


RESEARCH PAPER

GPR35 mediates Iodoxamide-induced migration inhibitory response but not CXCL17-induced migration stimulatory response in THP-1 cells; is GPR35 a receptor for CXCL17?

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BACKGROUND AND PURPOSE

GPR35 has long been considered an orphan GPCR, because no endogenous ligand of GPR35 has been discovered. CXCL17 (a chemokine) has been reported to be an endogenous ligand of GPR35, and it has even been suggested that it be called CXCR8. However, at present there is no supporting evidence that CXCL17 does interact with GPR35.

EXPERIMENTAL APPROACH

We applied two assay systems to explore the relationship between CXCL17 and GPR35. An AP-TGF- α shedding assay in GPR35 over-expressing HEK293 cells was used as a gain-of-function assay. GPR35 knock-down by siRNA transfection was performed in endogenously GPR35-expressing THP-1 cells.

KEY RESULTS

In the AP-TGF- α shedding assay, Iodoxamide, a well-known synthetic GPR35 agonist, was confirmed to be the most potent agonist among other reported agonists. However, neither human nor mouse CXCL17 had an effect on GPR35. Consistent with previous findings, G proteins $G\alpha_{i/o}$ and $G\alpha_{12/13}$ were found to couple with GPR35. Furthermore, Iodoxamide-induced activation of GPR35 was concentration-dependently inhibited by CID2745687 (a selective GPR35 antagonist). In endogenously GPR35-expressing THP-1 cells, Iodoxamide concentration-dependently inhibited migration and this inhibitory effect was blocked by CID2745687 treatment or GPR35 siRNA transfection. However, even though CXCL17 stimulated the migration of THP-1 cells, which is consistent with a previous report, this stimulatory effect of CXCL17 was not blocked by CID2745687 or GPR35 siRNA.

CONCLUSIONS AND IMPLICATIONS

The present findings suggest that GPR35 functions as a migration inhibitory receptor, but CXCL17-stimulated migration of THP-1 cells is not dependent on GPR35.

Abbreviations

AP-TGF- α , alkaline phosphatase fusion protein of TGF- α ; CXCL17, Chemokine (C-X-C motif) ligand 17; GPR35, G protein coupled receptor 35; LPA, lysophosphatidic acid; PGE₂, prostaglandin E₂

Introduction

Human **GPR35** was first described as an intronless GPCR gene encoding 309 amino acids (O'Dowd *et al.*, 1998), and subsequently, it was reported to be expressed in lungs, stomach, small intestine, colon, spleen and immune cells (including mast cells, basophils, eosinophils and invariant natural killer-like T cells) in man (Wang *et al.*, 2006; Fallarini *et al.*, 2010; Yang *et al.*, 2010). For a considerable time, GPR35 was considered an orphan receptor as no endogenous ligand had been discovered. Several endogenous molecules, such as lysophosphatidic acid, **kynurenic acid**, cGMP and reverse T3, were proposed as ligands (Wang *et al.*, 2006; Oka *et al.*, 2010; Jenkins *et al.*, 2011; Deng *et al.*, 2012; Southern *et al.*, 2013), but their potencies were too low (in the μM range) to support their roles as GPR35 ligands (Divorty *et al.*, 2015). Synthetic surrogate agonists, such as **zaprinast**, **pamoic acid**, compound 1, PSB-13253 and lodoxamide, have been successfully identified or developed (Taniguchi *et al.*, 2006; Jenkins *et al.*, 2010; Zhao *et al.*, 2010; Funke *et al.*, 2013; Neetoo-Isseljee *et al.*, 2013; Thimm *et al.*, 2013; MacKenzie *et al.*, 2014), and of these lodoxamide most potently interacts with human and rodent GPR35 (MacKenzie *et al.*, 2014). Selective synthetic antagonists for GPR35 were also developed, that is, CID-2745687 (also known as SPB05142 and ML194) and CID-2786812 (ML145) (Zhao *et al.*, 2010; Heynen-Genel *et al.*, 2010a,b; Jenkins *et al.*, 2012). GPR35 has been reported to couple to both $G_{\alpha_{i/o}}$ and $G_{\alpha_{13}}$ subunits and to recruit β -arrestin-2 (Wang *et al.*, 2006; Guo *et al.*, 2008; Ohshiro *et al.*, 2008; Jenkins *et al.*, 2010, 2011). Recently, **CXCL17** (a chemokine) was reported to be an endogenous ligand of GPR35, and GPR35 was proposed to be CXCR8 (Maravillas-Montero *et al.*, 2015). Furthermore, it was reported that CXCL17 increases the intracellular Ca^{2+} concentration in THP-1 cells (a human monocytic cell line) to promote chemotaxis at a concentration of 100 nM (Maravillas-Montero *et al.*, 2015). However, no supporting evidence has since been obtained with respect to the relationship between CXCL17 and GPR35, despite the fact that GPR35 is an emerging target in several pathological conditions, including inflammatory and cardiovascular diseases (Divorty *et al.*, 2015). The results presented by Maravillas-Montero *et al.* (2015) are intriguing but conflict with previous findings (Divorty *et al.*, 2015). Accordingly, verification of this ligand–receptor interaction by another laboratory or using another assay system is required (Im, 2004). Therefore, we used two assay systems to verify the matching of CXCL17 and GPR35. An AP-TGF- α shedding assay in GPR35 overexpressing HEK293 cells was used as a gain-of-function assay, and a GPR35 knock-down system, obtained by siRNA transfection, was applied to endogenously GPR35-expressing THP-1 cells as a loss-of-function assay.

