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Aurora kinase inhibitor tozasertib suppresses mast cell activation *in vitro* and *in vivo*

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Abstract

Background and Purpose: Mast cells (MCs) are important in allergic reactions. Here, we assess the anti-allergic effects of the anti-cancer drug tozasertib specifically regarding regulatory effects on MCs activation.

Experimental Approach: Tozasertib effects on MC degranulation was determined by measuring β -hexosaminidase and histamine release and by assessing morphological changes in RBL-2H3 and mouse bone marrow-derived mast cells (BMMCs) stimulated with mouse anti-dinitrophenyl (DNP)-immunoglobulin E (IgE)/DNP-human serum albumin (HSA) or human LAD2 cells activated with phorbol-12-myristate 13-acetate plus calcium ionophore (PMACI). Western blots were performed to detect the expression of molecules involved in NF- κ B, MAPK, and aurora kinase signaling. *In vivo* anti-allergic effects of tozasertib were determined in the murine IgE-mediated passive cutaneous anaphylaxis (PCA) and ovalbumin-induced active systemic anaphylaxis (ASA) models.

Key Results: Tozasertib treatment resulted in significantly decreased high-affinity IgE receptor (Fc ϵ RI) or PMACI-mediated degranulation in RBL-2H3 cells and in BMMCs or LAD2 cells as evidenced by β -hexosaminidase or histamine levels. Similarly, tozasertib prevented morphological changes in MCs, such as particle release and F-actin reorganization. In addition, tozasertib diminished the expression levels of phosphorylated (p)-NF- κ B p65, p-Erk1/2, p-p38 and p-Aurora A/B markedly, indicating that tozasertib can inhibit the signaling pathway mediating MC activation. Tozasertib attenuated IgE/Ag-induced PCA dose-dependently, as evidenced by reduced Evans blue staining. Similarly, tozasertib reduced body temperature levels and serum histamine levels in OVA-challenged ASA mice.

Conclusion and Implications: The aurora kinase inhibitor tozasertib suppressed MC activation *in vitro* and *in vivo*. Tozasertib may be a potential drug for targeting MC activation to treat allergic diseases or mastocytosis.

Abbreviations

PMACI, phorbol12-myristate 13-acetate plus calcium ionophore; IgE, Immunoglobulin E; PGs, prostaglandins; BMMCs, bone marrow-derived mast cells; DNP, dinitrophenol; HSA, human serum albumin; PCA, passive cutaneous anaphylaxis; OVA, ovalbumin; ASA, active systemic anaphylaxis; HRP, horse radish peroxidase; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK1/2, extracellular signal-regulated kinase 1/2; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; FITC, fluorescein isothiocyanate; Ag, antigen.

Introduction

Mast cells (MCs) are highly granulated tissue dwelling cells distributed throughout the body in connective tissues and at mucosal surfaces that are activated by immunoglobulin E (IgE) crosslinking of the high-affinity IgE receptor (FcεRI) on MC surfaces with exogenous allergenic molecules (Yu & Bement, 2007). Thus, MCs act as important effector cells of IgE-mediated immune responses and allergic inflammatory responses (Hanieh et al., 2017). Activated MCs can trigger cell degradation, which leads to the release of various preformed mediators (e.g. histamine and proteases) (Malacombe et al., 2006), newly synthesized lipids including leukotrienes (Sasaki & Yokomizo, 2019), prostaglandins, and inflammatory cytokines (Modena, Dazy, & White, 2016), that play important roles in allergic inflammation (Siraganian, 2003), which can lead to allergic diseases such as asthma, allergic rhinitis, urticaria, and allergic conjunctivitis (Kopec et al., 2006; Harvima et al., 2014). Therefore, MCs represent a potential therapy target to alleviate IgE-mediated allergic reactions in patients with allergic diseases.

Upon activation, MCs produce cytoplasmic granules with pro-inflammatory and allergic mediators that are released into the surrounding environment (Hu et al., 2018). Stimulation-induced wrinkling of the cell membrane facilitates the release of particles into the surrounding environment (Ohsawa & Hirasawa, 2012). Such changes in cell morphology during MC degranulation are controlled by actin microfilaments (Gilfillan & Tkaczyk, 2006; Montuschi & Peters-Golden, 2010). Meanwhile, crosslinked FcεRI leads to Ras activation and downstream mitogen-activated protein kinase (MAPK) pathway signaling that regulates

the synthesis of inflammatory factors (Kalesnikoff & Galli, 2008). In MCs, activation of MAPK extracellular signal-regulated kinase (ERK) has been shown specifically to trigger the production of inflammatory cytokines (Kaminska, 2005). Meanwhile, MC stimulation leads to the phosphorylation, ubiquitination, and then rapid degradation of I κ B α (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), which inhibits NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) (Li et al., 2002; Tornatore et al., 2012), a transcription factor that plays an important role in MC activation and inflammation (Lorentz et al., 2003; Kim et al., 2008). Thus, in theory, MCs could be regulated pharmacologically by enhancing I κ B α function or suppressing the NF- κ B signal pathway.

Drug repurposing, wherein new applications for existing or abandoned pharmacotherapies are developed, is currently one of the most important approaches to develop potential drugs for inhibiting MC activation in the treatment of MC-mediated allergic diseases (Wilkinson & Pritchard, 2015). Tozasertib (a.k.a. VX-680 and MK-0457; chemical structure shown in Fig. 1A.) is an aurora kinase inhibitor in phase II clinical trials for the treatment of various cancers with robust reported efficacy against chronic myelogenous leukemia (Falchook et al., 2015). It is known to inhibit cell growth and inflammation (Gizatullin et al., 2006; Ding et al., 2015). The mechanisms by which tozasertib affects MC activation, however, are unclear. Screening of potential target MC activation-targeting drugs requires *in vitro* and *in vivo* methods for testing anti-allergic effects (Wang et al., 2018).

The aim of the present study was to investigate how tozasertib affects MC activation *in vitro* and allergic responses *in vivo*. We examined MC activation by quantitating histamine and β -hexosaminidase release from IgE/antigen (Ag)-stimulated rat basophilic leukemia cells (RBLs) and bone marrow derived MCs (BMMCs) and in phorbol-12-myristate 13-acetate plus calcium ionophore (PMACI)-stimulated human LAD2 MCs (Gao et al., 2017), which were used to induce cell degranulation to trigger allergic inflammation in activated MCs as an *in-vitro* model of activated MCs (Yamada et al., 2019). We used the passive cutaneous anaphylaxis (PCA) mouse model with IgE/Ag induction and the ovalbumin (OVA)-induced active systemic anaphylaxis (ASA) mouse model to evaluate the inhibitory effects of

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tozasertib on IgE-mediated allergic reactions (Kee & Hong, 2017; Castillo-Courtade et al., 2015). To explore the mechanism of tozasertib effects on MC activation, we examined levels of inactive and activated NF- κ B p65 (p65), an important transcriptional regulator involved in MC degranulation (Kim et al., 2015), as well as inactive and activated levels of MAPKs [ERK, p38, and c-jun and N-terminal kinase (JNK)] (Li et al., 2014). If tozasertib inhibits MC degranulation and allergic disease presentation effectively, it could be a candidate for repurposing into an allergic inflammatory disease pharmacotherapy.

Materials and methods

Reagents

Dulbecco's Modified Eagle's medium, RPMI 1640, and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Tozasertib was purchased from MedChem Express (Monmouth Junction, NJ). Mouse anti-dinitrophenyl (DNP) IgE and DNP-human serum albumin (DNP-HSA) were purchased from Sigma (St. Louis, MO). Rabbit monoclonal IgG primary antibodies against ERK (RRID:AB_390779), phosphorylated ERK (p)-ERK (RRID: AB_2315112), p38 (RRID: AB_10999090), p-p38 (RRID: AB_2139682), JNK (RRID: AB_2250373), p-JNK (RRID:AB_823588), Aurora B (RRID: AB_10695307), p-Aurora A/B (RRID: AB_2061631) and anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (RRID: AB_10622025) were purchased from Cell Signaling Technology (Danvers, MA), and rabbit monoclonal primary antibodies against p65 (RRID: AB_776751) and I κ B α (RRID: AB_733068) were purchased from Abcam (Cambridge, MA). Rabbit anti-mouse IgG-HRP (horse radish peroxidase) (RRID: AB_2687626), mouse anti-rabbit IgG-HRP (RRID: AB_628497) and mouse monoclonal IgG primary antibodies against p-p65 (RRID: AB_2179156) and Aurora A (RRID: AB_667838) were purchased from Santa Cruz Biotechnology (Dallas, TX).

