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Anti-allergic effect of the Src family kinase inhibitor saracatinib

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The aim of this study was to evaluate the anti-anaphylactic and anti-allergic potentials of saracatinib, a Src family kinase inhibitor that was already shown to be safe in clinical trials when it was used as an anti-cancer drug. Using *in vitro* mast cell models, we found that saracatinib inhibited the degranulation response and cytokine production in RBL2H3 cells that were stimulated with IgE and antigen without affecting cell viability. Phosphorylation of Lyn, Akt, a PI3K substrate, and MAPKs including ERK, JNK, and p38, as well as the intracellular Ca^{2+} increase induced by this stimulation were also suppressed by saracatinib. This drug also inhibited symptoms in our established anaphylaxis mouse model, anaphylaxis-dependent spotted distribution of immune complex in skin (ASDIS). The intravenous injection of the mixture of IgE and antigen induced acute spotted distribution of immune complex in skin in hairless HR-1 mice, and its inhibition by intradermal injection of saracatinib was observed. Moreover, toluidine blue-stained skin sections indicated that the degranulation ratio of dermal mast cells was reduced in saracatinib-treated skin compared with vehicle-treated skin. Because only a few signaling inhibitors are used as anti-anaphylaxis and anti-allergic drugs, these results indicated the valuable suggestion that saracatinib and the Src family kinase inhibitors are good candidates for anti-anaphylaxis and anti-allergic drugs.

1. Introduction

Anaphylaxis is driven by allergic reactions that are categorized into two types by their mechanisms, including induced or not induced through immunological machinery (Reber et al. 2017). Based on the importance of various cell types, the reaction is also divided into mast cell-dependent and other cells-dependent phenomena (Subramanian 2016; Reber et al. 2017). Among them, anaphylaxis induced by IgE-mast cell-histamine axis, one of the immunological processes, presents the most important concern. Histamine and other mediators that are released from mast cells cause acute symptoms including urticaria with itching, nausea, vomiting, decreased blood pressure, dyspnea, and life-threatening shock (Reber et al. 2017). Although already-established symptomatic therapy such as adrenaline injection, treatment with histamine receptor antagonists, and steroid administration shows a remarkable effect (Muraro et al. 2014), it is valuable to determine new anti-anaphylactic compounds and identify new drugs, especially for some patients who do not benefit from the traditional drugs and have side effects from these medications.

"Drug repositioning" is a promising method to accelerate the development of a novel drug (Lotfi Shahreza et al. 2018). The method intends to switch a drug that is already used clinically for some diseases to treat other diseases. Methotrexate was originally developed as an anti-cancer drug and is now frequently used for rheumatoid arthritis. Tacrolimus was originally developed as an immunosuppressive drug to prevent graft rejection and it also effectively suppresses atopic dermatitis when applied to the skin. Its proven safety in clinical use is the main advantage of this method.

Thus, we investigated the anti-anaphylactic and anti-allergic effects of several molecular-targeted medications that are used to treat cancers. The c-kit inhibitor sunitinib effectively inhibits passive systemic anaphylaxis and suppresses symptoms of food allergy in mice (Yamaki and Yoshino 2012a). The mTOR inhibitor rapamycin and the JAK inhibitor ruxolitinib also suppress symptoms of food allergy in mice through decreasing immune responses (Yamaki and Yoshino 2012b, 2014). All three drugs inhibit IgE-induced activation of rat basophilic leukemia RBL2H3 cells (Yamaki and Yoshino 2012a, b, 2014). Afrin et al. (2015) then reported the therapeutic effect of sunitinib on allergic symptoms of patients in which mast cells worsen their disorder. These studies suggested the essential roles of the c-kit, JAK, and PI3K-Akt-mTOR signaling cascades in allergic reactions and their potential inhibitors as anti-allergic drugs.

The Src family kinases transmit stimulatory signals from various receptors to induce cancer cell proliferation (Espada and Martín-Pérez 2017) and IgE-dependent, FcεRI-mediated mast cell activation (Furumoto et al. 2005). The Src family kinase inhibitor saracatinib (Hennequin et al. 2006) was originally developed as an anti-cancer drug, but now, it has been repositioned to be developed as an anti-Alzheimer's disease drug in Phase 2 clinical trials. In this study, we intend to investigate the suppressive effect of clinically tolerated saracatinib on *in vitro* mast cell activation and *in vivo* anaphylactic reactions that are observed using the anaphylaxis-dependent spotted distribution of immune complex in skin (ASDIS) mouse model (Yamaki and Yoshino 2016) to clarify the putative importance of this kinase in allergic reactions and therapeutic efficacy of Src family kinase inhibitors. ASDIS is a unique phenomenon that is useful for examining anti-allergic effects of compounds.