Methods

AP-TGF- α shedding assay

HEK-293 cells (ATCC, Manassas, VA, USA) were cultured at 37°C in a 5% CO_2 humidified incubator and maintained in high glucose DMEM, containing 10% (v/v⁻¹) heat-inactivated

FBS, 100 U·mL⁻¹ penicillin, 50 $\mu\text{g}\cdot\text{mL}^{-1}$ streptomycin, 2 mM glutamine and 1 mM sodium pyruvate.

HEK-293 cells were seeded at a density of 2.0×10^5 cells·mL⁻¹ in a 12-well plate and, 16 h later, transfected with plasmids (an alkaline phosphatase fusion protein of TGF- α , human GPR35 or G_{α} proteins) for 24 h using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The next day, transfected HEK-293 cells were re-seeded in a 96-well plate, ligands were added in a concentration-dependent manner, and the plate was placed in an incubator for 1 h. Conditioned media were transferred into another empty 96-well plate and then p-NPP-containing solution was added to the conditioned media plate and to the cell plate. Plates were measured for 405 nm at 0 and 1 h after treatment of p-NPP-containing solution. The ratio of the two absorbances was used as a measure of GPCR activation.

Culture of THP-1 cells

Human THP-1 cells (a human monocytic leukaemia cell line) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT, USA), 100 U·mL⁻¹ penicillin, 50 $\mu\text{g}\cdot\text{mL}^{-1}$ streptomycin, 2 mM glutamine, at 37°C in a humidified 5% CO_2 incubator.

Migration assay

THP-1 migration was measured using 24-well Transwell inserts (6.5 mm) fitted with polycarbonate filters (5 μm pore size) (Corning Costar, Acton, MA, USA). THP-1 cells (1×10^6 cells in 150 μL of RPMI 1640 medium) were added to the upper chamber, and 600 μL of RPMI 1640 medium containing the indicated concentrations of lodoxamide or human CXCL17 was added to the lower chamber. Plates were incubated at 37°C in 5% CO_2 for 24 h, and the cells that migrated into the lower chamber were collected and counted using a haemocytometer.

Transfection

The following oligonucleotides were purchased from Bioneer (Korea); GPR35 siRNA sense 5'-CCACAAAAGCCAGGACUC U(dTdT)-3', antisense 5'-AGAGUCCUGGCCUUUUGUG G(dTdT)-3'. SN-1003 from Bioneer was used as scrambled siRNA. Briefly, THP-1 cells were seeded at a density of 1.0×10^5 cells in 12 well plates, and 24 h later, GPR35 siRNA (100 nM) was introduced to the cells using Lipofectamine LTX reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Non-silencing siRNA and siRNAs specific for GPR35 were transfected, and 48 h after transfection, target knock-downs were confirmed by RT-PCR and Western blotting.

