.B. performed the experiments; G.M. and E.B. analyzed and interpreted the data and wrote the manuscript; T. K. contributed to the discussion.

Acknowledgments

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Conflict of interest

Thomas Klein is a research employee of Boehringer Ingelheim Pharma.

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Figure Legends

Figure 1. DPP-4 expression and activity in immortalized human podocytes and mesangial cells. (A) Expression of the gene encoding for DPP-4 was evaluated in immortalized human podocytes and mesangial cells by RT-PCR analyses. Image is representative of five experiments run in duplicate. (B) DPP-4 activity in cell extracts, fresh, and podocyte-conditioned (5 days) media was evaluated by measuring the rate of fluorescence intensity increase, expressed as arbitrary units (A.U.) min^{-1} , normalized *per* μg of proteins. (C) DPP-4 expression in immortalized human podocytes was evaluated by Western blot analyses. Image is representative of five experiments run in duplicate. (D) Effects of increasing linagliptin concentrations (0.01-100 nM) on the enzymatic activity measured in immortalized human podocytes. Data are expressed as mean \pm SEM of five experiments run in duplicate. * $P < 0.05$ vs. podocytes.

Figure 2. Effects of glucose on DPP-4 activity in immortalized human mesangial cells. Immortalized human mesangial cells were exposed to high glucose concentration (4.5 g L^{-1}) up to 48 h. Enzymatic activity was evaluated in cell extracts by measuring the rate of

fluorescence intensity increase, expressed as arbitrary units (A.U.) min⁻¹, normalized *per* µg of proteins. Data are expressed as mean ± SEM of five experiments run in duplicate.

Figure 3. Effects of DPP-4 inhibitors on cell growth of immortalized human podocytes and mesangial cells. (A) Immortalized human podocytes and mesangial cells were exposed to linagliptin (30 nM; 1–5 days), and cell growth was measured by MTT assay. Data are expressed as cell growth *vs* control cultures (cells exposed to vehicle alone). (B) Immortalized human podocytes were exposed to increasing concentrations of either linagliptin (0.01–100 nM; 5 days) or sitagliptin (0.01–1000 nM; 5 days), and cell growth was measured as described. Data are expressed as mean ± SEM of six experiments run in triplicate. * $P < 0.05$ *vs.* control samples.

Figure 4. Effects of linagliptin on the expression of cyclin D1 and p27 in immortalized human podocytes. Immortalized human podocytes were exposed to linagliptin (100 nM) for 5 days then the expression of cyclin D1 and p27 was evaluated by Western blot analyses. Image is representative of five experiments run in duplicate.

Figure 5. Expression of components of the SDF-1-CXCR4/CXCR7 axes in immortalized human podocytes. (A) Expression of the gene encoding for SDF-1, CXCR4, and CXCR7 was evaluated in immortalized human podocytes by RT-PCR analyses. Image is representative of five experiments run in duplicate. (B) Release of SDF-1α by immortalized human podocytes was evaluated by measuring concentrations of this chemokine in fresh (0 days) and podocyte-conditioned (5 days) media by ELISA. Data are expressed as mean ± SEM of five experiments run in triplicate. * $P < 0.05$ *vs.* control samples.

Figure 6. Effects of AMD3100 on growth of immortalized human podocytes. (A) Immortalized human podocytes were exposed to increasing AMD3100 concentrations (0.01–1 µM; 5 days) in the absence or presence of linagliptin (1 nM), and cell growth was measured by MTT assay. Data are the mean ± SEM of five experiments run in triplicate. Combined effects were predicted by assuming Bliss independence.

