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Short Communication

The Pyrazolone Originally Reported to Be a Formyl Peptide Receptor (FPR) 2/ALX–Selective Agonist Is Instead an FPR1 and FPR2/ALX Dual Agonist

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Abstract. A pyrazolone compound acting as a formyl peptide receptor (FPR) 2/ALX–selective agonist has been reported, but its pharmacological activities on human FPRs (hFPRs) and mouse FPRs (mFprs) have not been well demonstrated. In this study, we found that this compound, designated as compound A, induced concentration-dependent calcium flux not only in Chinese hamster ovary (CHO) cells expressing hFPR2/ALX but also in cells expressing hFPR1, mFpr1, or mFpr2. It also induced the receptor internalization of hFPR1 and hFPR2/ALX and, accordingly, induced calcium influx and chemotactic responses in both human and mouse neutrophils. Our study revealed that compound A is in fact an FPR1 and FPR2/ALX dual agonist.

Keywords: formyl peptide receptor, neutrophil, chemotaxis

Formyl peptide receptor (FPR) 2/ALX, also known as FPRL1, belongs to a family of FPRs which are G_i protein-coupled receptors and is mainly expressed on neutrophils and monocytes (1, 2). This receptor has been implicated to play an important role in regulating inflammation, and FPR2/ALX agonists are expected to be novel anti-inflammatory and pro-resolution agents. Recently, several non-peptidyl small-molecule compounds acting as an FPR2/ALX agonist have been reported (3 – 8). Among them, the pyrazolone compound (compound 43 in reference 6 and designated as compound A in this report) was the most potent human FPR2/ALX (hFPR2/ALX) agonist ($EC_{50} = 0.044\ \mu M$) and was stated to have no activity for hFPR1, the closest receptor of hFPR2/ALX, up to $10\ \mu M$. In addition, this compound showed an inhibitory effect on human neutrophil chemotaxis against interleukin (IL)-8 and exerted an anti-inflammatory effect in mice. However, the pharmacological activities of compound A on hFPRs and mouse FPRs (mFprs) have not been well demonstrated.

In this study, we investigated the detailed agonistic activities of compound A for hFPR1 and hFPR2/ALX and their mouse homologous receptors mFpr1 and mFpr2, respectively (1). We evaluated the concentration-dependent agonistic activities of compound A for these receptors in aequorin assays. We further evaluated its agonistic activities in receptor internalization assays. In addition, we investigated its effects on freshly isolated human and mouse neutrophils in assays measuring calcium flux and chemotaxis.

Compound A [*N*-(4-chlorophenyl)-*N'*-(5-isopropyl-1-methyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl) urea] (Fig. 1A) was synthesized in the Medicinal Chemistry Research Laboratories at Daiichi Sankyo.

A calcium release assay based on the luminescence of mitochondrial aequorin in Chinese hamster ovary (CHO)-K1 cells transfected with $G_{\alpha 16}$, apoaequorin, and pcDNA3.1(+) constructs was performed as described previously (9). Briefly, CHO-K1 cells were transfected with a plasmid DNA encoding human $G_{\alpha 16}$ protein (Molecular Devices, Sunnyvale, CA, USA), plasmid DNA encoding apoaequorin (Invitrogen, Carlsbad, CA, USA), and plasmid DNA encoding one of the FPRs or pcDNA3.1(+) as a mock-transfection control using Lipofectamine 2000 (Invitrogen). The next day, the transiently transfected cells incubated with $2.5\ \mu M$ coelenterazine h were used for assays. The luminescence

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of aequorin was measured with FDSS 6000 (Hamamatsu Photonics, Shizuoka). The results are expressed as the percentage of the response to 100 μM adenosine triphosphate (ATP). Two peptides, fMLF (*N*-formyl-Met-Leu-Phe; Sigma-Aldrich, St. Louis, MO, USA) and WKYMVM (Trp-Lys-Tyr-Met-Val-Met-NH2; Tocris, Ellisville, MO, USA), were used as control agonists.