Statistics

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Results are expressed as the means \pm SEM for the number of determinations indicated. The statistical significances of differences were determined by ANOVA, and significance was accepted for P values < 0.01 . * Indicates significance compared to untreated

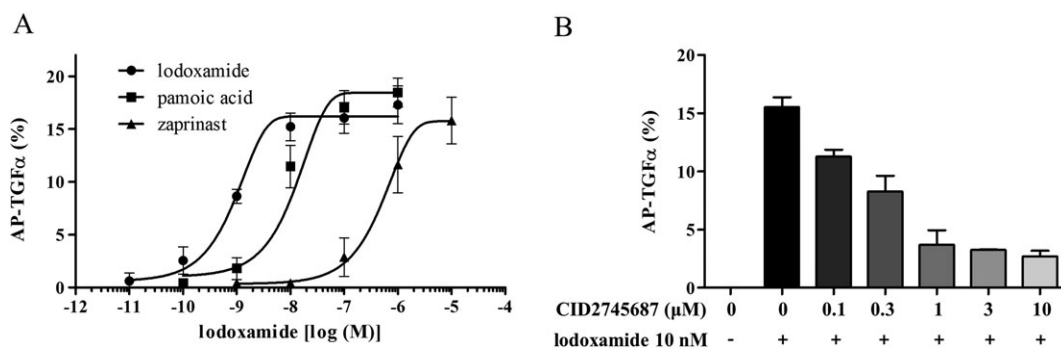


Figure 1

Lodoxamide induced the activation of GPR35, and this activation was inhibited by CID2745687 (a GPR35 selective antagonist). (A) Concentration–response curves for lodoxamide, pamoic acid and zaprinast as determined by the AP-TGF α shedding assay. Agonism of GPR35 agonists was examined in GPR35 coexpressed with a mixture of eight G α proteins in HEK-293 cells. Results are presented as the means \pm SEM of three individual experiments. (B) Inhibition of the lodoxamide-induced GPR35 activation by CID2745687. Antagonism by CID2745687 was tested in the presence of 10 nM of lodoxamide. Results are presented as the means \pm SEM of three individual experiments.

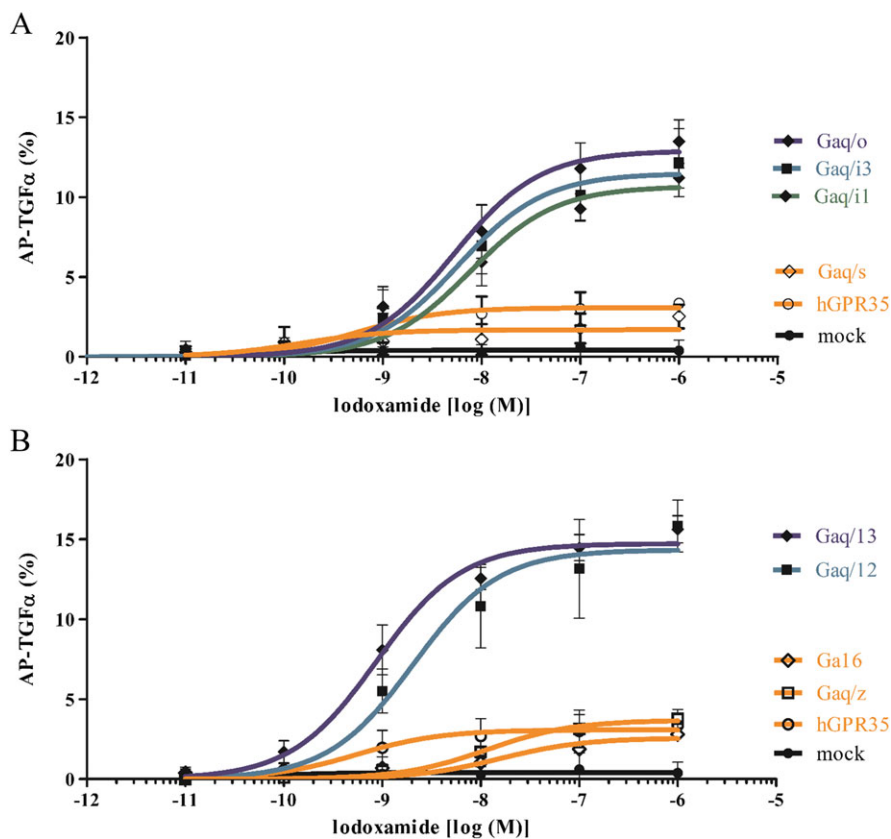


Figure 2

GPR35 coupling of G proteins. AP-TGF α release responses were measured in human GPR35-transfected HEK-293 cells, in which eight G α proteins were coexpressed individually. In G $\alpha_{q/11}$, G $\alpha_{q/i3}$, G $\alpha_{q/o}$, G $\alpha_{q/12}$ or G $\alpha_{q/13}$ -coexpressing cells, GPR35 induced potent AP-TGF α shedding responses, whereas G $\alpha_{q/s}$, G $\alpha_{q/z}$ or G α_{16} -coexpressing cells did not. Results are presented as the means \pm SEM of three individual experiments.

controls and [#] compared to lodoxamide-treated or negative control groups.