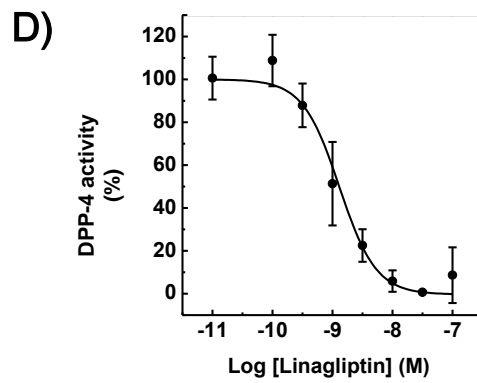
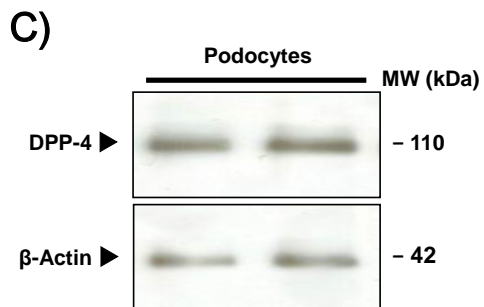
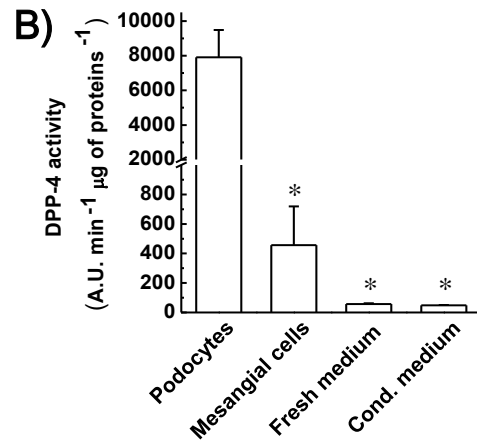
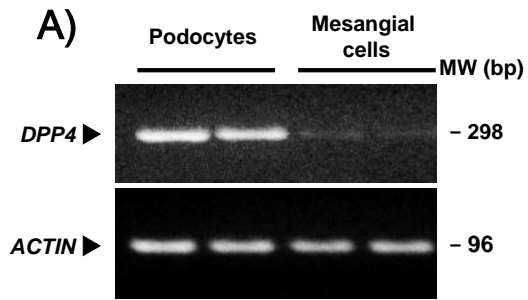
Figure 7. Effects of linagliptin and AMD3100 on ERK1/2 activation in immortalized human podocytes. Immortalized human podocytes were exposed to linagliptin (1 nM), AMD3100

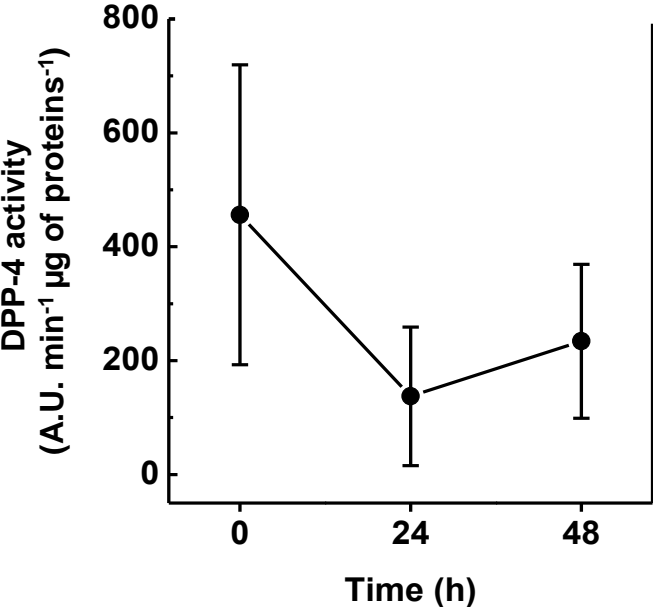
(0.3 μM) or both for 18 h. ERK1/2 activation was evaluated by Western blot analyses. Image is representative of five experiments run in duplicate.