Animals

Female Balb/c mice (6–8-wks old, 18–22 g) purchased from Guangdong Medical Laboratory Animal Center (Foshan, China) were bred in an aseptic-specified pathogen-free environment and kept in an environment with constant humidity ($55 \pm 10\%$) and temperature ($24 \pm 1^\circ\text{C}$). Mice were kept in open polypropylene cages with clean chip bedding under a 12-hour light/dark cycle with free access to food and water. Animal experiments were carried out according to protocols approved by the Animal Care and Use Committee of School of Medicine of Shenzhen University and were in compliance with the Guidelines on Animal Welfare of the School of Medicine of Shenzhen University. The health status of experimental mice was monitored twice daily and humane endpoints were used to determine if mice met the criteria for euthanasia before the study end point, namely weight loss exceeding 10–15% of body weight, lethargy, inability to stand, or anorexia.

PCA and ASA models were established as described in previous studies (Choi & Abraham, 2018; Ji et al., 2018). Mice were randomized into groups of equal size in preparation for researcher-blinded analyses. Our animal experiments are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath & Lilley, 2015) and the recommendations of the *British Journal of Pharmacology*.”

Euthanasia was performed by cervical dislocation under deep isoflurane anesthesia. Mouse femurs were collected to isolate BMMCs as described elsewhere (Caslin et al., 2018).

Cell culture

BMMCs were isolated from BALB/c mouse femurs and then cultured in complete RPMI-1640 with 100 U/ml penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10% FBS and 10 ng/ml interleukin-3, and 10 ng/ml stem cell factor. Flow cytometry detection of CD117 and FcεRI expression on cell surfaces after 4–6 weeks in culture indicate ≥95% MC purity (Caslin et al., 2018). LAD2 purchased from Otwo Biotech (ShenZhen, China) were cultured in Stem Pro-34 medium supplemented with 2.6% nutrient supplement (Thermo Fisher Scientific, Waltham, MA), 2 mM glutamine (Thermo Fisher Scientific, Waltham, MA), 100 ng/ml stem cell factor (GenScript, Nanjing, China), and antibiotics (penicillin/streptomycin, Thermo Fisher Scientific, Waltham, MA) (Kirshenbaum et al., 2014). RBLs (RBL-2H3 cell line) were obtained from Guangzhou Cellcook Biotechnology Co., Ltd (Guangzhou, China), cultured in complete Dulbecco's Modified Eagle's medium with 10% FBS, and then incubated in a humidified incubator at 37 °C, 5% CO₂ (Shao et al., 2018). The two human MC lines, namely human mast cell line 1 (HMC-1) and basophilic cell line KU812 (both from Guangzhou Cellcook Biotechnology Co., Ltd, Guangzhou, China), were cultured in RPMI 1640 medium with 10% FBS and then incubated in a humidified incubator at 37 °C in 5% CO₂.

Measurement of cell viability

Cell viability was measured with a Cell Counting Kit 8 (CCK-8) (MedChem Express,

Monmouth Junction) according to the manufacturer's protocol. Cells were seeded in 96-well culture plates at a density of 2×10^3 cells per well for 24 h and then incubated with different concentrations of Tozasertib for 24 h. Then, 10 μ l of CCK-8 solution was added to each well and incubated for 1 h at 37 °C. Absorbance was measured at 450 nm by a multi-well plate reader (BioRad, Hercules, CA).

Measurement of MC degranulation

BMMCs and RBLs were sensitized overnight with 100 ng/ml anti-DNP IgE and then treated with different concentrations of tozasertib for 1 h. After stimulation with 100 ng/ml of DNP-HSA Ag for 30 min, supernatants were collected separately to determine β -hexosaminidase levels. LAD2 cells were treated with different concentrations of tozasertib for 1 h, incubated with PMACI (10 μ M PMA and 500 nM CI) for 30 min, and the supernatant was collected to determine β -hexosaminidase levels (Park et al., 2018; Testai et al., 2016; Wang et al., 2017).

Detection of histamine release

IgE-sensitized BMMCs and RBLs were treated with different concentrations of tozasertib for 1 h. After Ag stimulation with 100 ng/ml of DNP-HSA for 20 min at 37 °C in 5% CO₂, the cells were centrifuged at 2000 $\times g$ for 10 min. The resultant supernatants were collected separately to determine histamine levels with histamine enzyme-linked immunosorbent (ELISA) kits (IBL, Hamburg, German) according to the manufacturer's instructions. The ELISAs were performed in triplicate; and reported data are means $\pm$ standard deviation (SD) from three independent experiments.

Toluidine blue staining

After stimulation with DNP-HSA or PMACI, RBL-2H3 and LAD2 cells, respectively, were incubated with 250 μ L of 4% paraformaldehyde/phosphate buffered saline (PBS) for 30 min at room temperature (RT). The mixture was discarded and the fixed cells were stained with 300 μ l of toluidine blue dye (1% w/v in 0.9% NaCl solution, pH 2.5) for 30 min. Images of

the stained cells were examined for heterochromatin particles with an inverted microscope (Carl Zeiss, Goettingen, Germany) (Kruger & Bloom, 1974; Fu, Ni, Wang, & Hong, 2018).

F-Actin microfilament staining

After stimulation with DNP-HSA or PMACI, RBL-2H3 and LAD2 cells, respectively, were washed with PBS and fixed with tissue fixative for 10 min at RT. The fixed cells were washed with PBS and then permeabilized with a 0.5% Triton X-100/PBS solution for 5 min. Thereafter, the cells were washed with PBS and incubated with 200 μ L of fluorescein isothiocyanate (FITC)-labeled phalloidin solution for 30 min at RT in the dark. The cells were observed under a fluorescence microscope (Carl Zeiss, Goettingen, Germany).

Flow cytometry

Stimulated cells pretreated or not with tozasertib were washed with PBS were labeled with PE-CD63 antibody (Miltenyi Biotec, Cologne, Germany), and transferred to a Cytoflex flow analyzer (Beckman Coulter, Indianapolis, IN) for flow cytometric analysis.

Western blot analysis

Stimulated cells were lysed in RIPA buffer (Beyotime, Beijing, China) with a protease-inhibitor cocktail (MedChem Express, Monmouth Junction, NJ). Protein concentrations were measured using a BCA protein kit (Beyotime, Beijing, China) and aliquots were separated by 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Merck Millipore, Billerica MA). The membranes were blocked with 5% bovine serum diluted in Tris-HCl buffered saline for 1 h at RT and incubated with primary antibody overnight at 4 °C, including anti-p-p65 (1:10000), anti-p65 (1:500), anti-I κ B α (1:1000), anti-p38 (1:2500), anti-p-p38 (1:1000), anti-ERK (1:1000), anti-phosphorylated ERK (1:2000), anti-JNK (1:1000), anti-p-JNK (1:1000), anti-Aurora A (1:500), anti-Aurora B (1:1000), anti-p-Aurora A/B (1:500) and anti-GAPDH (1:2000). GAPDH was used as a loading control. After washing with PBS, the membrane was incubated with HRP-conjugated secondary antibody (1:2000) for 1 h at RT. Immunoreactive

protein bands were visualized with a 1:1 ratio of chemiluminescent reagents (Meilun, Dalian, China) according to the manufacturer's protocol. The immune-related procedures comply with the recommendations made by the British Journal of Pharmacology (Alexander et al., 2018).

