Laboratory of Pharmacology, Kobe Pharmaceutical University, Kobe, Japan

PI3K/mTOR inhibitor dactolisib attenuates allergic response through inhibitions of the sensitization and mast cell activation

K. Yamaki*, Y. Tamura, T. Suzuki, Y. Uesaki, A. Dougan, Y. Koyama

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Corresponding author: Dr. Kouya Yamaki, Laboratory of Pharmacology, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe, Hyogo 658-8558, Japan.
yam@kobepharma-u.ac.jp

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The aim of this study was to evaluate the anti-allergic potentials of dactolisib, a dual PI3K/mTOR kinase inhibitor, on two important events for allergy: sensitization and the onset of anaphylactic symptoms. After sensitization with the antigen ovalbumin (OVA), five successive oral administrations of dactolisib effectively decreased serum anti-OVA antibody-an indicator of sensitization-levels in mice. In parallel with the antibody levels in their serum, anaphylactic rectal temperature decrease induced by the re-administration of OVA to dactolisib-treated mice was strongly diminished compared to that in vehicle-treated mice. The inhibitor also inhibited *ex vivo* splenic B cell activation indicated by the increase of phosphorylation of Akt, CD69 expression levels, and proliferation upon anti-B cell receptor antibody treatment, suggesting that suppressive effects of the inhibitor on B cell activation plays a role in its ability to decrease sensitization *in vivo*. We concurrently observed the anti-anaphylactic ability of dactolisib *in vivo* and *in vitro*. A single oral administration of the inhibitor attenuated the anaphylactic rectal temperature decrease induced in a mouse model of passive systemic anaphylaxis. In *in vitro* mast cell models, pretreatment with the drug inhibited the degranulation response and cytokine production in RBL2H3 cells triggered by IgE and antigens, without affecting cell viability. These results suggest that dactolisib, as well as other PI3K/mTOR inhibitors, might be a good candidate for anti-allergic drugs that exhibit both anti-sensitizing and anti-anaphylactic effects.

1. Introduction

Anaphylaxis is primarily induced by allergic reactions (Reber et al. 2017). Life-threatening phenomena, including vasodilation and dyspnea, are usually concomitant with skin and gastric symptoms such as urticaria and nausea. The complex event is mobilized through two subsequent phases: sensitization and onset. Antigen exposure at an earlier period activates antigen presentation by antigen-presenting cells to T lymphocytes through major histocompatibility complex (MHC) class II and T cell receptor interactions (Kwak et al. 2019). Activated T cells enhance the activation of antigen-specific B cells to produce antibodies that recognize the exposed antigen by classifying IgG, IgA, IgE, IgM, and IgD. Among them, IgE and IgG bind to FcεRI and FcγRIII on mast cells and basophils and induces the activation of signals to the cells upon antigen re-exposure, resulting in the release of chemical mediators such as histamine and allergic symptoms (Kanagaratham et al. 2020). Therefore, inhibiting certain events occurring in the sequential two phases is the key to patient relief and is the strategic target of the development of anti-allergic and anti-anaphylactic drugs (Estelle et al. 2010).

Signal transduction pathways control many biological reactions by modulating cellular functions including sensitization and onset. Among these pathways, the PI3K–Akt–mTOR pathway is one of the most important pathways regulating these two events. LY294002, a PI3Kα, β, and δ inhibitor, attenuates anti-allergen IgE production and allergic responses in airways upon antigen re-exposure (Saw et al. 2016). Similarly, mice expressing dominant–negative PI3Kδ did not exhibit an anaphylactic response (Ali et al. 2004). The Akt inhibitor SC79 attenuates passive systemic anaphylaxis (Su et al. 2014). In contrast, the mTOR inhibitor rapamycin inhibited sensitization in a food allergy mouse model by inhibiting sensitization; however, this was not through anti-anaphylactic effects (Yamaki et al. 2012). However, in clinical practice, drugs targeting the pathway have not been trialed and have not succeeded in coming into the market as anti-allergic drugs.

Dactolisib has been examined for its antitumor activity in relation to its inhibitory effects on PI3K and mTOR (Maira et al. 2008). *In vitro* research has proposed the potential of this compound to inhibit allergic onset by elucidating its inhibitory effects on the proliferation of human mastocytoma (HMC-1.1 cells) and degranulation of mast cells derived from human cord blood mononuclear cells (Blatt et al. 2012). However, anti-allergic effects of this drug *in vivo* have not been discovered. The effects of the drug on antibody production and B cell activation associated with sensitization events have not also been reported, although T cell activation in graft-versus-host disease development is inhibited by dactolisib in mice (Herrero-Sánchez et al. 2016).