2. Investigations and results

2.1. Effects of the Src family kinase inhibitor saracatinib on IgE/antigen-induced activation of RBL2H3 cells

Rat basophilic leukemia RBL2H3 cells are commonly used as an *in vitro* mast cell model (Jo and Park 2017). The cells were sensitized with purified anti-ovalbumin (OVA) monoclonal IgE (OE-1), and activated with OVA in the presence or absence of the indicated saracatinib concentrations. β-Hexosaminidase release induced by stimulation for 20 min was strongly decreased with saracatinib treatment in a dose-dependent manner (Fig. 1A) without exhibiting cytotoxicity (Fig. 1B). Saracatinib treatment also decreased interleukin (IL)-4 production at 4 h after the stimulation (Fig. 1C) with minimal modifications of cell viability (Fig. 1D).

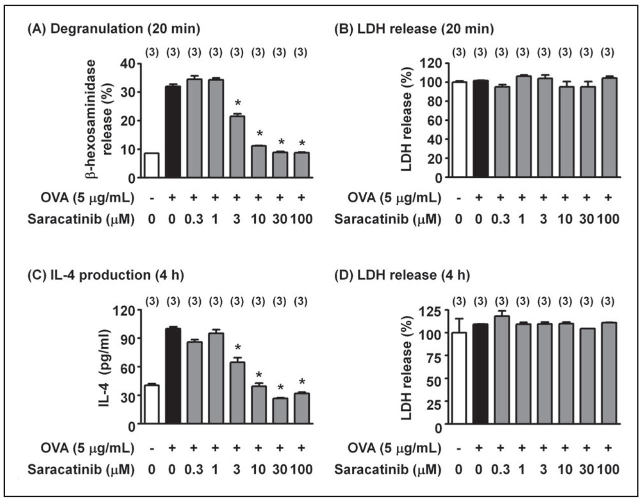

Fig. 1: Inhibitory effects of saracatinib on degranulation and IL-4 production of RBL2H3 cells stimulated by IgE and ovalbumin (OVA) without affecting cell viability. RBL2H3 cells were sensitized using anti-OVA monoclonal IgE OE-1 and then stimulated with OVA for 20 min (A) or 4 h (C) with or without the treatment of saracatinib. β-Hexosaminidase activity (A) and IL-4 concentration (C) in the cultured supernatants were measured. Cell viability was also determined at 20 min (B) and 4 h (D) by measuring LDH release into culture medium. Bars show the mean + SEM of three cultures. Parentheses above the columns also show the number of cultures. * p<0.05 vs. OVA alone, ANOVA followed by Dunnett's multiple comparison test. Similar results were obtained from two or more independent experiments.

2.2. Saracatinib suppresses key events, activates Lyn and phosphatidylinositol 3-kinase (PI3K), and increases intracellular Ca^{2+}

IgE/Ag stimulation activates kinases including Src family kinases such as Lyn and Fyn (Furumoto et al. 2005), which trigger subsequent intracellular signaling. To reveal the inhibitory effect of saracatinib on Src family kinases in IgE/Ag-stimulated RBL2H3 cells, the level of phosphorylated Lyn, an indicator of Lyn activation (Poderycki et al. 2010), was measured (Fig. 2). Treatment of saracatinib strongly reduced the IgE/Ag-induced increase of Lyn phosphorylation in dose-dependent manner. This result indicated that saracatinib decreased degranulation and *de novo* cytokine production by RBL2H3 cells through inhibition of Lyn and other Src family kinases, as shown in Fig. 1.