PCA model

Mice (6–8-wks old, 18–22 gram) were randomized into groups of equal size, with the researchers handling the mice blinded to their group designations. Mice (n = 5 per group) were injected intradermally with 500 ng of DNP-IgE into one ear. After 12 h, different concentrations of tozasertib were injected intraperitoneally. One hour later, 200 µl DNP-HSA (200 µl of a 5 mg/ml Evans blue solution containing 0.1 mg/ml of DNP-HSA) was injected into the tail vein. The mice were euthanized 1 h after DNP-HAS injection. The ears were removed and placed in 700 µl of formamide for extraction at 65 °C for 12 h. Dye absorbance was measured at 620 nm by a microplate reader (Thermo Fisher Scientific Inc, Waltham, MA). The ears were fixed with 4% formaldehyde and embedded in paraffin. Tissue sections were stained with toluidine blue as described in detail elsewhere (Ji et al., 2018).

ASA model

Age-matched mice (6–8-wks old) with body weight of 18–22 g were randomized into groups of equal size for researcher-blinded analysis. Mice (n = 5 per group) were sensitized on experimental day 0 and day 7 by intraperitoneal injection of 100 µg OVA and 2 mg alum in 200 µL PBS. From experimental day 9 to 13, tozasertib (50 mg/kg) and ketotifen (50 mg/kg) was injected intraperitoneally every 2 d. The animals were challenged with OVA injections (10 mg/kg) following sensitization. Ketotifen, an H1 blocker and MC stabilizer, was used as a positive control for inhibition of activated MCs. On day 14, the mice were challenged with 200 µg of OVA in PBS, and their rectal temperatures were measured 10 min later (Choi & Abraham, 2018). A tail-vein blood serum sample was collected from each mouse quantitative analysis of histamine release with a histamine ELISA kit.

Statistical analysis

All data are expressed as means $\pm$ SD. Kruskal–Wallis test was performed to determine the significant differences in vitro experiments by different treatments. One-way analyses of variance (ANOVAs) and Dunnett's post-hoc tests were performed to determine the statistical significance for the differences in vivo experiments among different treatment groups. Statistical analyses were performed in Prism 7 (GraphPad Software, La Jolla, CA) with a significance threshold of $p < 0.05$. Group size is the number of independent values, and that statistical analysis was done using these independent values. To control of the unwanted sources of variation, normalization of the data was also carried out. The normalized data was fold to control. The data and statistical analysis complies with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology and the data analysis were performed in a blinded manner (Curtis et al., 2018).

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 (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Fabbro, et al., 2017; Alexander, Kelly, et al., 2017).

Results

Tozasertib decreases degranulation of stimulated MCs

The chemical structure of tozasertib is shown in Figure 1A. The cytotoxic effects of tozasertib on RBL-2H3 cells and LAD2 cells were assessed with CCK-8 assays to ensure that post-tozasertib treatment effects on MC granule reduction were not due to induction of cell death. CCK-8 cytotoxicity assays showed that tozasertib did not affect cell viability at concentrations $\leq 100 \mu\text{M}$ in RBLs (Fig. 1B), LAD2 cells (Fig. 1C), HMC-1 cells (Suppl Fig. 1A), and KU812 cells (Suppl Fig. 1B). Thus, tozasertib concentrations $\leq 100 \mu\text{M}$ were considered appropriate for further study. Tozasertib inhibited β -hexosaminidase release, a hallmark of activated MCs, from IgE/Ag-stimulated RBLs and PMACI-stimulated LAD2 cells significantly and dose-dependently (Fig. 1D&E). Similarly, flow cytometric analysis showed that CD63 expression, another measure of MC degranulation, was suppressed by tozasertib treatment in activated RBLs and LAD2 MCs (Fig. 1F&G). Additionally, tozasertib inhibited histamine release from IgE/Ag-stimulated RBLs and PMACI-stimulated LAD2 cells significantly in a dose-dependently manner (Fig. 1H&I).

Tozasertib reduces morphological changes in stimulated MCs

Tozasertib effects on cytomorphology and the release of heterochromatin particles from RBL and LAD2 MCs were revealed by toluidine blue staining. Non-stimulated RBLs and LAD2 cells had elongated shapes and regular circular shapes, respectively. Both had visible intracellular stores of purple particles. IgE/Ag-stimulated RBLs and PMACI-stimulated LAD2 cells had irregular shapes and released purple particles. Both of these changes were attenuated by pretreatment with tozasertib in both cell lines (Suppl Fig.2). Moreover, actin may play a negative regulatory role in cell signaling, and its recombination is required for MC activation, specially F-actin (Porat-Shliom et al, 2013). FITC-phalloidin labeling of F-actin revealed F-actin based morphological changes in activated RBLs and LAD2 cells with or without tozasertib pretreatment relative to non-stimulated control cells (Suppl Fig.3). Note that non-stimulated RBLs showed spindle-shaped, F-actin with a uniform distribution

around the cells. Thus, suppression of morphological changes was associated with reduced F-actin cytoskeletal decomposition in tozasertib-pretreated MCs.

Tozasertib suppresses activation of NF- κ B and MAPKs in MCs

Levels of p-p38, p-ERK, and p-JNK were significantly increased in IgE/Ag-stimulated RBLs and PMACI-stimulated LAD2 cells. Tozasertib pretreatment attenuated phosphorylation of these kinases in stimulated RBLs and LAD2 cells (Fig. 2A&B). MC stimulation was associated with increased phosphorylation of NF- κ B/p65 and degradation of I κ B α compared to non-stimulated controls, and tozasertib treatment inhibited both of these stimulation effects (Fig. 2C&D).

Tozasertib has an inhibitory effect on IgE/Ag-stimulated BMMCs

Tozasertib did not affect primary BMMC viability significantly at concentrations in the range of 0–100 μ M (Fig. 3A), affirming that tozasertib was not cytotoxic in our experiments. Tozasertib inhibited the release of β -hexosaminidase and histamine in BMMCs activated with IgE/Ag stimulation in a dose-dependent manner (Fig. 3B&C). Western blot analyses of signal pathway molecules showed that tozasertib pretreatment inhibited activation of NF- κ B and MAPKs (p38, JNK, and ERK) in BMMCs, as evidenced by decreased levels of p-p38, p-JNK, p-ERK1/2, p-p65, and I κ B α (Fig. 3D&E). The effects of tozasertib on NF- κ B and MAPK signaling molecules exhibited dose dependence in IgE/Ag-stimulated BMMCs. These results indicated that tozasertib suppressed activation of IgE/Ag-stimulated BMMCs through down-regulation of NF- κ B and MAPK pathways.

Activated MCs stimulated with IgE/Ag or PMACI showed high levels of Aurora kinase activity in RBLs (Suppl Fig. 4A), BMMCs (Fig. 3F) or LAD2 cells (Suppl Fig. 4B), as evidenced by decreased levels of p-Aurora A and p-Aurora B in western blot assays. Additionally, tozasertib inhibited the phosphorylation of Aurora kinase A/B dose-dependently in IgE/Ag-stimulated RBLs (Suppl Fig. 4A), BMMCs (Fig. 3F) or PMACI-stimulated LAD2 cells (Suppl Fig. 4B), indicating that tozasertib inhibition of Aurora kinase activity in activated MCs may contribute to suppression of MC activation.

Tozasertib alleviates allergic reactions in PCA mice

Given that tozasertib down-regulated the release of allergic mediators *in vitro*, we used PCA of the ear to evaluate tozasertib effects on anaphylaxis *in vivo*. When ears were injected with 4% Evans blue dye mixed with Ag in PCA tests, tozasertib reduced allergic responses in DNP-HSA-sensitized PCA mice compared to the PBS-injected control group as reflected by reduced Evans blue dye density in the injected ear (Fig. 4A), reduced ear thickness (Fig. 4B&C), and diminished extrusion of dye from the injected ear (Fig. 4D).