Materials

Lodoxamide was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), and kynurenic

acid was from Sigma-Aldrich (St. Louis, MO, USA). Recombinant mouse CXCL17 and human CXCL17 were purchased from R&D system (Minneapolis, MN, USA), and zaprinast, CID-2745687 and pamoic acid were from Tocris (Ellisville, Missouri, USA). Plasmids for the AP-TGF α shedding assay, that is, an alkaline phosphatase fusion protein of TGF α ,

human GPR35 and eight $G\alpha$ proteins, were kindly provided by Dr Junken Aoki at Tohoku University. The antibody for GPR35 (NBP2-24640) was purchased from Novus Biologicals (Littleton, CO, USA).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

Results

Lodoxamide, but not CXCL17, activates GPR35 in HEK293 cells

As a gain-of-function assay, GPR35 was overexpressed in HEK293 cells and its activity was measured by AP-TGF- α shedding assay. AP-TGF- α shedding assay is generally considered the best system to test or verify functions of almost all GPCRs (Inoue *et al.*, 2012). Lodoxamide (a synthetic GPR35 agonist) was found to be a more potent agonist than other reported

agonists, such as pamoic acid, kynurenic acid and zaprinast (Figure 1A). EC₅₀ values for lodoxamide, pamoic acid, zaprinast and kynurenic acid were 1 nM, 9 nM, 0.7 μ M and 4.1 mM respectively. Furthermore, the lodoxamide-induced activation of GPR35 was concentration-dependently inhibited by CID2745687 (a selective GPR35 antagonist) (Figure 1B). AP-TGF- α shedding was observed when GPR35 was co-transfected with $G\alpha_{q/11}$, $G\alpha_{q/13}$, $G\alpha_{q/o}$, $G\alpha_{q/12}$ or $G\alpha_{q/13}$, but not with $G\alpha_{q/s}$, $G\alpha_{q/z}$ or $G\alpha_{16}$ (Figure 2), which suggests GPR35 can couple to $G\alpha_{11}$, $G\alpha_{13}$, $G\alpha_o$, $G\alpha_{12}$ or $G\alpha_{13}$, which is consistent with the previously reported G protein coupling character of GPR35 to $G\alpha_{i/o}$ and $G\alpha_{13}$ (Divorty *et al.*, 2015). However, neither human nor mouse CXCL17 activated GPR35 (Figure 3), which contrasts with the previous report by Maravillas-Montero *et al.* (2015).

Lodoxamide inhibits the migration of THP-1 cells through GPR35, but CXCL17-stimulated migration is not dependent on GPR35

THP-1 cells were used for the loss-of-function assay, because they express GPR35 endogenously and because its involvement in the action of CXCL17 action was observed only in these cells (Maravillas-Montero *et al.*, 2015). We observed