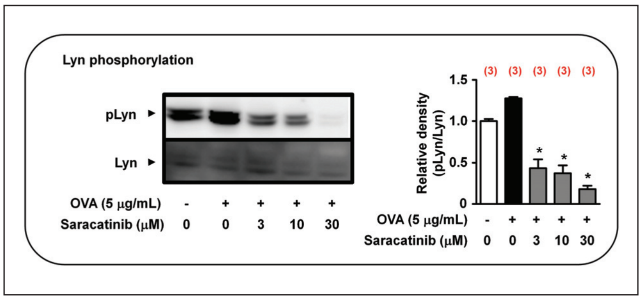

Fig. 2: Saracatinib inhibits Lyn phosphorylation in RBL2H3 cells. OE-1-sensitized RBL2H3 cells were stimulated with OVA for 15 min with or without saracatinib treatment. The cell lysate was applied to Western blotting analysis to detect phosphorylated Lyn (pLyn) level and Lyn level. Bars show the mean + SEM of three independent experiments. * p<0.05 vs. OVA alone, ANOVA followed by Dunnett's multiple comparison test. Parentheses above the columns also show the number of samples from independent experiments.

Among several molecules that were located downstream of the Src family kinases, PI3K activation and intracellular Ca^{2+} increase are critical for both degranulation and cytokine production (Tan et al. 2017). Thus, we next examined the effects of saracatinib on IgE/Ag-induced Akt phosphorylation as an indicator of PI3K activity

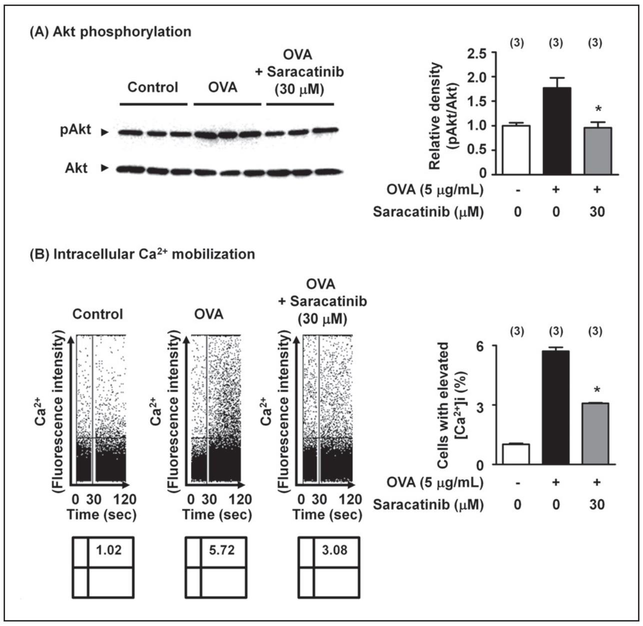

Fig. 3: Saracatinib suppressed Akt phosphorylation and intracellular calcium mobilization in stimulated RBL2H3 cells. (A) OE-1-sensitized RBL2H3 cells were stimulated with OVA for 15 min with or without saracatinib treatment. The cell lysate was applied to Western blotting analysis to detect phosphorylated Akt (pAkt) level and Akt level. (B) OE-1-sensitized RBL2H3 cells were incubated with saracatinib and stimulated with OVA for 90 s. Fluo-3-derived fluorescence, as an indicator of the intracellular Ca^{2+} level, was monitored. Bars show the mean + SEM of three cultures. Parentheses above the columns also show the number of cultures. * p<0.05 vs. OVA alone, ANOVA followed by Dunnett's multiple comparison test. Similar results were obtained from two independent experiments.