Tozasertib reduces allergic reactions in OVA-induced ASA mice

Following the OVA challenge, the body temperatures of OVA-sensitized ASA mice (see Fig. 5A for model development schedule) were significantly lower than those of control mice not subjected to OVA sensitization, and this difference was obviated in OVA-sensitized mice given a tozasertib or ketotifen (positive control) pre-challenge treatment (Fig. 5B). Note that 50 min after the challenge induction, at the nadir of the response curve when the mean rectal temperature for the OVA-sensitized group was 31 °C, the mean rectal temperature of the tozasertib and ketotifen pretreated OVA-sensitized groups were similar to that of the non-sensitized control group at 37 °C. Concomitantly, serum histamine levels increased after the OVA challenge and those increases were significantly suppressed by tozasertib (Fig. 5C).

Discussion

The present study showed that tozasertib reduced FcεRI and PMACI-mediated activation and degranulation in MCs as evidenced by decreased β-hexosaminidase release, CD63 expression, heterochromatin particle release, cytomorphological change, and F-actin degradation, relative to non-stimulated control groups. These cell-level effects of tozasertib were accompanied by diminished levels of activated MAPKs and NF-κB/p65 on a molecular level, indicating that tozasertib inhibited intracellular signaling associated with MC activation. Furthermore, *in vivo*, tozasertib attenuated IgE/Ag-induced PCA in mice as well as OVA challenge-induced changes in body temperature and serum histamine levels in ASA model mice.

Tozasertib is primarily known as an inhibitor of Aurora kinases (K_i values of 0.6 nM, 18 nM, and 4.6 nM, respectively for A, B, and C type (Harrington et al., 2004), though it may also interact with other molecules (Martens et al., 2018; Seymour et al., 2014). It has been shown to have anti-proliferation, cell death-promoting, and inflammation inducing effects on various cell types. For example, recently, tozasertib was shown to inhibit proliferation and migration of human umbilical vein endothelial cells as well as angiogenesis (Sun et al., 2019). Interestingly, tozasertib has been shown to transform macrophages from an inflammatory (M1) phenotype to an anti-inflammatory (M2) phenotype, thus suppressing autoimmune disease pathogenesis (Ding et al., 2015). Here, we showed tozasertib inhibition of MC activation *in vitro* and *in vivo* for the first time, and report new evidence regarding likely molecular mechanisms.

We tested tozasertib effects on two modes of activation of MCs, which act as effector cells in the development of allergic responses: IgE/Ag stimulation or PMACI induction (Theoharides & Kalogeromitros, 2006). IgE/Ag stimulation involves IgE crosslinking with FcεRI cell surface receptors (Peavy & Metcalfe, 2008), whereas PAMCI induction involves activation of surface G protein-coupled receptors and consequent influx of calcium, which activates downstream signaling (Chen et al., 2017). Both methods generated MC degranulation, as evidenced by histamine and β-hexosaminidase release. We demonstrated tozasertib inhibition of both signs of degranulation that was similar to the anti-allergic effects of dexamethasone (Yamada et al., 2019).

MC degranulation is accompanied by F-actin rearrangement and concomitant changes in cell morphology (Frigeri & Apgar, 1999; Gilfillan & Tkaczyk, 2006). Membrane structure changes in activated MCs have been shown to be consequent to the crosslinking of cell surface receptors by external antigens (Briggs, 1963), the redistribution of actin filaments (Deng et al., 2009), and decoupling of the cytoskeleton from the plasma membrane (Draber et al., 2012). The present inhibition of MC activation by tozasertib *in vitro* appeared to be similar to those of coptisine, a cytotoxic alkaloid used in traditional Chinese medicine, shown to prevent granule release and F-actin reorganization (Fu et al., 2018).

Tozasertib alleviated the allergic response-related MC activation in the PCA and ASA models. The range of tozasertib doses selected (50–70 mg/kg) were appropriate for clinical application. Previously, Harrington et al. (2004) showed that tozasertib (75 mg/kg twice daily for 13 d) reduced tumor size significantly in a human xenograft model with acute myeloid leukemia HL-60 cells. Importantly, the body weight of the mice treated with tozasertib showed only a very small reduction compared with the control group, indicating that tozasertib was well tolerated in the body (Brondfield et al., 2015). In addition, the low dose of tozasertib (12.5 mg/kg) also reduced tumor growth significantly, suggesting that the drug has favorable pharmacodynamics *in vivo*. In our study, we treated PCA or ASA model mice with 50 mg/kg or 75 mg/kg tozasertib, doses which were not cytotoxic and were not highly effective. However, the clinical trial phase II of tozasertib for cancer therapy was discontinued due to adverse toxicity effects, including febrile neutropenia, stomatitis, gastrointestinal toxicity, hypertension, and fatigue (Falchook et al., 2015). Subsequently, some researchers have focused on modifying tozasertib's structure with the goals of reducing toxicity and improving pharmacodynamic activity (Long et al., 2018). Thus, modification of tozasertib is a promising strategy for the development of a safe, effective anti-allergic drug. Moreover, clinical trials aimed at determining the efficacy and toxicity of tozasertib in the treatment of allergic diseases.

Activation of the MAPK signaling pathway has been shown to be positively associated with MC activation (Avila et al., 2012). Because activation of MAPKs, such as ERK, JNK, and p38 signal, can upregulate inflammatory mediators at the transcriptional and translational

levels (Kaminska, 2005), inhibition of MAPK signaling in MCs should weaken allergic symptoms. In addition, the transcription factor NF- κ B is a well-known regulator of inflammatory and immune responses, and activated NF- κ B signaling has been shown to promote the expression of inflammatory factors in MCs (Lin et al., 2017). Thus, targeted inhibition of NF- κ B signaling can also suppress MC activation. For example, roxatidine was reported to attenuate MC-mediated allergic inflammation via inhibition of NF- κ B and p38 MAPK activation (Lee et al., 2017). Additionally, resveratrol inhibited MC degranulation by down-regulating MAPK and NF- κ B signaling pathways (Zhang et al., 2019). Similarly, our results showed that tozasertib blocked MAPK and NF- κ B activation in activated MCs, as evidenced by down-regulation of the phosphorylation of ERK, JNK, p38, and p65. Moreover, Aurora kinase A activity is positively associated with MAPK and NF- κ B activity. Inhibition of Aurora kinase A with tozasertib or silencing RNAs has been shown to down-regulate MAPK signaling, leading to inhibition of nasopharyngeal tumor cell invasion (Wan et al., 2008). Additionally, Aurora kinase A has been shown to promote inflammation through upregulation of NF- κ B activity via phosphorylation of I κ B α (Yao et al., 2009). Inhibition of aurora kinase A has also been shown to reduce phosphorylated NF- κ B p65 expression leading to inhibited NF- κ B activity (Sun et al., 2007). Interestingly, in the present study, we showed that aurora kinase activity was upregulated in activated MCs that were stimulated with IgE/Ag treatment and that tozasertib inhibited aurora kinase activity in activated MCs, resulting in an anti-allergy effect. These findings suggest that aurora kinase A may be an important target for MC activation, a possibility that requires further experimental confirmation.

Due to the targeting specificity limitations of small molecule inhibitors, further work is needed to determine the specific target of tozasertib responsible for the suppression of MC activation. In addition to inhibiting Aurora A/B/C kinases, tozasertib can also suppress FLT3 and BCR-ABL kinases (Cheetham et al. 2007). More in-depth research is needed to resolve how different tozasertib concentrations and different tozasertib-targeted effector cells contribute to tozasertib's pharmacological effects. Importantly, our pharmacology and molecular analysis of MC activation findings are confirmatory of an inhibitory effect of

tozasertib on MC activation, although further experiments are needed to determine the exact target of tozasertib that mediates this inhibition. The present findings provide a reference for exploring a molecular target for inhibition of MC activation.

Conclusion

Taken together, we found that the aurora kinase inhibitor tozasertib inhibits MC activation *in vitro* by inhibiting MAPK and NF- κ B signaling pathways, and alleviates MC activation mediated allergic responses *in vivo* by inhibiting the function of MCs. Therefore, tozasertib may be a potential drug for targeting MC activation to treat MC activation mediated allergic diseases or mastocytosis.