In this study, we examined the effect of dactolisib on the two events, namely sensitization and anaphylactic onset, in mice. To explore the inhibitory effect of the drug on sensitization, intravenous antigen administration inducing sensitization was followed by peroral dosing of dactolisib for 5 d. To investigate the protective effect of the drug against anaphylaxis upon allergen challenge, IgE-sensitized mice were intravenously challenged with the antigen after a single oral administration of dactolisib. Some of the mechanisms underlying these effects of the inhibitor were revealed by experiments with *in vitro* B cell and mast cell cultures.

2. Investigations and results

2.1. Effects of the PI3K/mTOR inhibitor dactolisib on sensitization events and following active systemic anaphylaxis

Sensitization to allergens occurs accidentally (e.g., bee sting) or chronically (e.g., pollen and house dust mites). Therefore, therapeutic interventions were administered after allergen exposure. From this point of view, mice were immunized by a model allergen ovalbumin (OVA) on day 0 followed by five daily peroral doses of dactolisib (Fig. 1A).

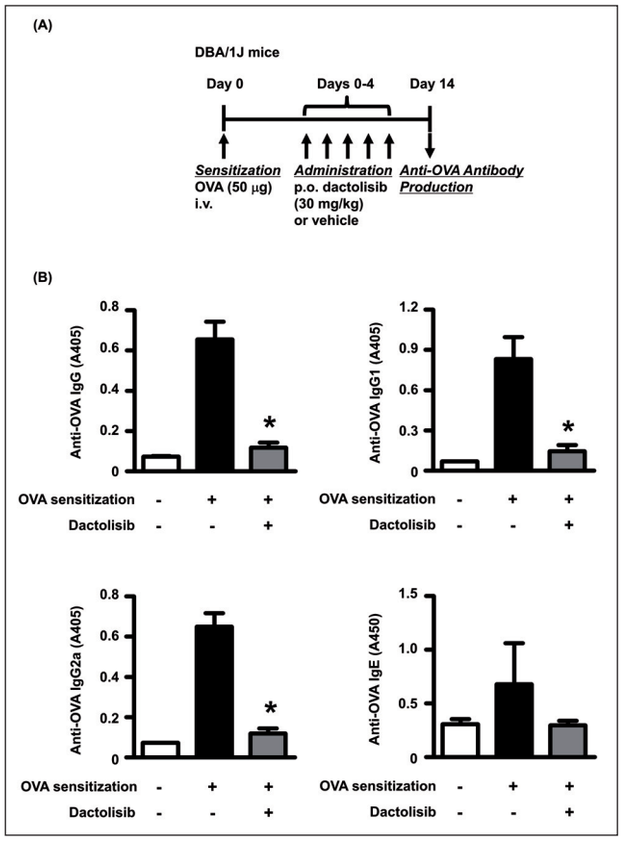


Fig. 1: Inhibitory effect of oral dactolisib on allergen sensitization in mice. As indicated in (A), DBA/1J mice were intravenously injected with ovalbumin (OVA) on day 0. Subsequently, dactolisib was orally administered for 5 d from day 0 to 4. On day 14, sera were obtained, and the anti-OVA IgG, IgE, IgG2a, and IgG1 levels were measured by enzyme-linked immunosorbent assay (ELISA) (B). Bars represent the mean ± SEM of six to seven mice. * $p < 0.05$ vs. OVA injection, Mann–Whitney *U*-test. Similar results were obtained in two independent experiments.