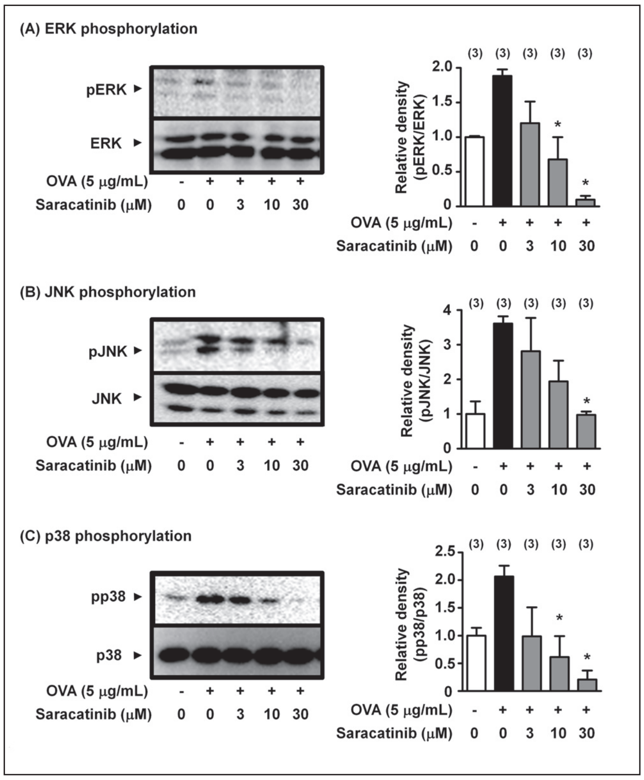

Fig. 4: Inhibition of MAPKs activation in RBL2H3 by saracatinib. OE-1-sensitized RBL2H3 cells were stimulated with OVA for 15 min with or without saracatinib treatment. The cell lysate was applied to Western blotting analysis to detect phosphorylated ERK (pERK) level and ERK level (A), as well as pJNK, JNK (B), pp38, and p38 (C). Bars show the mean + SEM of three independent experiments. * p<0.05 vs. OVA alone, ANOVA followed by Dunnett's multiple comparison test. Parentheses above the columns also show the number of samples from independent experiments.

(Fig. 3A) and intracellular Ca^{2+} level (Fig. 3B). Quiescent RBL2H3 cells possessed moderate Akt phosphorylation, and IgE/Ag stimulation for 15 min upregulated the phosphorylation level. Saracatinib almost completely blunted the increasing effect of IgE/Ag on Akt phosphorylation and reversed it to the basal level. However, even in the presence of the inhibitor, there was a substantial intracellular Ca^{2+} increase induced by this stimulation within a few minutes.