Declarations

Ethics approval and consent to participate

All studies involving mice were performed according to protocols approved by the Animal Care and Use Committee of the School of Medicine of Shenzhen University.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests exist.

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 as stated in the BJP guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Authors' contributions

Conceived and designed the study: JKM and CJJ. Analyzed the data: ZLN and CJJ.

Performed the studies: ZLN, HYB and SYT. Contributed reagents /materials/analysis tools:

CJJ and JKM. Wrote the manuscript: ZLN, CJJ and JKM.

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Supplementary Figure 1. Cytotoxicity of tozasertib on human mast cell line HMC-1 cells and basophilic cell line KU812 cells. Cell viability assays were conducted to assess cytotoxic effects of different concentrations of tozasertib on HMC-1 cells and KU812 cells. HMC-1 (A) and KU812 cells (B) were incubated with tozasertib at the indicated doses (0–100 μ M) for 24 h. Cell viability was determined by CCK-8 assays. The data shown are means $\pm$ SD of three independent experiments; * $p < 0.05$ vs. non-treated activated cells.

Supplementary Figure 2. Tozasertib effects on toluidine blue staining in stimulated RBLs and LAD2 cells. RBLs or LAD2 cells were pretreated with tozasertib for 1 h, and then stimulated with DNP-HSA or PMACI for 30 min. Cells were fixed in 4% paraformaldehyde and then stained with toluidine blue dye. Images of the stained RBLs (A) and LAD2 cells (C) were examined and captured with an inverted microscope. Arrows in the figure indicate irregular cell morphology and the extracellular release of granules. (B, D) Means $\pm$ SD of three independent experiments are shown; * $p < 0.05$ vs. activated cells without treatment.

Supplementary Figure 3. Tozasertib effects on FITC-phalloidin labeled F-actin in stimulated RBLs and LAD2 cells. RBLs or LAD2 cells were pretreated with tozasertib for 1 h, and then stimulated with DNP-HSA or PMACI for 30 min. These cells were fixed with 4% paraformaldehyde and stained with FITC-phalloidin. Images of stained RBL-2H3 (A) or LAD2 (C) cells were examined and captured with a fluorescence microscope. Arrows in the figure indicate that the cells morphology became irregular due to disassembly of the F-actin cytoskeleton. (B, D) Means $\pm$ SD of three independent experiments are shown; * $p < 0.05$ vs. non-treated activated cells.

Supplementary Figure 4. Tozasertib inhibits aurora kinase activity in activated RBL-2H3 cells and LAD2 cells. IgE-sensitized RBL-2H3 cells or PMACI-stimulated LAD2 cells were pre-incubated with indicated concentrations of tozasertib for 1 h, then stimulated with DNP-HSA for 10 min. Western blot analysis of aurora kinase molecules (Aurora A, Aurora B, p-Aurora A and p-Aurora B) in activated RBL-2H3 or LAD2 cells.

Figure 1

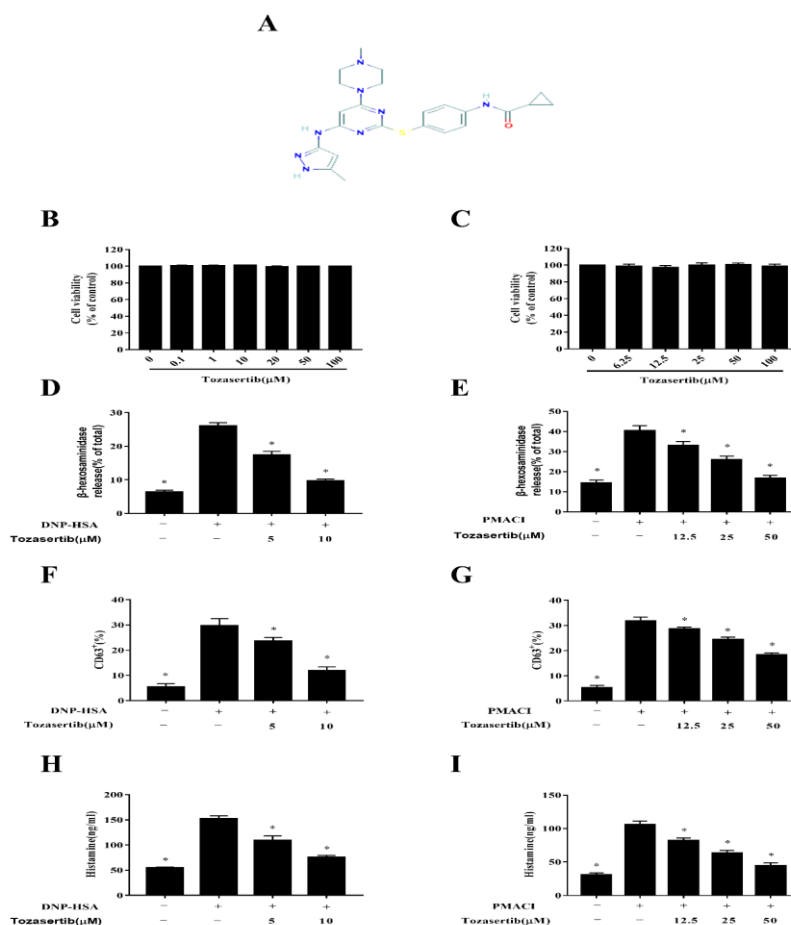


Figure 1. Chemical structure of tozasertib and its anti-allergic effects in activated RBL-2H3 cells and LAD2 cells. (A) Chemical structure of tozasertib. Cytotoxic effect of different concentrations of tozasertib on RBL-2H3 cells using cell viability assay. RBL-2H3 cells (B) and LAD2 cells (C) were incubated with tozasertib at a dose of 0–100 μ M for 24 h. Cell viability was determined with CCK-8 assays. β -hexosaminidase levels were detected. RBL-2H3 cells (D) and LAD2 cells (E) were pretreated with tozasertib for 1 h and then stimulated with DNP-HSA and PMACI for 30 min. β -hexosaminidase quantities in the cell supernatant and lysate were measured. CD63 expression by activated RBL-2H3 cells (F) or LAD2 cells (G) treated with tozasertib was determined by FACS analysis. Histamine levels in activated RBL-2H3 cells (H) or LAD2 cells (I) treated with tozasertib were determined with histamine ELISA kits. Means $\pm$ SD of three independent experiments are shown; * p < 0.05 vs. non-treated activated cells.