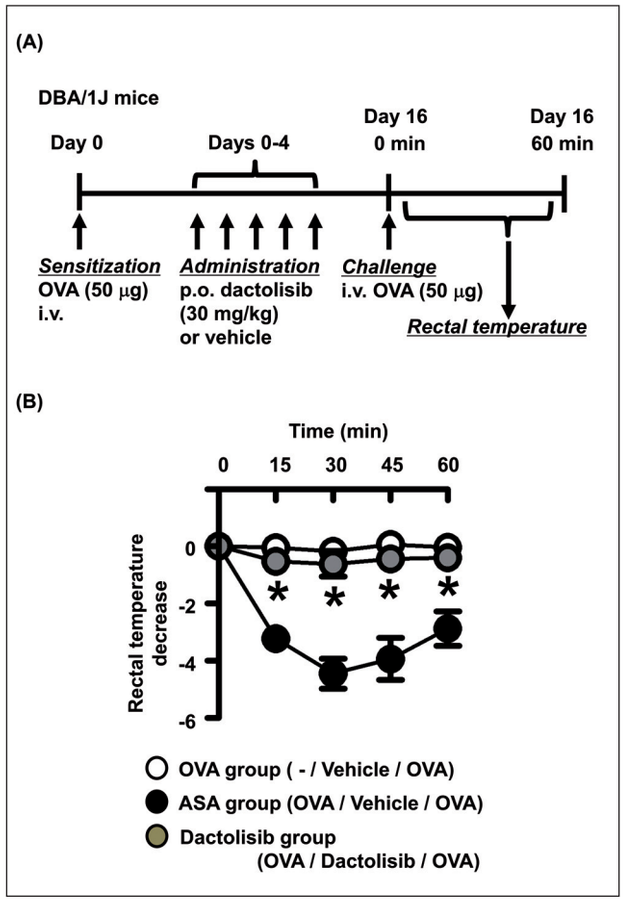


Fig. 2: Attenuation of active systemic anaphylaxis (ASA) in mice treated with dactolisib at the sensitization period. Similar to the experiments shown in Figure 1, DBA/1J mice were intravenously injected with ovalbumin (OVA) on day 0 (A). Subsequently, dactolisib was orally administered for 5 d from day 0 to 4. On day 16, the mice were challenged with an intravenous injection of OVA to induce rectal temperature decrease as an indicator of active systemic anaphylaxis (ASA). Bars represent the mean ± SEM of four to ten mice. * $p < 0.05$ vs. ASA group, Mann–Whitney *U*-test. Similar results were obtained in two independent experiments.

On day 14, anti-OVA IgG, an indicator of general sensitization, was increased by OVA immunization and significantly decreased by dactolisib treatment (Fig. 1B, upper left). The indicator of Th2 response/allergic sensitization, anti-OVA IgG1, was significantly diminished by dactolisib administration (Fig. 1B, upper right), while the immunization-dependent increase in anti-OVA IgE, another indicator of Th2 response as well as allergic sensitization, showed a decreasing tendency in the presence of the drug (Fig. 1B, lower right). The indicator of Th1 response, anti-OVA IgG2a, was also decreased by the inhibitor (Fig. 1B, lower left).

On day 16, the mice were challenged with an intravenous OVA injection (Fig. 2A). The rectal temperature decrease owing to anaphylaxis caused by the challenge was almost completely abolished by dactolisib treatment (Fig. 2B), in parallel with the decrease in antibody production (Fig. 1B).

These results indicate that temporal consecutive treatments with dactolisib effectively prevent antibody production, leading to allergic predisposition, even after antigen exposure.

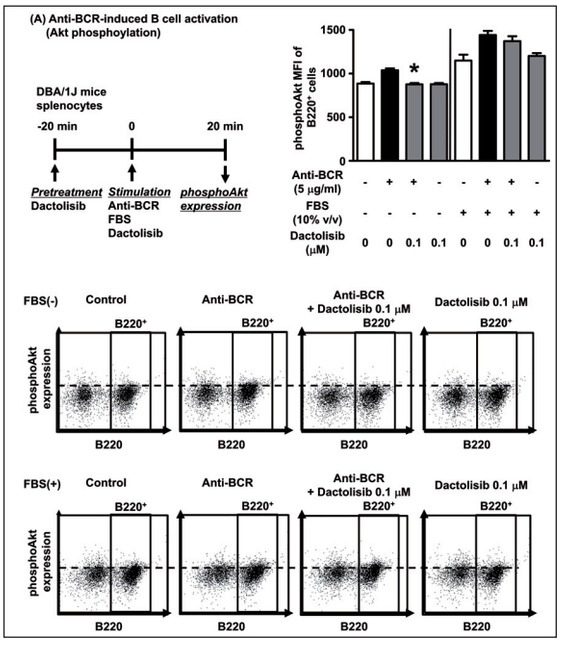
2.2. Effects of dactolisib on anti-B cell receptor (BCR)-induced B cell activation and splenocyte proliferation

The activation of B cells, which differentiate into plasma cells and produce antibodies, is a key step in establishing sensitization to antigens. Therefore, we next investigated the possibility that dactolisib inhibits B cell activity *in vitro*.

Stimulation of splenic B cells with anti-BCR antibodies mimics antigen-dependent BCR stimulation. This stimulation increased the phosphorylation of Akt, a molecule located directly downstream of PI3K, cultured either with or without fetal bovine serum (FBS) (Fig. 3A). When the cells were cultured without serum, PI3K inhibitor dactolisib at a concentration of 0.1 μM almost completely blunted anti-BCR-induced Akt phosphorylation. This result indicated that dactolisib inhibited anti-BCR-dependent activation (Fig. 3A). The decreasing tendency of the anti-BCR-induced phosphorylation of Akt was also observed in dactolisib-treated splenic B cells cultured with FBS-containing medium (Fig. 3A).