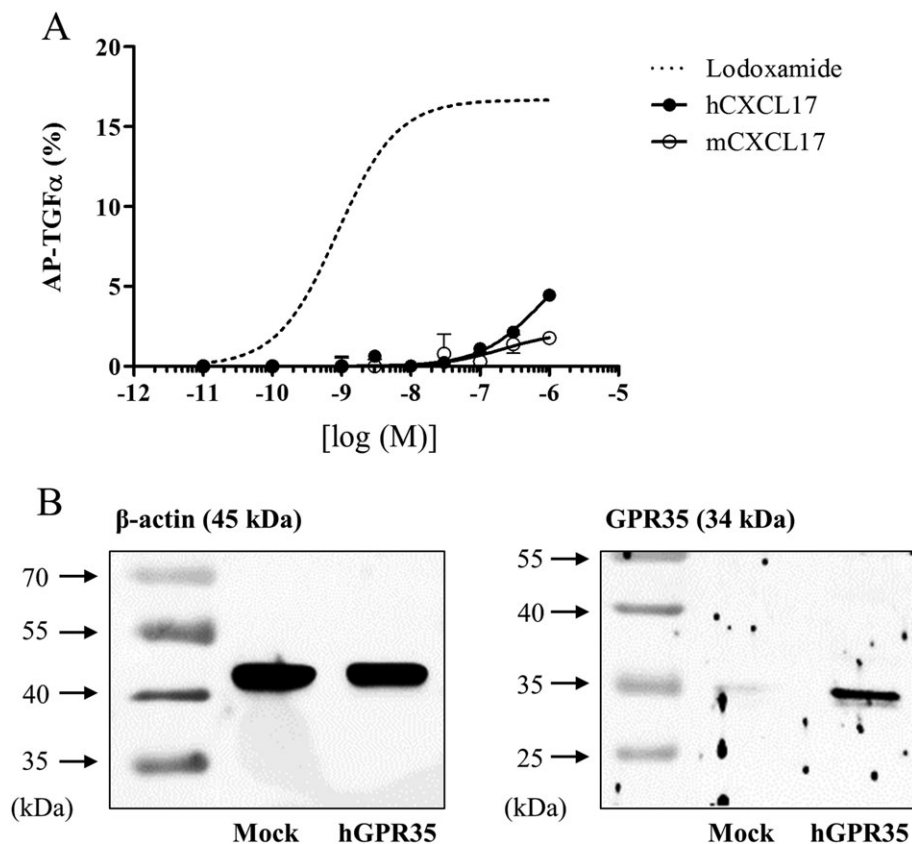


Figure 3

Human and mouse CXCL17 responses as determined by the AP-TGF- α shedding assay. (A) Effects of human and mouse CXCL17 in the AP-TGF- α shedding assay. Release of AP-TGF- α by both human and mouse CXCL17 was measured in HEK-293 cells transfected with GPR35 and a mixture of eight $G\alpha$ proteins. Results are presented as the means \pm SEM of three individual experiments. Concentration-response curve for lodoxamide was duplicated from Figure 1 for a comparison. (B) HEK-293 cells were transfected with plasmids of human GPR35 or mock plasmid along with AP-TGF- α and G proteins 24 h, and then, GPR35 proteins (B) were evaluated by Western blotting.