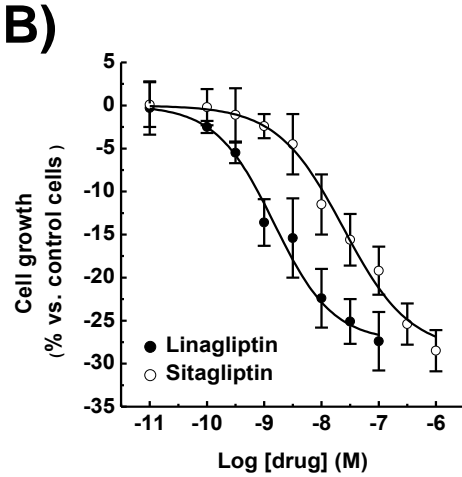
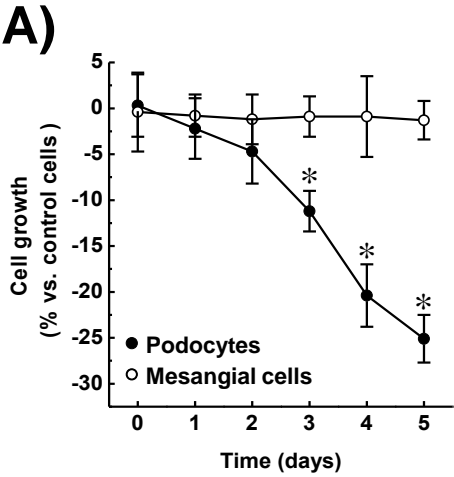
Figure 8. Effects of AZD6244 on growth of immortalized human podocytes. Immortalized human podocytes were exposed to increasing AZD6244 concentrations (0.1–10 μM ; 5 days) either in the absence or presence of linagliptin (1–100 nM), and cell growth was measured by MTT assay. Data are the mean \pm SEM of five experiments run in triplicate. Solid lines represent the combined observed effects. Dashed lines represent the combined effects predicted by assuming Bliss independence.

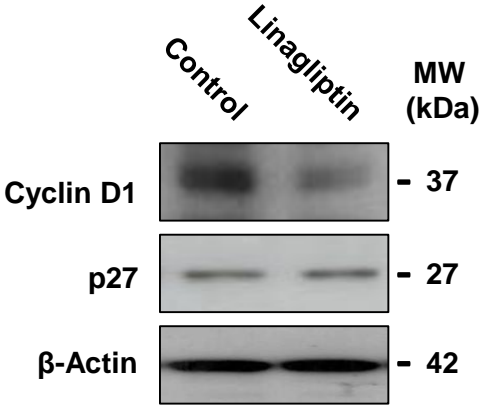
Figure 9. A proposed mechanistic model to interpret the effects of linagliptin and AMD3100 on the growth of immortalized podocytes. (A) DPP-4 is highly expressed by podocytes, and it likely participates to the SDF-1-CXCR4/CXCR7 signalling both by mediating the local processing of SDF-1 and interacting with CXCR4. (B) Decrease of podocyte proliferation could result from the perturbation of the SDF-1 signalling which is altered by both linagliptin and AMD3100, in a DPP-4-dependent and DPP-4-independent manner, respectively.