Anti-BCR stimulation induced the expression of the activation marker CD69 on the surface of cultured splenic B cells (Fig. 3B). The upregulation of CD69 expression by BCR stimulation was lowered by the presence of 0.1 μM dactolisib (Fig. 3B). Moreover, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) analysis revealed that BCR stimulation-induced splenocyte proliferation was inhibited by the drug in a dose-dependent manner (Fig. 3C). It was previously shown that B cells proliferate mainly within CFSE-loaded splenocyte cultures upon stimulation with an anti-BCR antibody (Yamaki et al. 2022).

Thus, dactolisib inhibited allergic predisposition in mice, as shown in Fig. 1, at least partly through the direct modulation of B cell activity. It should be also noted that 0.1 μM dactolisib decreased MTT formazan formation below non-treated control level. Thus, serum-dependent activation was induced in parallel with anti-BCR-dependent activation and caused MTT formazan increase. Dactolisib might inhibit both activation mechanisms.



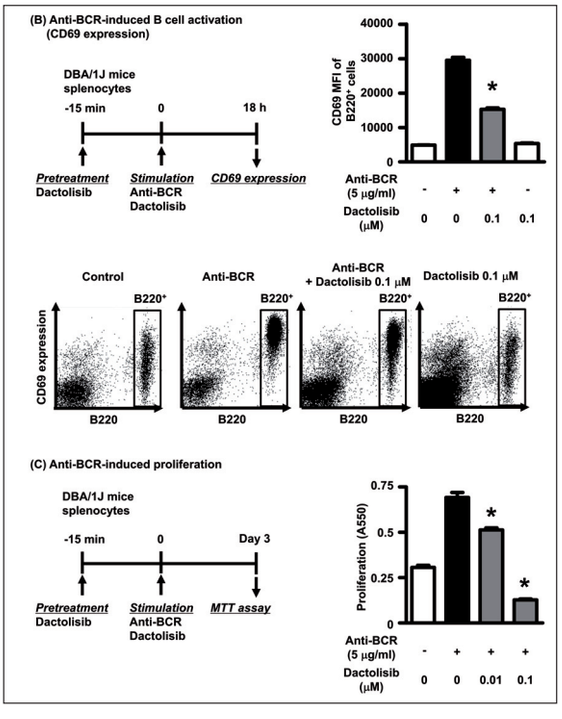


Fig. 3: Inhibition of B cell receptor (BCR)-dependent activation of splenic B cells by dactolisib (A) Splenocytes were stimulated with anti-BCR for 20 min with or without dactolisib treatment. The cells were fixed, permeabilized, and then stained with Alexa488-labeled anti-phosphoAkt and PE-labeled anti-B220. The levels of phosphorylated Akt in the square in the dot plots (B220$^+$ cells) were calculated and shown as a bar graph. Dotted lines in the dot plots are drawn at the same level as a reference for comparison of 8 dot plots. Bars show the mean + SEM of quadruplicated cultures. * $p < 0.05$ vs. anti-BCR group, one-way analysis of variance followed by Dunnett's multiple comparison test. Similar results were obtained from two independent experiments. (B) Splenocytes were stimulated with anti-BCR for 18 h with or without dactolisib treatment. The cells were stained with PE-Cy7-labeled anti-CD69 and PE-labeled anti-B220. The levels of CD69 expression in the square in the dot plots (B220$^+$ cells) were calculated and shown as a bar graph. Bars show the mean + SEM of quadruplicated cultures. * $p < 0.05$ vs. anti-BCR group, one-way analysis of variance followed by Dunnett's multiple comparison test. Similar results were obtained from two independent experiments. (C) Splenocytes were stimulated with anti-BCR for 3 d with or without dactolisib treatment. Cell proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) analysis. Bars show the mean + SEM of quadruplicated cultures. * $p < 0.05$ vs. anti-BCR group, one-way analysis of variance followed by Dunnett's multiple comparison test. Similar results were obtained from two independent experiments.