Agonist-induced internalization of the receptors was measured as described extensively in a previous report (10). Briefly, human embryonic kidney (HEK) 293 cells stably expressing hFPR1- or hFPR2/ALX-enhanced–green fluorescent protein (EGFP) fusion protein were seeded and incubated in a poly-lysine–coated plate overnight. The cells were stimulated with agonists at 37°C for an hour and fixed with paraformaldehyde. The nuclei of the cells were dyed with Hoechst 33342. The fluorescence intensities of the confocal images were measured with a plate reader (IN Cell Analyzer 1000; GE Healthcare, Little Chalfont, Buckinghamshire, UK), and the receptor internalization was quantified using the granularity analysis algorithm of the IN Cell software. The number of granules per nucleus, a parameter calculated by the software, is shown as an internalization index.

All the experiments using human materials were reviewed and approved by the Institutional Ethical Committee of Daiichi Sankyo. Venous blood anticoagulated with sodium heparin was obtained from healthy volunteers. The neutrophils were purified by sequential preparation of sedimentation with dextran sulfate and density-centrifugation with Ficoll-Paque (GE Healthcare). The erythrocytes were removed by hypotonic shock. In the experiments using mice, male Balb/c mice of five to seven weeks of age were purchased from Charles River Laboratories Japan (Kanagawa). All the animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Daiichi Sankyo. Mouse neutrophils were isolated from bone marrow cells by density centrifugation with Histopaque-1077 and Histopaque-1119 (Sigma-Aldrich). The purity of isolated neutrophils were routinely >95%, as assessed by light microscopic analysis of the cells stained with Diff-Quick (Wako Pure Chemical Industries, Osaka), and >98% viable, as assessed by a trypan blue exclusion test.

In the measurement of the calcium flux, isolated human neutrophils were incubated with 4 μM Fluo 4-acetoxymethyl ester (AM) and 0.16% pluronic acid at 37°C for 30 min. After washing the cells, they were stimulated with compound A. The calcium flux was measured with a flow cytometer as changes of the fluorescence intensity in the neutrophils. To study the calcium flux in mouse neutrophils, bone marrow cells were labeled with phycoerythrin-conjugated anti-Gr-1 monoclonal antibody and then incubated with Fluo 4-AM and pluronic acid as described above. The cells were stimulated with compound A and the calcium flux in the Gr-1$^+$ neutrophil population was analyzed.

A chemotaxis assay was performed using HTS Transwell-96 plates with 3-μm pores (Corning, Lowell, MA, USA). Isolated human or mouse neutrophils were incubated with Calcein-AM at 37°C for 30 min. Chemotaxis of neutrophils was induced at 37°C for 1 h (human) or 30 min (mouse). The migrated cells were lysed with sodium dodecyl sulfate and the resultant fluorescence intensity was measured with a plate reader. Cytotoxity was evaluated by the Alamar Blue staining method (11).

In the beginning, we investigated the concentration-dependent agonistic activities of compound A for hFPR1, hFPR2/ALX, mFpr1, and mFpr2 by aequorin assays. We confirmed that compound A induced a concentration-dependent calcium flux in hFPR2/ALX-expressing cells (Fig. 1B). Unexpectedly, compound A also induced a calcium flux in hFPR1-expressing cells with potency similar to that in hFPR2/ALX-expressing cells. Compound A did not induce a calcium flux in mock-transfected control cells (data not shown). Furthermore, we investigated the agonistic activities of compound A for mFpr1 and mFpr2 and it induced calcium mobilization in both mFpr1- or mFpr2-expressing cells. Thus compound A acted as a dual agonist for FPR1 and FPR2/ALX in humans and for Fpr1 and Fpr2 in mice.

To further confirm the agonistic activity of compound A on hFPR1 and hFPR2/ALX, we next investigated whether compound A would induce receptor internalization using cells expressing hFPR1- or hFPR2/ALX-EGFP. Upon stimulation with compound A, the cells showed receptor internalization, which was detected as granule formation (Fig. 2A). Compound A induced the internalization of both hFPR1 and hFPR2/ALX in a concentration-dependent manner (Fig. 2B). The responding patterns of the three ligands, compound A, fMLF, and WKYMVM, were similar between the aequorin and internalization assays. From these results, we concluded that compound A is a potent FPR1 and FPR2/ALX dual agonist.