Figure 2

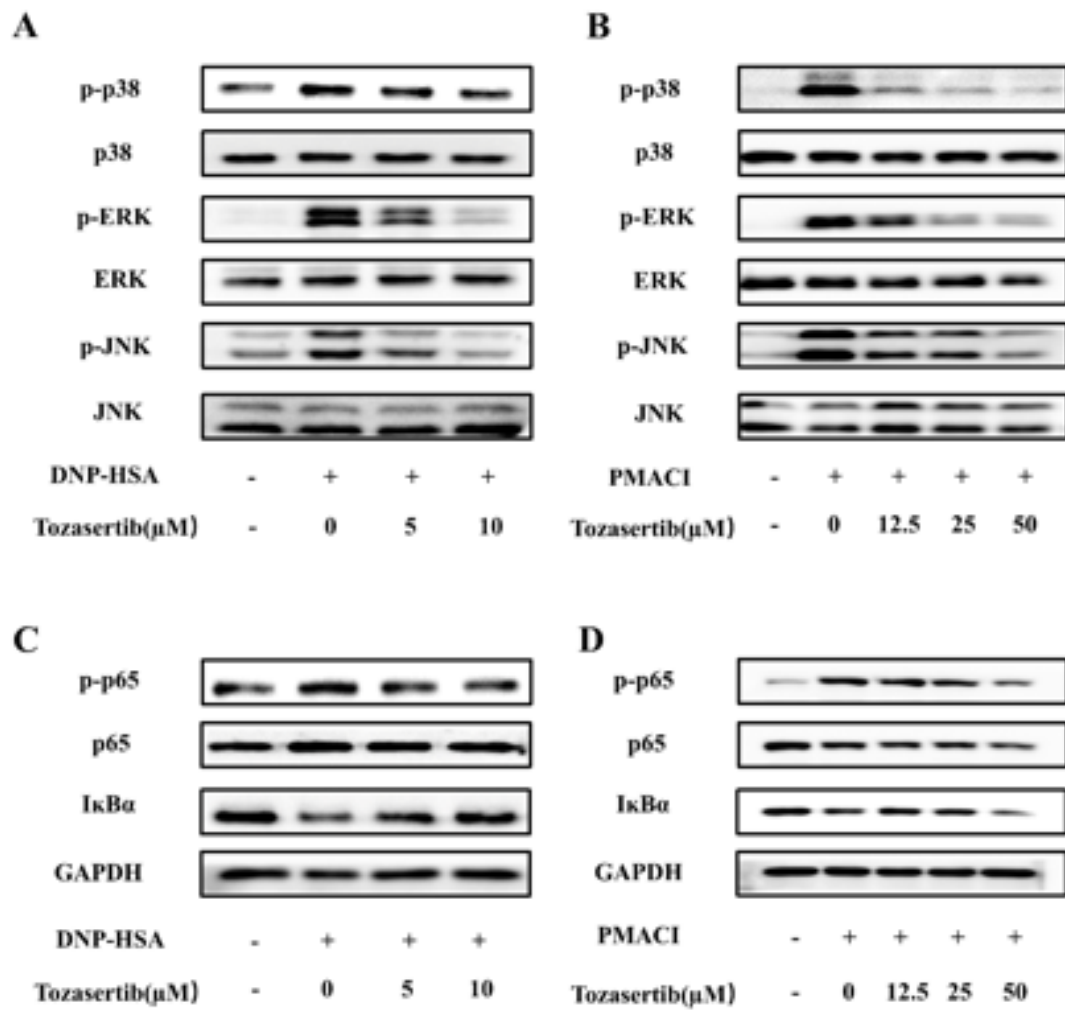


Figure 2. Tozasertib inhibits the activation of MAPK signaling and NF- κ B pathway in activated RBL-2H3 cells or LAD2 cells. IgE-sensitized RBL-2H3 cells or PMACI-stimulated LAD2 cells were pre-incubated with indicated concentrations of tozasertib for 1 h, then stimulated with DNP-HAS or PMACI for 10 min. Western blot analysis of the molecules of MAPK signaling (p-p38, p38, p-ERK, ERK, p-JNK, and JNK) expressed in activated RBL-2H3 cells (A) or LAD2 cells (B). As well, western blot analysis of the molecules of NF- κ B pathway (p-p65, p65, and I κ B α) expressed in activated RBL-2H3 cells (C) or LAD2 cells (D).

Figure 3

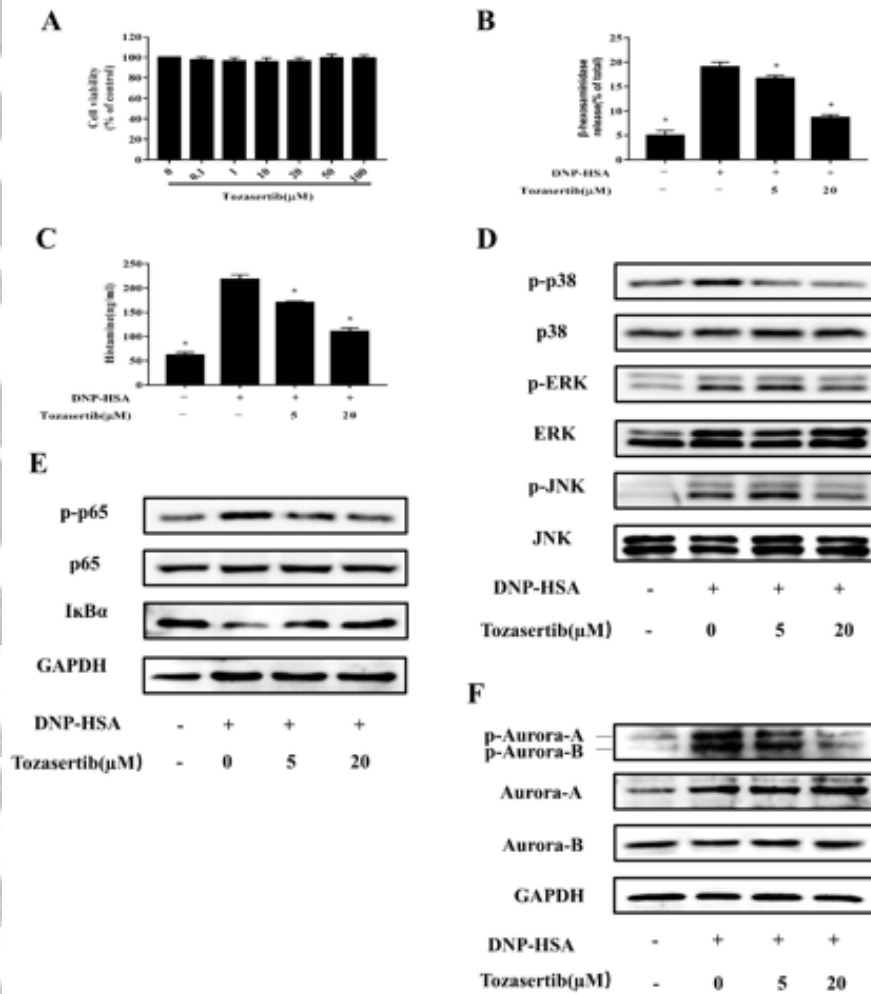


Figure 3. Inhibitory effects of tozasertib on IgE/Ag-stimulated BMMCs. (A) BMMCs were incubated with tozasertib at the indicated dose of 0–100 μM for 24 h. Cell viability was assessed with CCK-8 assays. (B) IgE-sensitized BMMCs were pretreated with tozasertib for 1 h, and then stimulated with DNP-HSA for 30 min. β-hexosaminidase levels in BMMC supernatant and lysate were measured. (C) Histamine levels of activated BMMCs treated with tozasertib were determined with histamine ELISA kits. Western blots of total lysate samples from activated mast cells demonstrating tozasertib treatment (0 μM, 5 μM, and 20 μM) inhibition of MAPK signaling (p-p38, p38, p-ERK, ERK, p-JNK, and JNK) activation (D), NF-κB pathway (p-p65, p65 and IκBα) activation (E), and Aurora kinase activation (A and B, non-phosphorylated and phosphorylated forms) (F). Means ± SD of three independent experiments; *p < 0.05 vs. non-treated activated cells.