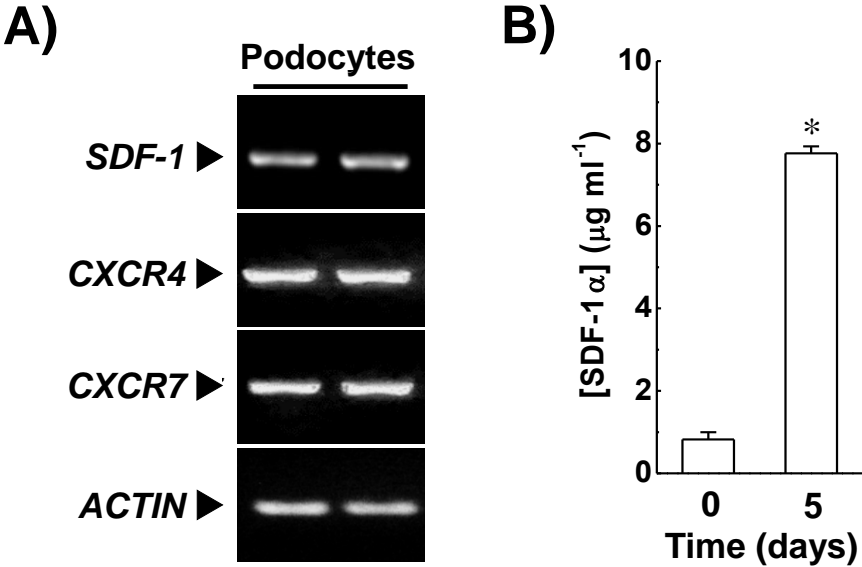
Miglio et al_Figure_1



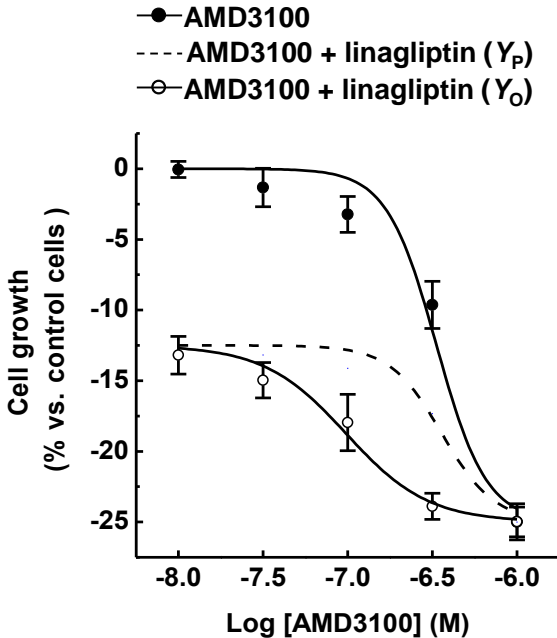




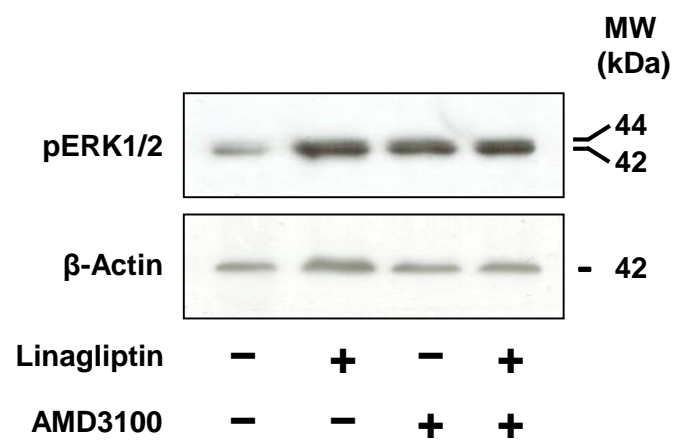




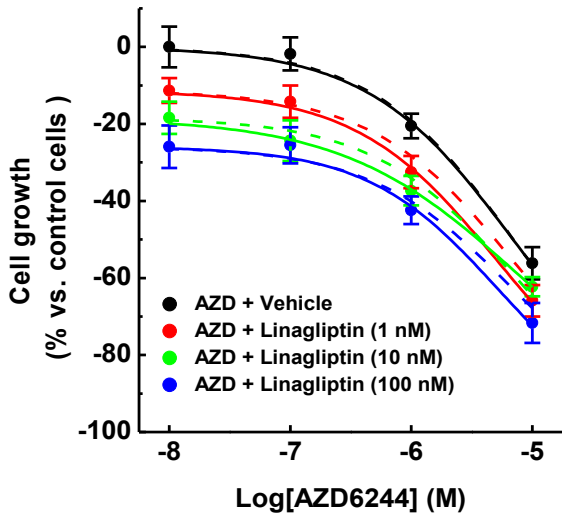
Miglio et al_Figure_6



Miglio et al_Figure_7



Miglio et al_Figure_8



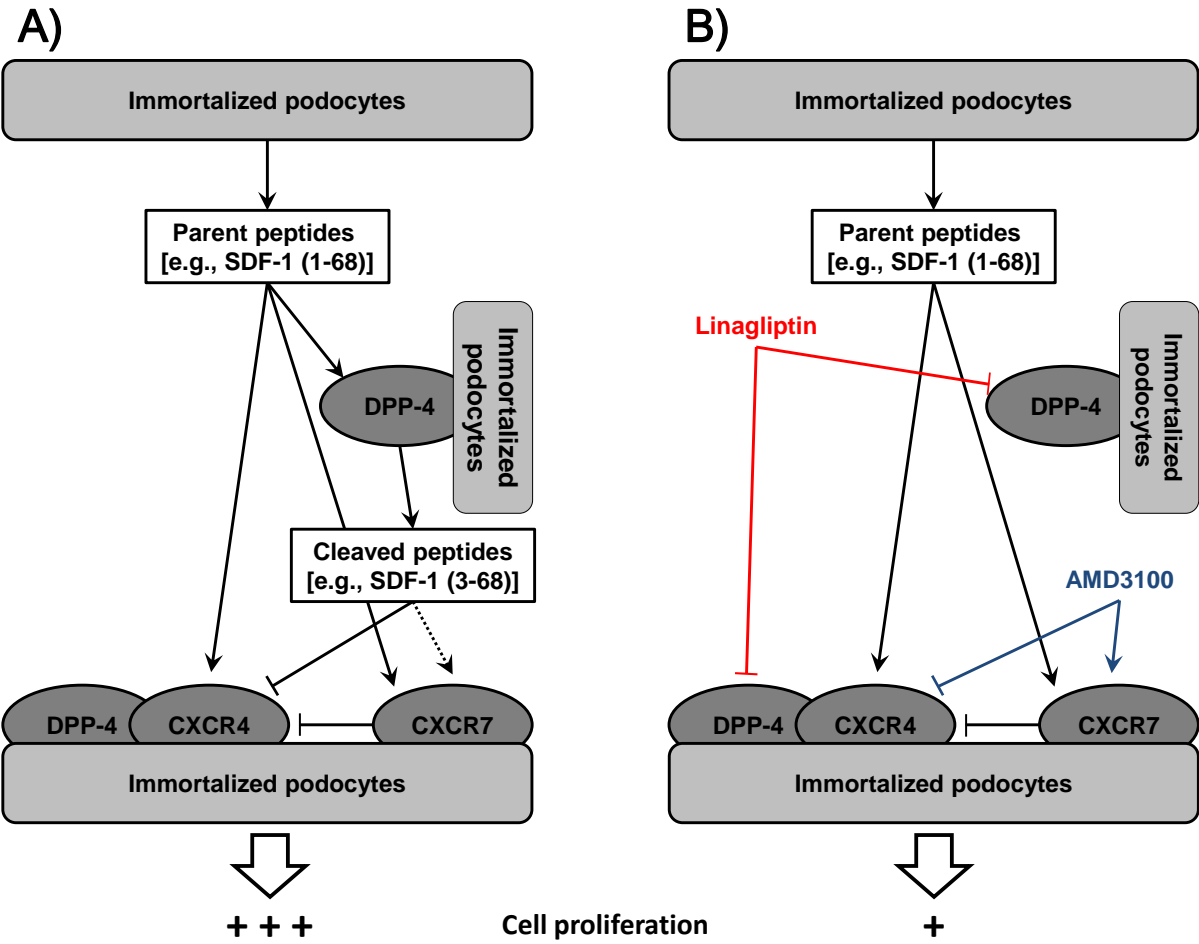


Table 1. Effects of linagliptin of cell cycle progression.

Cell cycle phase	Vehicle	Linagliptin (nM)	
		1	100
Sub-G ₁	4.8 ± 0.3	5.0 ± 0.3	4.8 ± 0.3
G ₀ /G ₁	56.7 ± 3.0	63.2 ± 1.4	66.7 ± 1.7 ^a
S	16.0 ± 0.9	13.4 ± 0.4	10.9 ± 0.5 ^a
G ₂ /M	22.5 ± 2.1	18.4 ± 0.9	17.7 ± 1.4

Immortalized human podocytes were exposed to linagliptin for 5 days, then they were harvested, stained with propidium iodide, and examined by flow cytometry to determine the percentage of cells in the different phases of the cell cycle. Data are the mean ± SEM of five experiments run in duplicate.

^a*P* < 0.05 vs vehicle alone.

Table 2. Combined effects of AZD6244 and linagliptin on podocyte growth.

[Linagliptin] (nM)	pIC ₅₀	
	<i>Y_P</i>	<i>Y_O</i>
-	5.2	5.2
1	5.2	5.3
10	5.2	5.1
100	5.2	5.3

Immortalized human podocytes were exposed to either AZD6244 alone or AZD6244 + linagliptin for 5 days, then cell growth was evaluated. Data are the mean ± SEM of five experiments run in duplicate.