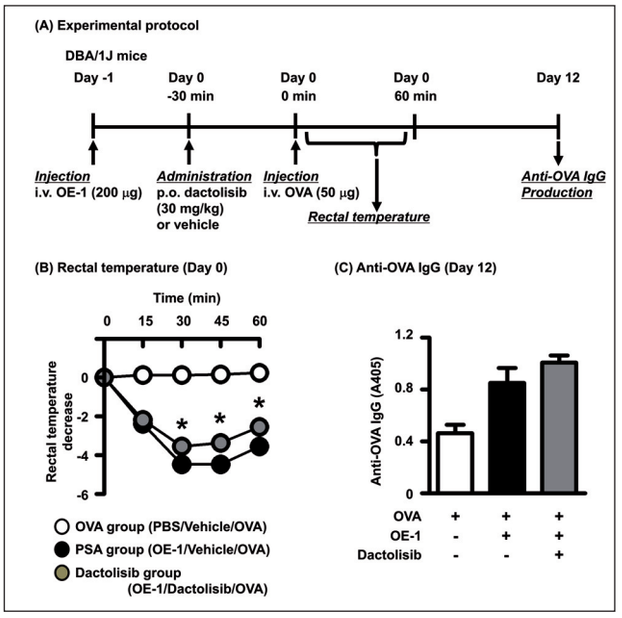


Fig. 4: Anti-anaphylactic effect of dactolisib in passive systemic anaphylaxis (PSA) model DBA/1J mice. Mice were intravenously injected with anti-ovalbumin (OVA) monoclonal IgE and OE-1. The next day, dactolisib was orally administered 30 min before OVA injection to induce PSA. The mice were challenged with an intravenous injection of OVA to induce rectal temperature decrease as an indicator of active systemic anaphylaxis (ASA) (A). After 12 d, sera were obtained and the anti-OVA IgG levels were measured by enzyme-linked immunosorbent assay (ELISA) (B). Bars represent the mean ± SEM of four to ten mice. * $p < 0.05$ vs. ASA group, Mann–Whitney *U*-test. Similar results were obtained in two independent experiments.

2.3. Effects of dactolisib on onset event, namely anaphylactic temperature decrease, in passive systemic anaphylaxis (PSA)

Following sensitization, re-exposure to the antigen induces antigen-specific IgE-dependent mast cell activation and anaphylactic reactions. Experimentally, passively administered IgE and subsequent allergen challenge induces anaphylactic responses, which is called the PSA model. Because this model causes anaphylactic reactions without sensitizing events, it has an advantage in investigating drugs against onset events associated with mast cells and chemical mediators such as histamine.

To reveal the inhibitory effect of dactolisib on allergic onset, we used the PSA model. A single dose of dactolisib administered 30 min before allergen challenge (Fig. 4A) weakly but significantly inhibited the OE-1/OVA-induced rectal temperature decrease (Fig. 4B), suggesting a novel anti-anaphylactic effect of the drug *in vivo*.

The IgE/antigen complex induces massive antibody production against the antigen compared to that induced by the antigen alone (Heyman 2014). The enhancement machinery is beneficial for inducing efficient immune reactions (e.g., preventing infections), but is detrimental to allergic situations. The single dose of dactolisib did not diminish IgG antibody production following anaphylactic inhibition (Fig. 4C), in contrast to the inhibition of antibody production by consecutive administrations of the drug (Fig. 1B).

These results indicate that a single administration of dactolisib exerted an inhibitory effect on the onset of anaphylaxis without modulating the following immune responses, such as antibody production. This indicates that the drug is not immunotoxic. Alternatively, a single administration is insufficient to attenuate the boosting effect (e.g., antibody production) and prevent the exacerbation of allergic predisposition induced by anaphylaxis.

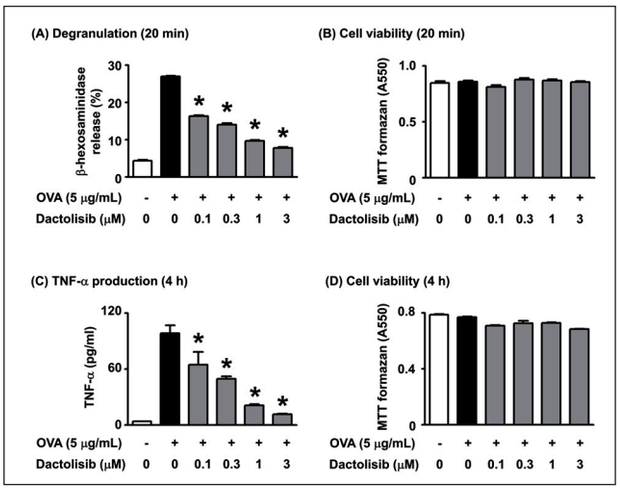


Fig. 5: Inhibitory effects of dactolisib on degranulation and TNF-α production of RBL2H3 cells stimulated by IgE and ovalbumin (OVA) without affecting cell viability. RBL2H3 cells were sensitized using anti-OVA monoclonal IgE OE-1 and then stimulated with OVA for 20 min (A) or 4 h (C), with or without dactolisib treatment. β-Hexosaminidase release (A) and IL-4 concentration (C) were measured in the cultured supernatants. Cell viability was determined at 20 min (B) and 4 h (D) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) analysis. Bars show the mean ± SEM of three cultures. * $p < 0.05$ vs. OVA alone, one-way analysis of variance followed by Dunnett's multiple comparison test. Similar results were obtained from two or more independent experiments.