To study the functional consequences of stimulating freshly isolated human and mouse neutrophils with an FPR1 and FPR2/ALX dual agonist, we investigated the effects of compound A on calcium mobilization and chemotactic response in neutrophils. Stimulation with compound A induced calcium mobilization in both human and mouse neutrophils (Fig. 3A). Compound A

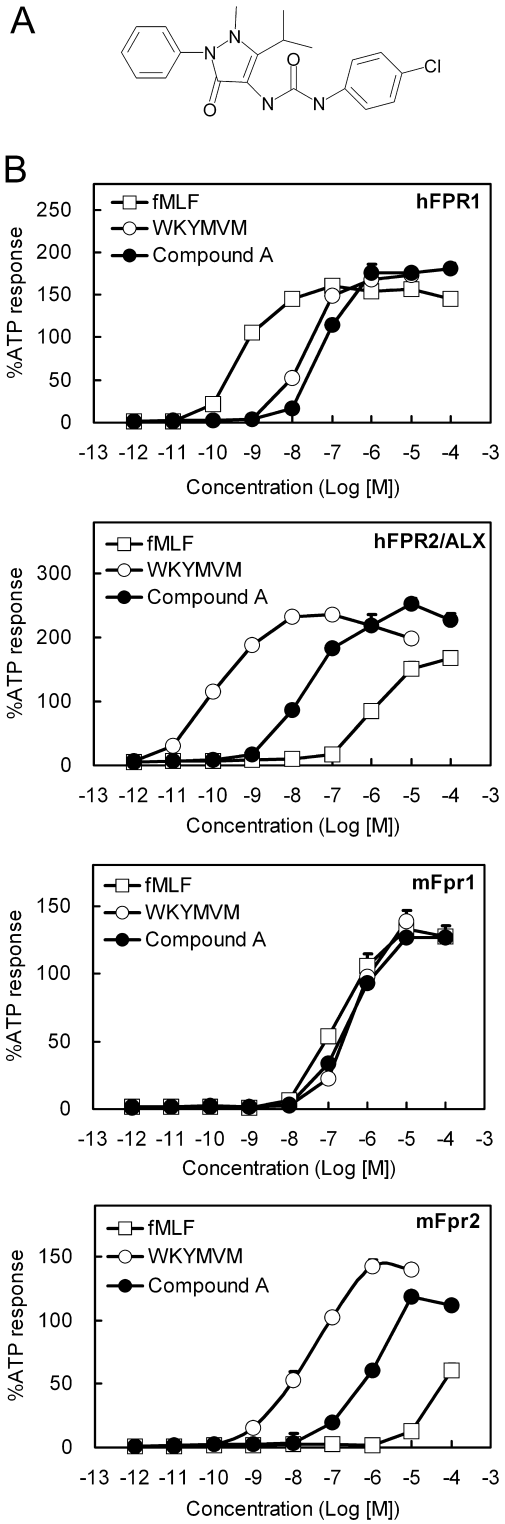

Fig. 1. Compound A induced calcium mobilization in cells expressing hFPR1, hFPR2/ALX, mFpr1, or mFpr2. A) Chemical structure of compound A is shown. B) CHO-K1 cells were transiently transfected with one of the FPRs (hFPR1, hFPR2/ALX, mFpr1, or mFpr2), $G_{\alpha 16}$, and apoaequorin. Ca^{2+} mobilization was determined by the luminescence of the aequorin. Data (mean ± S.E.M.) are expressed as the percentage of the response to 100 μM ATP. The experiment was performed in duplicate and repeated three times with similar results.