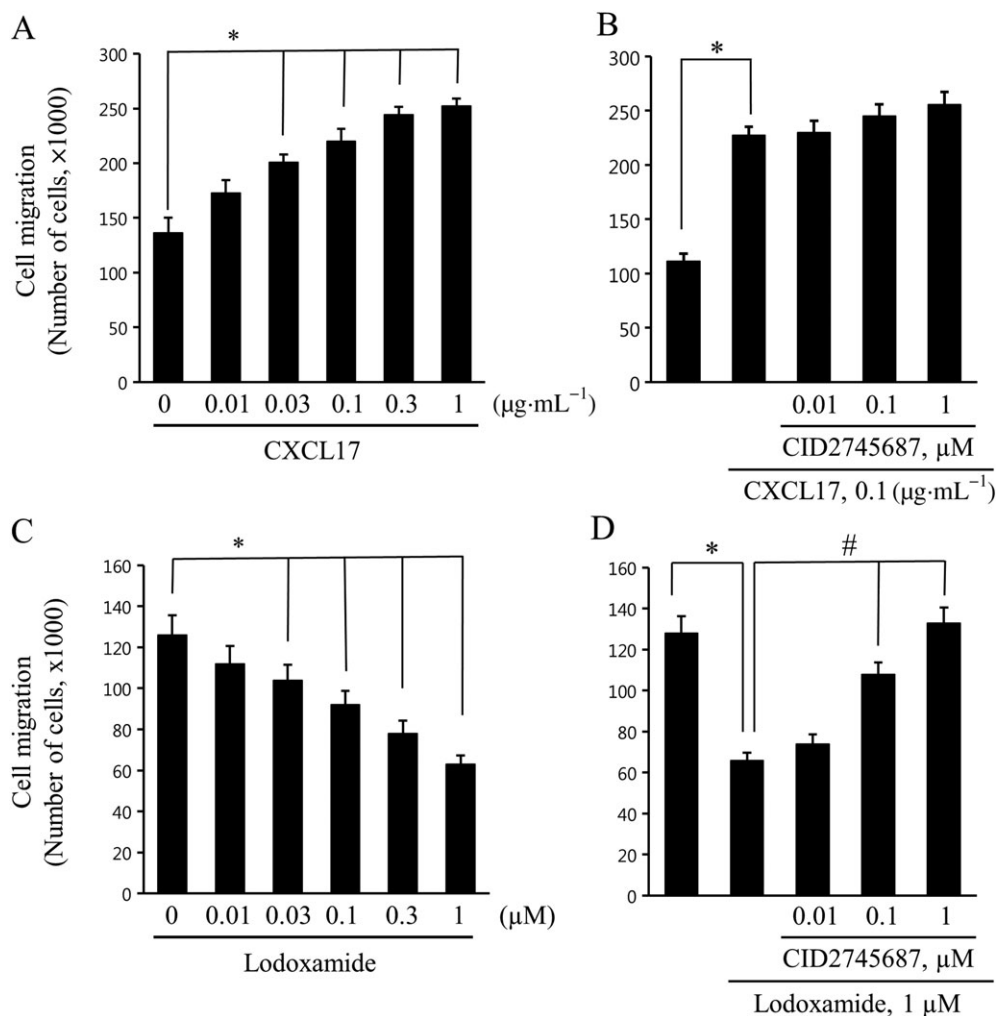


Figure 4

Migration responses to CXCL17 or lodoxamide in THP-1 cells. (A and C) THP-1 cells were added to upper Transwell chambers and allowed to migrate for 24 h toward lower chambers containing the indicated concentrations of CXCL17 or lodoxamide. (B and D) THP-1 cells were pretreated with the indicated concentrations of CID2745687 for 1 h and then added to the upper chambers and allowed to migrate for 24 h toward lower chambers containing $0.1 \mu\text{g}\cdot\text{mL}^{-1}$ CXCL17 or $1 \mu\text{M}$ lodoxamide. Results are presented as the means \pm SEM of three or four individual experiments.

that CXCL17 stimulated the migration of THP-1 cells (Figure 4A), which is consistent with the previous findings (Maravillas-Montero *et al.*, 2015). However, the CXCL17-induced stimulation of migration was not blocked by treatment with the GPR35 antagonist CID2745687 (Figure 4B) or by GPR35 siRNA transfection (Figure 5C). In contrast, lodoxamide inhibited the migration of THP-1 cells in a concentration-dependent manner (Figure 4C), and this inhibition by lodoxamide was blocked by CID2745687 treatment (Figure 4D) or GPR35 siRNA transfection (Figure 5D). These findings suggest that GPR35 functions as a migration inhibitory receptor for lodoxamide in THP-1 cells and that CXCL17 stimulates migration in a GPR35-independent manner.

Discussion

Confirmation of ligand–receptor matching by another laboratory is important, because mismatching can cause

considerable confusion, as exemplified by the mismatching of OGR-1 and GPR4 with sphingosylphosphorylcholine and lysophosphatidylcholine respectively (Xu and Casey, 1996; Zhu *et al.*, 2001, 2005; Bektas *et al.*, 2003). However, when several assay systems support a ligand–receptor matching result, even in a single report, the result is usually reliable, for example, for the LPA-GPR23 interaction (GPR23 was later renamed as the LPA₄ receptor) (Noguchi *et al.*, 2003; Im, 2004; Lee *et al.*, 2007; Kihara *et al.*, 2014).

In the case of CXCL17-GPR35 matching, Maravillas-Montero *et al.* reported that CXCL17 promotes the chemotaxis of THP-1 cells in a pertussis toxin-sensitive manner and that chemotaxis was enhanced in PGE₂-treated THP-1 cells (Maravillas-Montero *et al.*, 2015). These findings support the notion that CXCL17 can induce chemotaxis and that its receptor might be coupled to pertussis toxin-sensitive G $\alpha_{i/o}$ proteins (Maravillas-Montero *et al.*, 2015). Our data support the stimulatory effect of CXCL-17 on migration in THP-1 cells. However, GPR35 was not found to be involved.