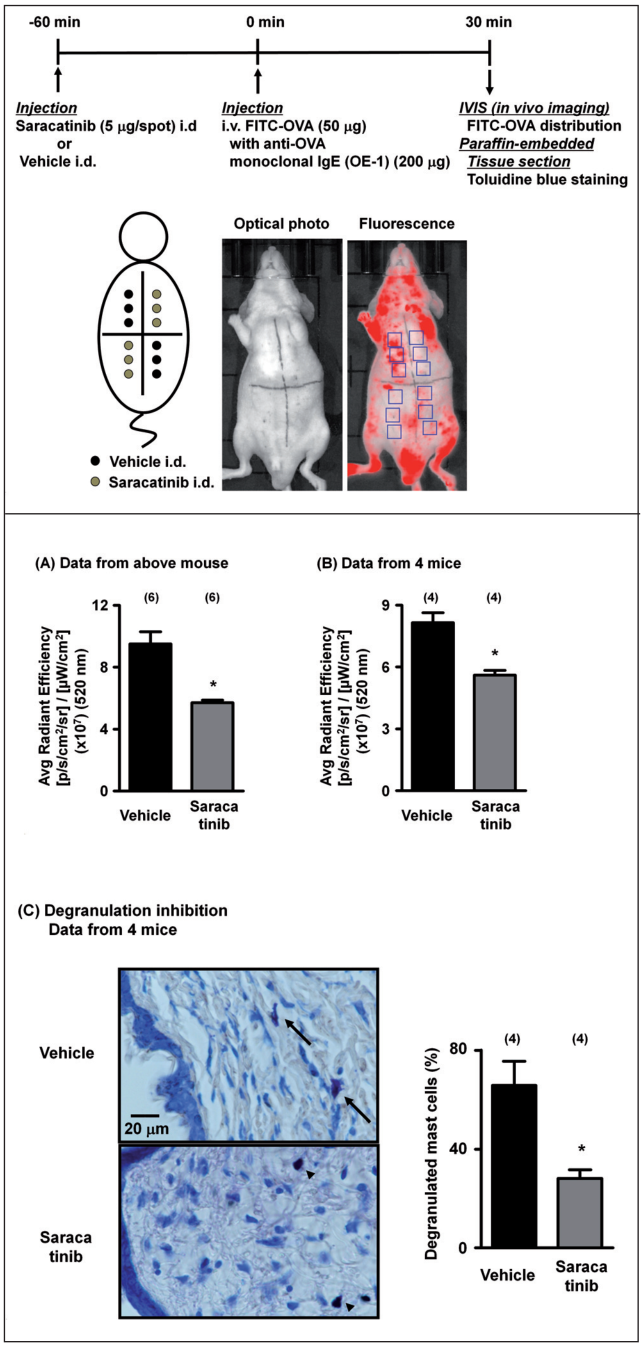

Fig. 5: Anti-anaphylactic effect of saracatinib in mouse skin. Hairless HR-1 mice were intradermally injected with saracatinib or vehicle. After 1 h, the mice were intravenously injected with OE-1 mixed with FITC-OVA to induce ASDIS. (A) The mean average radiant efficiency (ARE) was calculated from the indicated positions of saracatinib- and vehicle-injected skin. Bars show the mean + SEM of six positions. Parentheses above the columns also show the number of positions. (B) The mean ARE was calculated from saracatinib- and vehicle-injected skins from four mice. Bars show the mean + SEM of four mice. Parentheses above the columns also show the number of mice used. (C) Skin treated with saracatinib and vehicle from four mice were fixed with formalin and embedded in paraffin. The sections were stained with toluidine blue to judge mast cell degranulation in the skin. The arrow indicates degranulated mast cells. The arrowhead indicates a resting, non-degranulated mast cell. Bars show the mean + SEM from 10-14 fields of skin sections in each of four mice. Parentheses above the columns also show the number of mice used. * $p<0.05$ vs. vehicle, Student's *t*-tes

2.3. *IgE/antigen-induced phosphorylation of mitogen activated kinases (MAPKs) are modulated by saracatinib*

MAPKs receive the activation signals from PI3K and intracellular Ca^{2+} of IgE/Ag-stimulated RBL2H3 cells and mediate cytokine production following to self-phosphorylation (Draber et al. 2016). The Src family kinase inhibitor saracatinib similarly inhibited phosphorylation of representative MAPKs including ERK, JNK, and p38 induced by IgE/Ag stimulation (Fig. 4A, B, and C).

2.4. *In vivo anti-allergic effect of saracatinib examined using the ASDIS model previously established the passive systemic anaphylaxis model with distinctive skin symptoms*

For measurement of the anti-anaphylactic effect of saracatinib, we used the previously established ASDIS model, which is an anaphylactic mouse model (Yamaki and Yoshino 2016). The HR-1 hairless mouse with ASDIS displays a spotted distribution of immune complex in the skin, which is observed using *in vivo* imaging in parallel with anaphylaxis. There are some advantages to using this model, which are as follows: (1) Based on Russell and Burch's "3R principles," (Aske and Waugh 2017) all steps of the experiment were performed under anesthesia to relieve pain in the animals; (2) Skin symptoms with anaphylaxis can be induced without surgery on their skin to prevent artificial results; and (3) Topical application of the drug enables a comparison with its effect to vehicle within one animal.

Intradermal saracatinib injection prevented the anaphylactic reaction in skin in ASDIS mice (Fig. 5A and B). Toluidine staining revealed that the increased degranulation ratio of mast cells in skin of the saracatinib-treated area was lower compared with the vehicle-treated area in same mouse (Fig. 5C).

3. Discussion

Although the saracatinib kinase inhibition profile suggests that saracatinib exhibits anti-anaphylactic effects, research to clarify these effects has not been performed. In this study, we first demonstrated the anti-anaphylactic ability of saracatinib both *in vitro* and *in vivo*.

In vitro experiments with RBL2H3 cells revealed that saracatinib inhibited IgE/Ag-dependent degranulation and cytokine production through blunting the activations of intracellular signaling including Lyn, PI3K and MAPKs and increasing intracellular Ca^{2+}. The dual function of Lyn on mast cell activation/inhibition is an interesting issue that was reported by Xiao et al. (2005). They used anti-dinitrophenyl (DNP) IgE with antigens that contained oligomeric (DNP3-BSA) or multivalent (DNP21-BSA) epitopes to stimulate bone marrow-derived mast cells. However, we used anti-OVA monoclonal IgE and OVA, which consisted of a monomer and oligomers, resulting in monomeric to oligomeric epitopes. (This was the presumed structure based on OVA monomer and oligomers results from Western blotting.) Although it is difficult to compare the results that were obtained from those two different experimental conditions, weaker stimulation by anti-DNP IgE/DNP3-BSA in the Xiao's experiments seemed to be relatively similar to our stimulation that was triggered by OE-1/OVA. Our results might reveal the underlying phenomenon that low intensity stimulation by OE-1/OVA positively regulated mast cell activity through Lyn activation, and this activation was reduced by its inhibitor saracatinib. This suggests that inhibition of Lyn by saracatinib mediates its anti-allergic effect, which is consistent with Lyn's positive roles (Siraganian 2003). In addition to Lyn, Fyn is another Src family kinase, which is also a direct target of saracatinib. Based on the inhibitory profile of saracatinib and the roles of Fyn in mast cells (Furumoto et al. 2005), Fyn should be inhibited by saracatinib. This inhibition by saracatinib participated in the suppressing effect on mast cell function in our experiments. Taken together, inhibition of Src family kinases shows the main pharmacological mechanism of the inhibitor on mast cell activation.