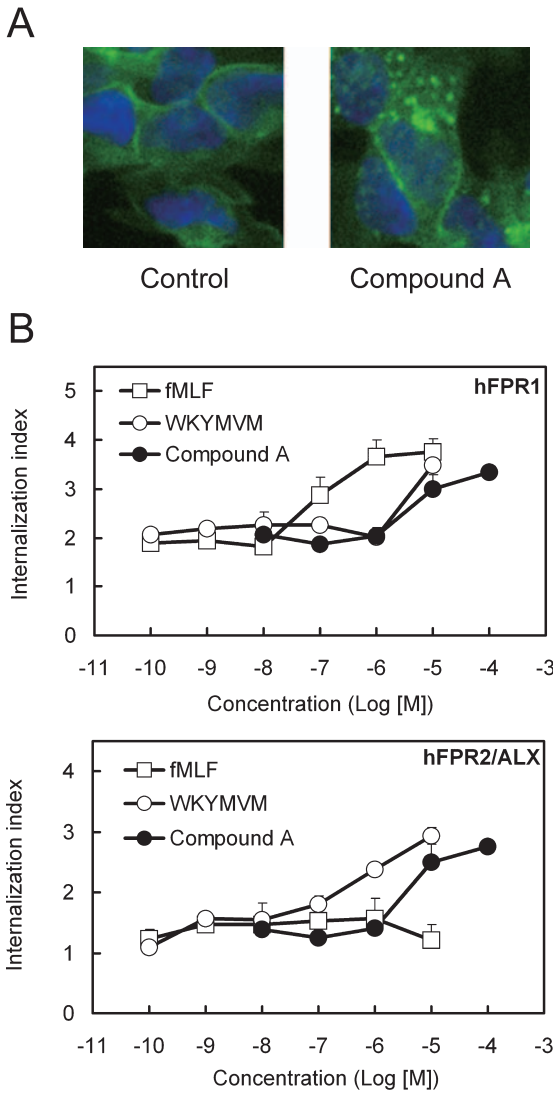

Fig. 2. Compound A induced receptor internalization of hFPR1 and hFPR2/ALX. HEK293 cells expressing hFPR1- or hFPR2/ALX-EGFP were treated with compound A, fMLF, or WKYMVM at various concentrations. After an hour, the cells were fixed, stained with Hoechst 33342 for nuclei, and analyzed with an automated cell imager. A) Typical results of images from the vehicle (0.1% DMSO in medium)-treated control and 10 μM compound A–treated hFPR1-expressing cells are depicted. B) Data (mean ± S.E.M.) are expressed as the internalization index, which was calculated as described in the text. The results of the control cells are 2.2 ± 0.1 for hFPR1 and 1.4 ± 0.1 for hFPR2/ALX. The experiment was performed in triplicate and repeated twice with similar results.

also induced chemotactic responses in both human and mouse neutrophils with a typical bell-shaped dose–response curve (Fig. 3B). We assessed the cytotoxicity of compound A to human and mouse neutrophils by using Alamar Blue (11), a redox indicator, and no obvious cytotoxicity was observed up to 10 μM (data not shown). Thus, compound A induced the activation and chemotaxis of both human and mouse neutrophils.