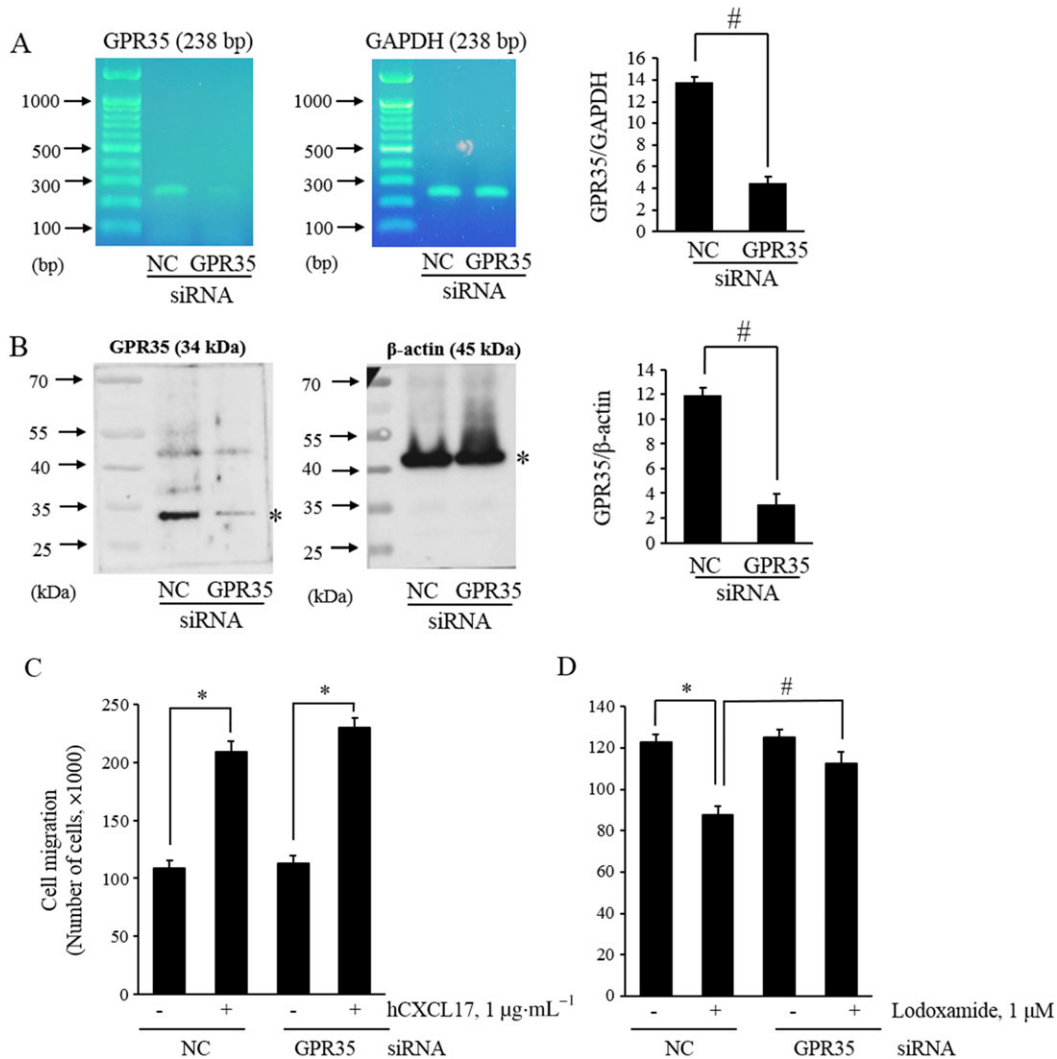


Figure 5

GPR35-dependence of migration response to lodoxamide in THP-1 cells. (A and B) THP-1 cells were transfected with scrambled siRNA as negative control (NC) or GPR35 siRNA (GPR35) for 48 h, and then, the expressions of GPR35 mRNA (A) or protein (B) were evaluated by RT-PCR or Western blotting. (C and D) Transfected THP-1 cells were added to the upper chambers and allowed to migrate for 24 h towards the lower chambers containing 0.1 $\mu\text{g}\cdot\text{mL}^{-1}$ CXCL17 or 1 μM lodoxamide. Results are presented as the means \pm SEM of three individual experiments.

Increased expression of GPR35 in PGE₂-treated THP-1 cells and reduced expression of GPR35 in the lungs of CXCL17 knockout mice were suggested to be indirect evidence of CXCL17-GPR35 matching (Maravillas-Montero *et al.*, 2015), but these increased or decreased expression levels of GPR35 might have occurred coincidentally due to variable responses to CXCL17 (Maravillas-Montero *et al.*, 2015).