2.4. Effects of dactolisib on the IgE/antigen-induced activation of RBL2H3 cells

Mast cells release histamine and other mediators responsible for anaphylactic induction. Thus, we investigated whether dactolisib inhibits mast cell activation *in vitro*.

Dactolisib (≥0.1 μM) strongly inhibited β-hexosaminidase (an indicator of degranulation) release from OE-1-sensitized and OVA-stimulated RBL2H3 cells (Fig. 5A) without affecting cell viability (Fig. 5B). These results confirmed the inhibitory effect of dactolisib on IgE-dependent mast cell degranulation in human mast cell experiments (Blatt et al. 2012) and explained a possible reason for the *in vivo* anti-anaphylactic effect revealed in this study (Fig. 4B). The inhibitory effects of dactolisib on mast cell activation were also observed in the later response, namely, tumor necrosis factor-a (TNF-a) production (Fig. 5C), with minimal cytotoxicity (Fig. 5D).

These results indicate that IgE-dependent mast cell activation was effectively blocked by dactolisib, which might be associated with the attenuating effect of the drug on anaphylaxis in mice *in vivo*.

3. Discussion

As anaphylaxis is acute and life-threatening, the establishment of preventive and therapeutic options is desired. To this end, many studies have identified compounds that modulate immune cell and mast cell functions *in vitro*, such as cell cultures; however, the *in vivo* effects of most of these compounds have not been clarified. Unfortunately, anaphylaxis is a complex phenomenon occurring in humans and other animals; thus, animal experiments are unavoidably required to develop anti-anaphylactic drugs for human use.

In this study, we focused on the *in vivo* effects of the PI3K/mTOR inhibitor dactolisib. The beneficial effect of dactolisib on the two phases of anaphylaxis was demonstrated in mice for the first time. Sensitization to allergen mediated by immune cells and the onset of anaphylaxis caused by mast cells upon allergen re-exposure, the two essential events for anaphylactic response, were significantly inhibited by dactolisib treatment. Additional *in vitro* experiments showed the direct inhibitory effects of dactolisib on the B cell and mast cell activation associated with the two events.

IgE and IgG1 are Th2-dependent antibodies (Coffman et al. 1986; Isakson et al. 1982). IgE and IgG1 induce mast cell activation and *in vivo* anaphylactic responses (Shade et al. 2015; Silva et al. 2008) upon antigen exposure. Dactolisib almost completely abolished antigen-specific antibody responses. The increase in anti-OVA IgG1 levels induced by OVA immunization suggested that allergic predisposition in immunized mice was strongly prevented by dactolisib administration just after exposure to OVA (Fig. 1B). The anti-sensitizing effect of dactolisib was also suggested by the inhibition of anaphylactic responses induced by re-exposure to the allergen (Fig. 2B), in parallel with the decreasing tendency of anti-OVA IgE levels caused by dactolisib in OVA-immunized mice (Fig. 1B). In addition, the total anti-OVA IgG and Th1-dependent antibody anti-OVA IgG2a (Snapper and Paul 1987) levels in sensitized mice were strongly suppressed by the drug (Fig. 1B), indicating that dactolisib has the ability to broadly inhibit immune responses, B cell activation, and Th1/Th2 responses. Our *in vitro* experiments revealed a direct inhibitory effect of dactolisib on B cell activation, especially phosphorylation of Akt (Fig. 3A, B and C). Zhu et al. (2019) reported that Akt1 and Akt2 isoforms in B cells are responsible for germinal center (GC) formation and antibody production in response to immunization. Thus, inhibitory effects of dactolisib on B cell activation may explain the decrease of the anti-OVA antibody levels observed *in vivo*. In parallel, the inhibitory effect of dactolisib on T cell activation may have participated in the suppression of antibody production in our *in vivo* study. Dactolisib is also known to attenuate Th1 cytokine IFN-γ and Th2 cytokine IL-4 production by anti-CD3/CD28-stimulated human peripheral blood T cells (Herrero-Sánchez et al. 2016).

Our results suggest that a single administration of dactolisib inhibited the onset of anaphylaxis in the *in vivo* PSA model (Fig. 4B). Experiments with RBL2H3 cells showed a preventive effect of the drug on IgE-induced degranulation (Figure 5A), similar to the results shown by Blatt et al. (2012) using human mast cell lines and peripheral blood-derived mast cells. We also explored the downregulation of TNF-α production by dactolisib (Fig. 5C), which may modulate subsequent immune responses. Unfortunately, we could not obtain evidence of considerable cytokine production inhibition by mast cells *in vivo* by a single oral dose of the drug during anaphylaxis, resulting in the attenuation of the production of the immune response indicator anti-OVA IgG on day 12 (Fig. 4B); however, five doses of dactolisib strongly suppressed antibody production (Fig. 1B).