An *in vivo* study using our distinctive anaphylactic mouse model, ASDIS (Yamaki and Yoshino 2016), indicated that the inhibitor suppresses an anaphylactic reaction in the skin in parallel with decreasing mast cell degranulation in the tissue. The limitation of this study is that the results did not show the importance of Src family kinase inhibition by saracatinib in our *in vivo* anaphylactic ASDIS model. In a future study, we intend to resolve this issue.

Cytokines, especially IL-4, which are released from activated mast cells, positively regulate mast cell functions and worsen the allergic reaction that occurs thereafter (McLeod et al. 2015). Thus, inhibiting IL-4 release by saracatinib as well as degranulation showed its regulatory effects on the allergic predisposition in addition to decreasing the acute anaphylactic reactions. Because JNK (Sun et al. 2015) and p38 (Hirasawa et al. 2000) have an essential role for IgE/Ag-induced IL-4 production in mast cells, general suppressive effects of saracatinib on JNK and p38 activation should be responsible for the decreased cytokine production.

The saracatinib concentrations that were used in *in vitro* experiments in the present study were similar to those in serum of cancer patients treated with the drug for 21 days in clinical trials (C_{max}, 247-922 ng/mL, which is the same as 0.45-1.7 μM, at a dose 175 mg/day for 21 days; Fujisaka et al. 2013). Compared with the concentration (C_{max}), saracatinib 5 μg/spot is thought to be a high dose at the injection site, but the systemic dose is comparatively lower (for 25 g/mouse). Only a few studies have focused on the injection site concentration and/or metabolism. Because the systemic half-life of the drug is long (about 40 h) (Fujisaka et al. 2013), saracatinib metabolism *in vivo* within several hours can be ignored. Rather, Proulx et al. (2010) reported that 2/3 of intradermally injected indocyanine green-containing liposome (which was not a molecule that was similar to saracatinib, but it was a macromolecule) diffused from the injected site after 4 h. This indicated that intradermally injected saracatinib substantially and rapidly diffused from the injection site within 1 h. This may partly explain the reason why the relatively high amount of saracatinib was required, which caused an inhibitory effect in our experiment. However, this does not deny, and rather it may increase, the possibility that the Src family kinase inhibitor is an anti-allergy drug. Our present study first reported that the clinically tolerated, Src family kinase inhibitor saracatinib exerted anti-anaphylactic and anti-allergic effects both *in vivo* and *in vitro*. Other researchers also investigated and suggested the potentials for the synthetic compound furaltadone (Nam et al. 2018) and naturally occurring compound nujiangexanthone A (Lu et al. 2016), both of which have inhibitory activity for Src family kinases. Suppressive effects of Chk inhibitor AZD7742 (Park et al. 2018) and the EGFR tyrosine kinase inhibitor WZ3146 (Park et al. 2019) on the Src family kinases, including Lyn and Fyn, were proposed to play a role in its anti-anaphylactic effects. These observations suggest the pivotal role of the Src family kinases in allergic symptoms and confirm that the Src family kinase inhibitors could be a drug for allergy treatment. The relative importance of each Src family kinase in mediating mast cell functions might vary among individuals. Because saracatinib is multi-targeted inhibitor toward Src family kinases including Lyn and Fyn, the drug may be effective against allergy in a wide range of patients with the different important balances of kinases, as mentioned above. In addition, the action of saracatinib is quite different from anti-allergic agents that are already used clinically such as anti-histamines and steroids. This characteristic of saracatinib enables it to be co-administered with other drugs.

4. Experimental

4.1. Animals and cell lines

HR-1 mice (males, 8–14 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed in a controlled environment and given standard chow and water *ad libitum*. All animal experiments were approved by the Animal Ethics Committee of Kobe Pharmaceutical University. RBL2H3 cells were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan. OE-1-producing hybridoma was previously established in our laboratory (Yamaki and Yoshino 2009).