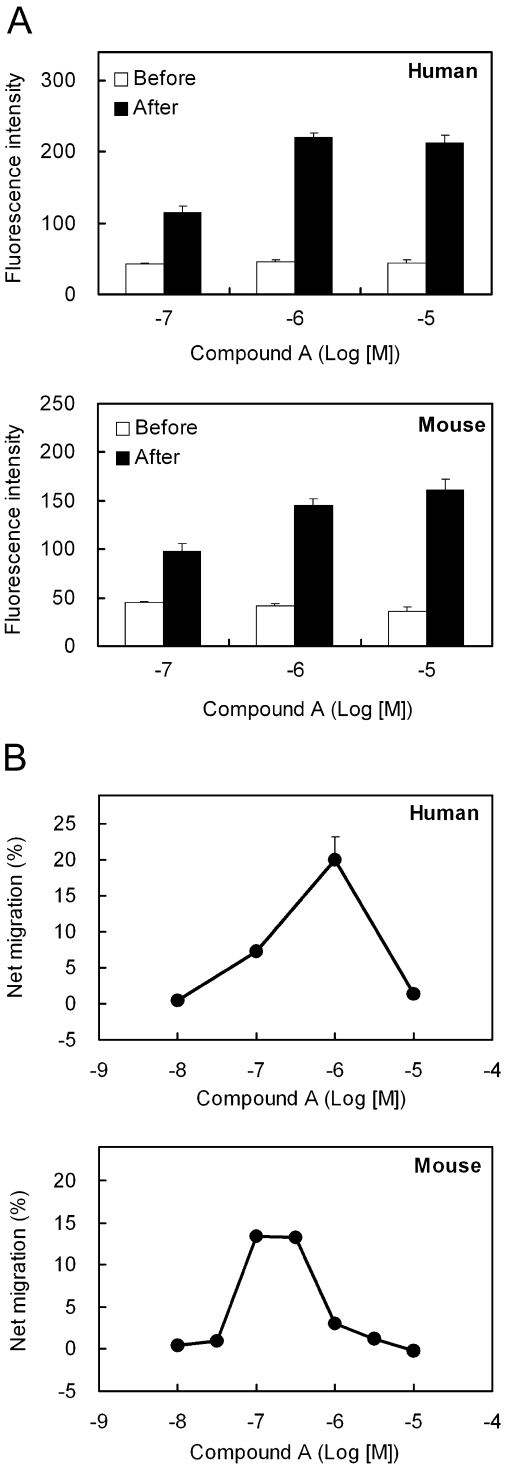

Fig. 3. Compound A induced calcium flux and chemotactic response in human and mouse neutrophils. A) Ca^{2+} mobilization in human or mouse neutrophils was measured as the fluorescence intensity of calcium indicator Fluo 4 by flow cytometry before (white bar) and after (black bar) stimulation with various concentrations of compound A. Data (mean ± S.E.M.) are expressed as the mean fluorescence. B) Chemotaxis assays were performed using a Transwell. Chemotaxis of human or mouse neutrophils was induced with various concentrations of compound A. Data (mean ± S.E.M.) are expressed as the percentage of net migrated cells. All experiments were performed in duplicate and repeated twice with similar results.

In this study, we showed that compound A had agonistic activities for FPR1 and FPR2/ALX in humans and Fpr1 and Fpr2 in mice. Compound A also induced activation and chemotaxis of neutrophils in both humans and mice.

Compound A was originally reported to be an hFPR2/ALX-selective agonist with no activity for hFPR1 up to 10 μM (6). To our surprise, compound A showed both hFPR1 and hFPR2/ALX agonistic activities. This discrepancy might be due to the different types of G-protein used in the respective aequorin assays; they used $G_{\alpha 15}$ and we used $G_{\alpha 16}$, which might influence the sensitivity of the calcium signal in the aequorin assay. We used the two well-known peptide ligands fMLF and WKYMVM as controls in our assay system and their potency and selectivity of responses against hFPR1, hFPR2/ALX, mFpr1, and mFpr2 were consistent with previously reported ones (1, 2, 12). Moreover, we confirmed the agonistic activities of compound A for hFPR1 and hFPR2/ALX in the receptor internalization assays. Therefore, we thought our assay system was appropriately validated and that the dual agonistic activity of compound A for both FPR1 and FPR2/ALX in humans and Fpr1 and Fpr2 in mice was accurately determined.

We showed for the first time that compound A induced human and mouse neutrophil chemotaxis, which was consistent with its agonistic activities for hFPR1 and mFpr1 because the receptors are well known to mediate the chemotactic responses of neutrophils (1). It was reported that compound A inhibited human neutrophil chemotaxis induced by IL-8 or fMLF (6). This inhibition might be due to a competing attraction between chemoattractants in the lower chamber and compound A in the top chamber of the Transwell. All the other biological effects of this compound reported previously (6, 7) should be reconsidered as well.

In summary, we revealed that compound A acted as a dual agonist for FPR1 and FPR2/ALX in humans and for Fpr1 and Fpr2 in mice. A great deal of effort has been made to obtain small molecule FPR2/ALX-selective agonists that might have anti-inflammatory and pro-resolution effects. Since compound A turned out to be a dual agonist for FPR1 and FPR2/ALX, highly-selective and potent agonists for FPR2/ALX are still awaited.

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