Figure 4

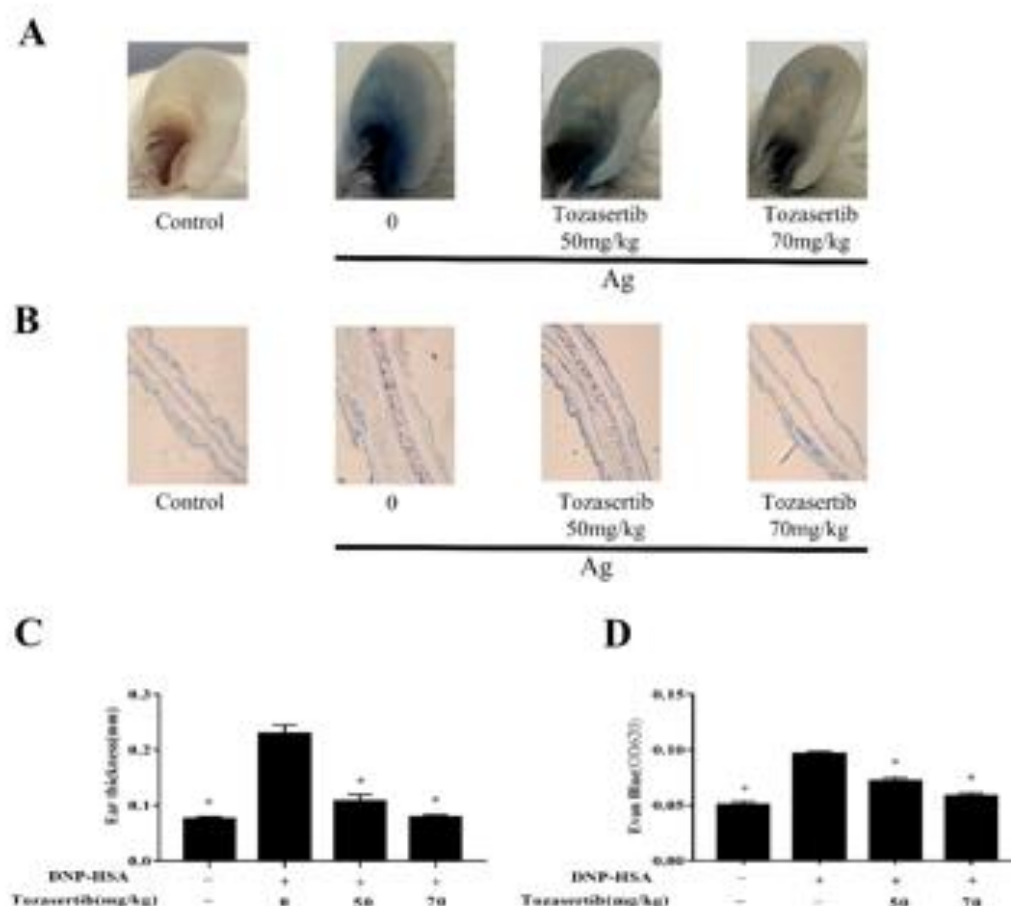


Figure 4. Anti-allergic effects of tozasertib on IgE/Ag-stimulated PCA. Mice, sensitized with 0.5 μ g of anti-DNP IgE for 24 h were administered tozasertib intraperitoneally for 1 h and then were challenged with DNP-HSA. (A) The ears of mice with absorbed Evans blue were photographed (n = 5 per group). (B) Images of representative toluidine blue-stained ears. (C) Measurement of ear thickness. (D) Spectrophotometer detection of dye extracted from each ear. Means $\pm$ SD of independent experiment are shown; n=5. *p < 0.05 vs. non-treated mice.

Figure 5

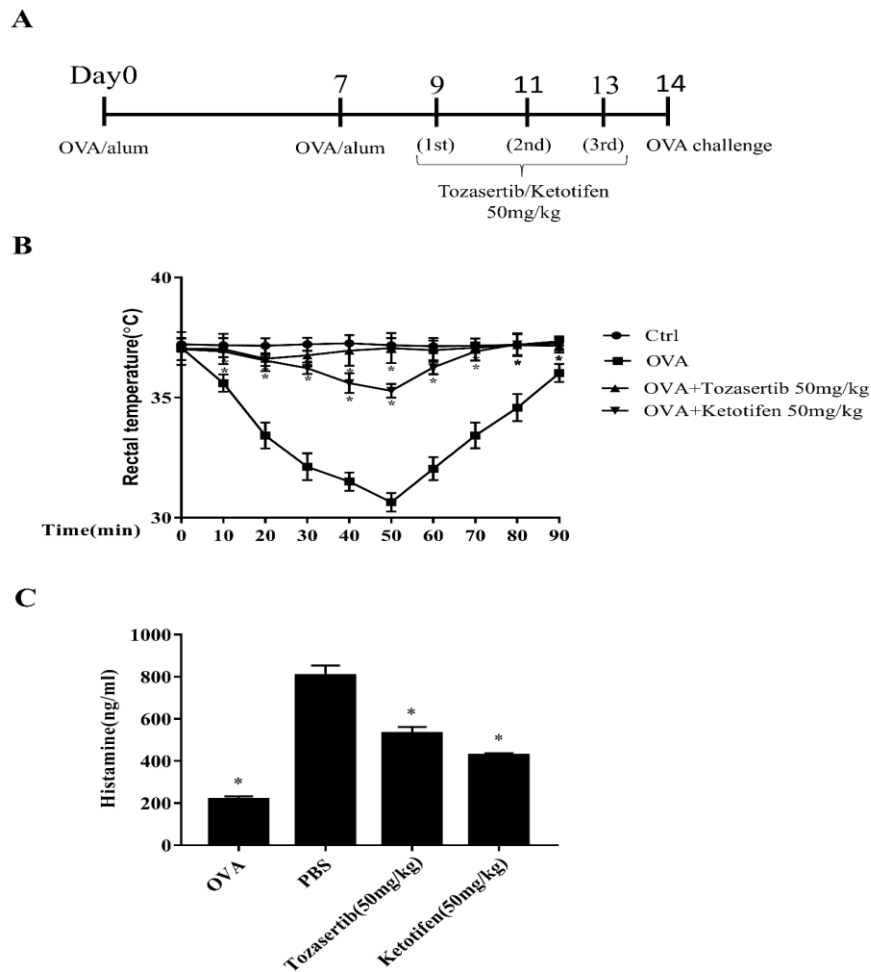
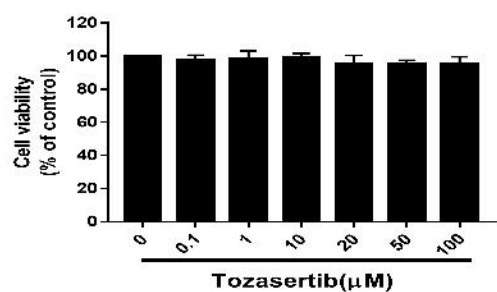


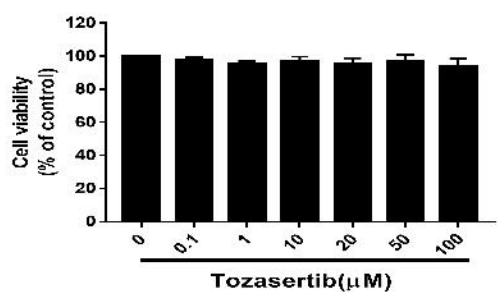
Figure 5. Anti-allergic effects of tozasertib on OVA-induced ASA. (A) ASA mouse model protocol (n = 5 per group) based on sensitization with intraperitoneal OVA (100 μ g with 2 mg alum adjuvant in 200 μ L of PBS). Allergic response was assessed with 200- μ g OVA challenge with or without intraperitoneal tozasertib pre-challenge treatment. Ketotifen treatment was used as a positive control. (B) Means ($\pm$ SD) rectal temperatures measured every 10 min after the OVA challenge from three independent experiments; * p < 0.05 vs. activated group without tozaertib or ketotifen pretreatment. (C) Levels of released histamine in the serum from groups treated or not treated with tozasertib determined by ELISA. Means $\pm$ SD of independent experiment are shown; n=5. * p < 0.05 vs. non-treated mice.