We previously demonstrated that the mTOR inhibitor rapamycin effectively inhibits anti-allergen antibodies in a mouse food allergy model but failed to suppress PSA (Yamaki et al. 2012). Shin et al. (2018) also reported that the immunosuppressive effect of mTOR inhibitors strongly diminished immune responses. *In vitro* studies have shown that mTOR plays an important role in B cell activation (Zeng et al. 2020; Inui et al. 2002) but not in the degranulation response of mast cells (Yamaki et al. 2012). In contrast, PI3K inhibitors have been shown to attenuate both immune response (Saw et al. 2016) and anaphylactic shock in mice (Collmann et al. 2013). An *in vitro* study showed that PI3K participates in the activation of both B cells (Venkataraman et al. 1998) and mast cells (Collmann et al. 2013). Thus, dactolisib is thought to inhibit anaphylactic onset through its inhibitory effect on PI3K and have an immunosuppressive effect through the inhibition of both PI3K and mTOR. Confirmation of the target molecules of dactolisib for the prevention of sensitization and anaphylaxis onset will be established in future studies.

A discrepancy in the effective concentrations of dactolisib was observed between two cultures, namely mouse splenic B cells (Fig. 3) and RBL2H3 cells (Fig. 5). Dactolisib has been reported to show variable efficacy, namely an IC_{50} between 0.025 μM and 0.8 μM, in inhibiting proliferation of different myeloma cells (McMillin et al. 2009). Proliferation of human mast cell lines are inhibited by relatively lower doses (0.025 μM to 0.25 μM) of dactolisib, while relatively higher doses (1 μM) of the drug was required to attenuate the proliferation of mononuclear cells in human bone marrow (Blatt et al. 2012). This discrepancy is thought to be due to the difference in cell types.

In conclusion, considering the results of this study, dactolisib is a potential allergy treatment, capable of both preventing sensitization and attenuating anaphylactic symptoms upon allergen exposure. These characteristics are suitable for anti-allergic drug shearing with glucocorticoids, which can inhibit sensitization by blunting B cell activation (Dennis et al. 1987), leading to antibody production (Puigneró et al. 1995) and mast cell activation (Daëron et al. 1982), leading to anaphylaxis (Puigneró et al. 1995) causing anaphylactic symptoms. Our results support the use of PI3K/mTOR dual inhibitors as preventive and therapeutic agents for immune diseases, including allergies, as mentioned in some reviews (Foster et al. 2012; Wu and Mohan, 2009).

4. Experimental

4.1. Animals and cell lines

DBA/1J mice (male, 8–14 weeks old) were purchased from Charles River Japan Inc. (Yokohama, Japan). The animals were housed in a controlled environment and provided with 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. The OE-1-producing hybridoma has been previously established in our laboratory (Yamaki and Yoshino 2009).

4.2. Immunization, sequential dactolisib administration, and subsequent active systemic anaphylaxis induction

Mice were immunized with an intravenous injection of 50 µg OVA in phosphate-buffered saline (PBS) on day 0. After immunization, dactolisib (30 mg/kg body weight) was suspended by sonication in distilled water with 0.5% methylcellulose/0.2% TWEEN80 and orally administered daily from day 0 to day 4. On day 14, the mice were sacrificed, and sera were obtained for the measurement of anti-OVA levels with the enzyme-linked immunosorbent assay (ELISA) as indicators of sensitization. Alternatively, mice were challenged with the intravenous administration of 50 µg of OVA to induce active systemic anaphylaxis. Rectal temperature decrease was measured at 0, 15, 30, 45, and 60 min after the challenge as an indicator of anaphylactic symptoms. The dose of dactolisib used (30 mg/kg) was selected based on previous reports (Bellozi et al. 2019; Maira et al. 2008) and the treatment did not alter body weight gain (data not shown).

4.3. ELISA

Serum anti-OVA IgG, IgG1, and IgG2a levels were measured using an OVA-coated 96-well ELISA plate (Corning Inc., Kennebunk, ME, USA). The wells were blocked with 1% casein in PBS, and then the serum samples were diluted adequately (for IgG and IgG1 measurements, 1:5000 to 1:50000 dilutions; for IgG2a measurement, 1:50 to 1:500 dilutions). After 1 h of incubation at room temperature and several subsequent washes, the wells were incubated with a detection antibody—alkaline phosphatase-labeled anti-mouse IgG, IgG1, or IgG2a. After 1 h incubation at room temperature and several subsequent washes, the substrate *p*-nitrophenylphosphate was added to the wells and the absorbance of the wells was measured at 405 nm.