4.2. Degranulation assay

RBL2H3 cells (2.5×10^5 cells/well of 24 well plate; Corning, Corning, NY, USA) were cultured in RPMI1640 medium that was supplemented with 10% fetal bovine serum and antibiotics with 1 μg/mL of OE-1 for 18 h. The OE-1-sensitized RBL2H3 cells were then pretreated with the indicated concentrations of saracatinib (Cayman Chemical, Ann Arbor, MI, USA) for 15 min. To induce degranulation, the cells were stimulated with OVA (Merck KGAa, Darmstadt, Germany) and the indicated concentrations of saracatinib for 20 min. The supernatants were mixed with *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (Merck KGAa) in a 96-well plate and kept at 37 °C for 2 h. After adding the bicarbonate buffer, the absorbance at 405 nm of the resulting yellow solution was measured as an indicator of the degranulation ratio.

4.3. IL-4 production assay

OE-1-sensitized RBL2H3 cells were pretreated with the drug and were stimulated with OVA with saracatinib for 4 h. Then, IL-4 concentrations in the supernatants were measured using an IL-4 ELISA kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), in accordance with the kit instructions.

4.4. Cell viability assay

After culturing the cells, medium containing 0.5 mg/mL MTT (Merck KGAa) was added to the wells with the cells and cultured for an additional 4 h. The medium was then removed, and the resident violet products were dissolved with dimethylsulfoxide (Fujifilm Wako Pure Chemical Co., Osaka, Japan). The absorbance at 550 nm of the solution was measured as an indicator of cell viability, namely mitochondrial oxidative activity.

4.5. Western blotting detecting phosphorylation of kinases

Lyn, Akt, ERK, JNK, and p38 phosphorylation levels were measured with Western blotting, as reported elsewhere. Briefly, RBL2H3 cells (1×10^6 cells/35 mm dish) were pretreated and stimulated as above for 15 min. The proteins in the cell lysates were separated in 8% or 12% polyacrylamide gel with sodium dodecylsulfate and blotted to a PVDF membrane. The antibody to phosho-Lyn was purchased from Thermo Fisher Scientific, Inc. Anti-Lyn was purchased from Bioss (Woburn, MA, USA). Antibodies to phospho-Akt, Akt, phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, and p38 were purchased from Cell Signaling Technology (Danvers, MA, USA) and they were used to detect each protein in the membrane, in accordance with the manufacturer's instructions. The chemiluminescence on the membrane was detected by LAS4000 (General Electric Company, Boston, MA, USA).

4.6. Measurement of intracellular Ca^{2+} level

RBL2H3 cells (6.6×10^6 cells/15 mL tube) were incubated in PBS with the calcium indicator FLUO-3-AM (4 mM; Dojindo Laboratories, Kumamoto, Japan) for 30 min at 37°C with mild mixing. Then the cells were washed and dispersed in RPMI1640 medium. The aliquots of the cell suspension were pretreated with saracatinib for 15 min and stimulated with OVA. From 30 s before to 150 s after the stimulation, FLUO-3-derived fluorescence was measured by FACScalibur (BD Biosciences, San Jose, CA, USA).

4.7. ASDIS in HR-1 mice

HR-1 mice were intravenously injected with 200 μg OE-1 mixed with 50 μg FITC-OVA (Thermo Fisher Scientific, Inc.) to induce an anaphylactic response in ASDIS mice. To measure the inhibitory effect of saracatinib, the drug (2% in phosphate-buffered saline containing 0.1% dimethylsulfoxide) was intradermally injected 1 h before ASDIS induction. The dotted fluorescence in skin was detected by IVIS Lumina XRMS Series III (PerkinElmer, Inc., Waltham, MA, USA). For quantification of ASDIS, average radiant efficiency (ARE) within 1×1 cm areas of interest was calculated with Living Image software (Perkin Elmer, Inc.).

4.8. Toluidine blue staining of skin sections

Vehicle-injected and saracatinib-injected areas of the skin were fixed with 10% formalin (Fujifilm Wako Pure Chemical Co.). Then, the tissues were embedded in paraffin (Fujifilm Wako Pure Chemical Co.) and cut into sections that were 4-mm in diameter to measure the mast cell degranulation ratio using toluidine blue staining (Muto Pure Chemicals Co., Tokyo, Japan).

4.9. Statistics

The Student's *t*-test was used for comparisons between two groups. Dunnett's *post hoc* test was only performed for multiple-group comparisons when the *p* value for a one-way analysis of variance (ANOVA) was less than 0.05. A *p* value of less than 0.05 was considered to be significant.

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Conflicts of interest: none declared.

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