Maravillas-Montero *et al.* also reported that CXCL17 increases the intracellular Ca²⁺ concentration in human THP-1 cells (Maravillas-Montero *et al.*, 2015). Furthermore, CXCL17-induced intracellular Ca²⁺ increase was observed in GPR35-transfected BA/F3 cells and HEK293 cells (Maravillas-Montero *et al.*, 2015). However, these cells were not treated with a GPR35 antagonist or subjected to GPR35 knock-down to confirm that GPR35 mediated this CXCL17-induced Ca²⁺ response (Maravillas-Montero *et al.*, 2015). The report of

Maravillas-Montero *et al.* contrasts with data from previous studies, in which GPR35-activation by other ligands did not promote coupling to G α_q pathway (Jenkins *et al.*, 2011; 2012; Mackenzie *et al.*, 2011; Maravillas-Montero *et al.*, 2015). Similarly, it was reported that lysophosphatidic acid induced and increase in Ca²⁺ in GPR35-expressing HEK293 cells (Oka *et al.*, 2010), although this was contradicted by subsequent reports (Oka *et al.*, 2010; Southern *et al.*, 2013; Divorty *et al.*, 2015). In addition, we did not observe a CXCL17-induced increase in intracellular Ca²⁺ in THP-1 cells, although we did observe that ATP increased Ca²⁺ in these cells (Supporting Information Figure S1).

As mentioned above, pharmacological evidence for CXCL17-GPR35 matching was inadequate in the previous study and was not supported by our findings. To summarize, CXCL17 did not activate GPR35 in the AP-TGF- α shedding

assay, which provided a means of validating the effects of other GPR35 activators. Furthermore, CXCL17-promoted migration was not blocked by CID-2745687 (a GPR35 selective antagonist) or by GPR35 knock-down in THP-1 cells. However, Iodoxamide inhibited migration in these THP-1 cells, and this inhibitory effect was blocked by CID-2745687 and by GPR35 knock-down. Therefore, our findings indicate that GPR35 functions as a migration inhibitory receptor and that CXCL17 stimulates migration in a GPR35-independent manner in THP-1 monocytic cells.

There are two isoforms of human GPR35, that is, the short GPR35a and long GPR35b (Okumura *et al.*, 2004). As shown in Supporting Information Figure S2, GPR35a is mainly expressed in THP-1 cells and we used GPR35a. Because Iodoxamide has been used as a mast cell stabilizer, we tested the selectivity of Iodoxamide for GPR35 by applying the AP-TGF- α shedding assay in H₁ histamine or LPA₁ lysophosphatidic acid receptor overexpressing HEK293 cells. As shown in Supporting Information Figure S3, the H₁ and LPA₁ receptors were confirmed to be functional, but Iodoxamide did not activate either receptor.

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Author contributions

S.-J.P. conducted the experiments of Figures 1 and 2. S.-J.L. conducted the experiments of Figures 3 and 4. S.-J.P. and S.-Y.N. conducted the experiments for the Supporting Information figures. D.-S.I. planned the experiments, analyzed data and wrote the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

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Figure S1 Effects of lodoxamide, CXCL17, and APT on intracellular Ca²⁺ concentrations in THP-1 cells. A) Representative [Ca²⁺]_i traces of THP-1 cells treated with lodoxamide (100 nM or 1 mM), hCXCL17 (100 nM), mCXCL17 (100 nM), or ATP (100 mM). Arrows indicate when compounds were added. B) Histogram of Ca²⁺ responses. Results are presented as the means \pm SEs of three independent experiments.

Figure S2 THP-1 cells express GPR35a. RT-PCR was conducted to detect GPR35a and GPR35b. THP-1 cells expressed mainly GPR35a and we used GPR35a. Primer sequences are followings. hGPR35a Forward GTGTTTCGTGGTCTGCTTCC T, hGPR35b Forward GTCCTTGCGTCTCTCTGACC, hGPR35 Reverse GAGAGTCCTGGCTTTTGTGG.

Figure S3 Lodoxamide did not induce activation of H1 histamine or LPA1 lysophosphatidic acid receptor. Concentration-response curves of histamine (A), LPA (B), and lodoxamide (C and D) as determined by the AP-TGF α shedding assay in H1 or LPA1 coexpressed with a mixture of eight G α proteins in HEK-293 cells. Results are presented as the means \pm SEs of 3 individual experiments.