An anti-IgE-coated 96-well ELISA plate was used for anti-OVA IgE measurements. The wells were blocked with superblock blocking buffer in PBS (Thermo Scientific, Rockford, IL, USA) and then serum samples were diluted five-fold and added to the wells. After 1 h incubation at room temperature and several subsequent washes, biotinylated OVA was added to the wells. After 1 h of incubation and several subsequent washes, horseradish peroxidase-labeled anti-biotin IgG was added to the wells. After 1 h of incubation and subsequent washes, 3,3',5,5'-tetramethylbenzidine substrate was added to the wells. After the emergence of a blue color, the reaction was stopped by adding phosphoric acid and the absorbance of the wells was measured at 450 nm.

4.4. Measurements of phosphorylated Akt expression, CD69 expression and proliferation of B cells

For measurement of phosphorylated Akt expression, splenocytes obtained from DBA/1J mice were harvested in a 1.5 mL tube (Greiner Bio-One International GmbH, Kremsmünster, Germany) in RPMI1640 medium supplemented with or without 10% FBS. The cells (2×10^6 cells/well) were pretreated with the indicated concentrations of dactolisib for 15 min and then stimulated with 5 µg/mL anti-mouse IgM antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 20 min. After incubation, the cells were fixed with Lyse/Fix buffer (BD Biosciences, San Diego, CA, USA), permeabilized with Perm buffer III (BD Biosciences), and stained with Alexa488-labeled anti-phosphoAkt (pS473) (BD Biosciences) and PE-labeled anti-B220 antibodies (Biolegend, San Diego, CA, USA) for 30 min at 4 °C, followed by fluorescence-activated cell sorting analysis.

For measurement of CD69 expression and MTT assay, splenocytes (2×10^6 cells/well) obtained from DBA/1J mice were harvested in a 48-well plate (Corning, Corning, NY, USA) in RPMI1640 medium supplemented with 10% FBS and antibiotics. The cells were pretreated with dactolisib for 15 min and then stimulated with anti-mouse IgM antibody for 18 h (CD69 expression) or 3 d (MTT assay) in the presence of the inhibitor. After incubation, the cells were recovered and stained with PE-Cy7-labeled anti-mouse CD69 and PE-labeled anti-B220 antibodies for 30 min at 4 °C, followed by fluorescence-activated cell sorting analysis. Alternatively, cells were used for the MTT assay to measure cell proliferation.

4.5. Passive systemic anaphylaxis

The mice were sensitized with an intravenous injection (200 µg) of anti-OVA IgE (OE-1). The next day, sensitized mice were orally administered 30 mg/kg dactolisib. After 30 min, the mice were challenged with an intravenous injection (50 µg) of OVA to induce passive systemic anaphylaxis. The decrease in rectal temperature was measured at 0, 15, 30, 45, and 60 min after the challenge.

4.6. Degranulation assay

RBL2H3 cells (2.5×10^5 cells/well in a 24-well plate; Corning, Corning, NY, USA) were cultured in RPMI1640 medium supplemented with 10% FBS and antibiotics with 1 µg/mL OE-1 for 18 h. The OE-1-sensitized RBL2H3 cells were pre-treated with the indicated concentrations of dactolisib for 15 min. Cells were stimulated with OVA (Merck KGAa, Darmstadt, Germany) in the presence of the indicated concentrations of dactolisib for 20 min. The supernatants were mixed with *p*-nitrophenyl-N-acetyl-b-D-glucosaminide (Merck KGAa) in a 96-well plate and incubated at 37 °C for 2 h. After adding bicarbonate buffer, the absorbance of the resulting yellow solution was measured at 405 nm.

4.7. TNF-α production assay

OE-1-sensitized RBL2H3 cells were pre-treated with dactolisib and stimulated with OVA and dactolisib for 4 h. TNF-α concentrations in the supernatants were measured using a TNF-α ELISA kit (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions.

4.8. MTT assay

After culturing, medium containing 0.5 mg/mL MTT (Merck KGAa) was added to the wells and the cells were cultured for an additional 4 h. The medium was then removed and the resident violet products were dissolved in dimethyl sulfoxide (Fujifilm Wako Pure Chemical Co., Osaka, Japan). The absorbance of the solution was measured at 550 nm wavelength.

4.9. Statistics

The Mann–Whitney *U*-test was used for comparisons between the two groups. Dunnett's *post hoc* test was only performed for multiple-group comparisons when the *p*-value for one-way analysis of variance was less than 0.05. The *p*-value significance was set at $p < 0.05$.

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