Supplementary Figure 1

A

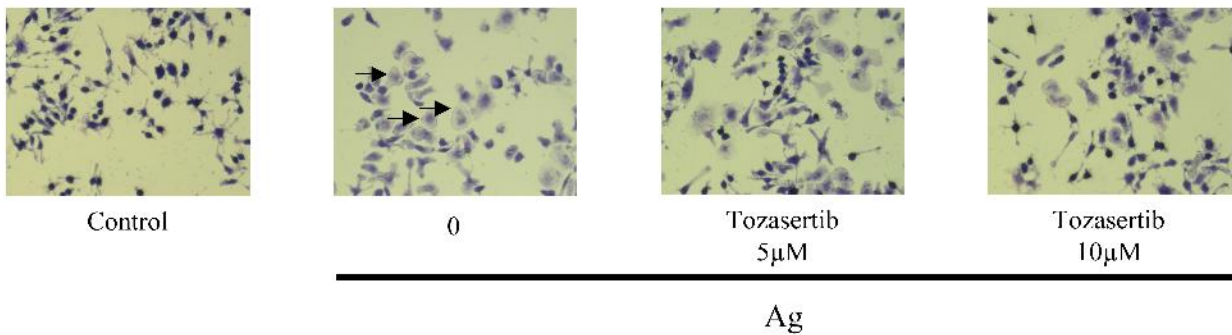


B

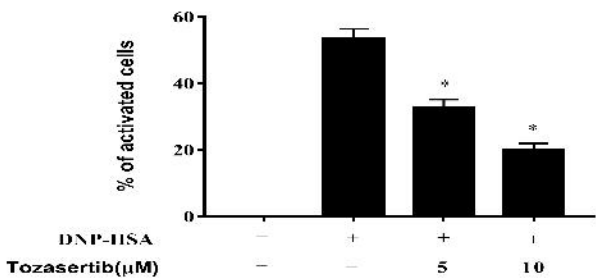


Supplementary Figure 2

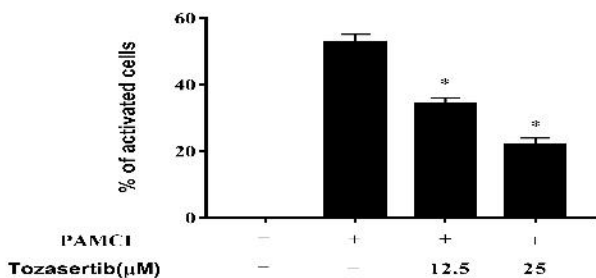
A



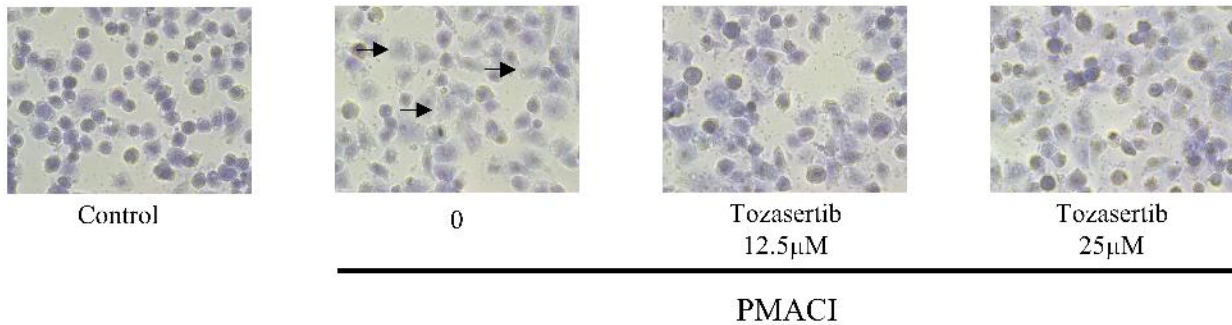
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D

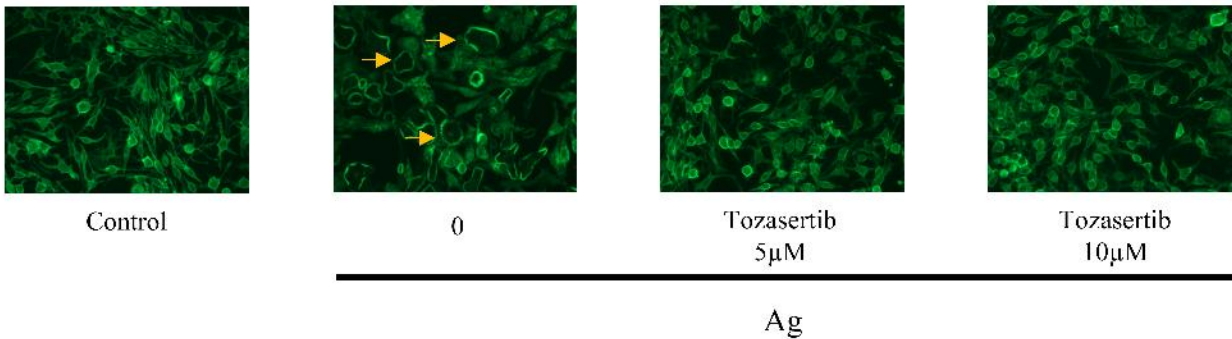


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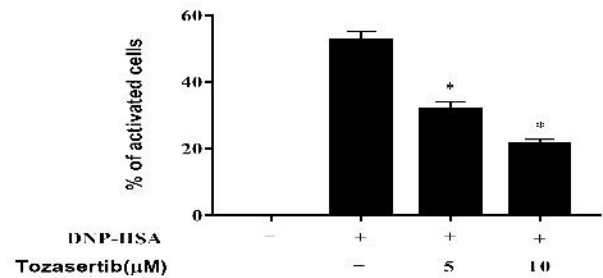


Supplementary Figure 3

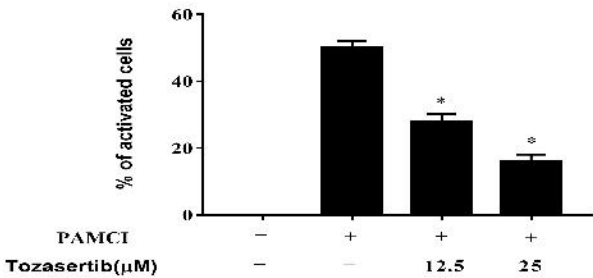
A



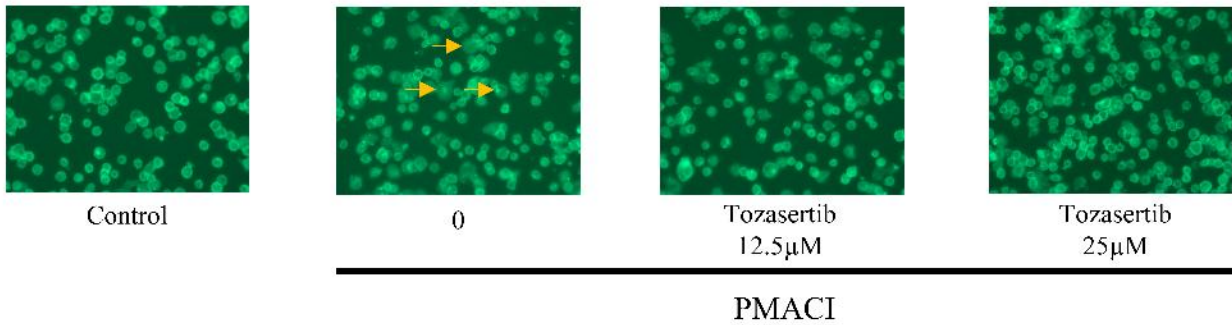
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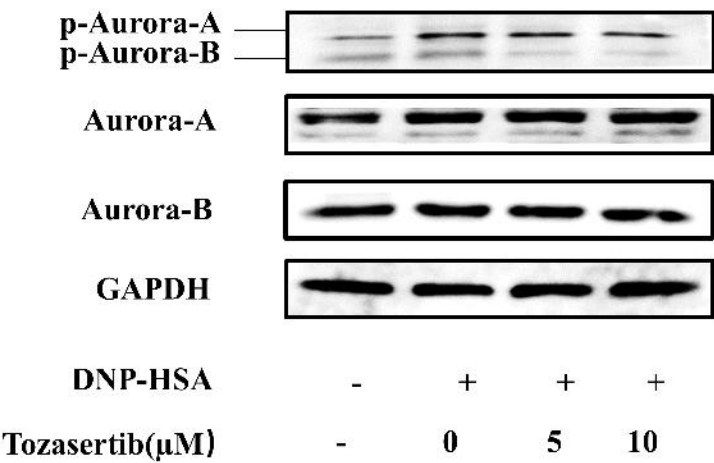


B



Supplementary Figure 4

A



B

