Bletinib ameliorates neutrophilic inflammation and lung injury by inhibiting Src family kinase phosphorylation and activity

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Abstract

BACKGROUND AND PURPOSE Neutrophil overactivation is crucial in the pathogenesis of acute lung injury (ALI). Bletinib (3,3'-dihydroxy-2',6'-bis(p-hydroxybenzyl)-5-methoxybibenzyl), a natural bibenzyl first extracted from Bletilla striata in 1983, has anti-inflammatory, antibacterial, and antimitotic potential. In this study, we evaluated the therapeutic effects of Bletinib in human neutrophilic inflammation and lipopolysaccharide (LPS)-mediated ALI. EXPERIMENTAL APPROACH We assessed integrin expression, superoxide anion production, degranulation, neutrophil extracellular trap (NET) formation, and adhesion in activated human neutrophils through flow cytometry, spectrophotometry, and immunofluorescence microscopy. Moreover, phosphorylation of Src family kinases (SFKs) and downstream proteins was evaluated through immunoblotting. Finally, a murine LPS-induced ALI model was used to investigate the potential therapeutic effects of Bletinib treatment. KEY RESULTS In activated human neutrophils, Bletinib reduced degranulation, respiratory burst, NET formation, adhesion, migration, and integrin expression; suppressed the enzymatic activity of SFKs, including Src, Lyn, Fgr, and Hck; and inhibited the phosphorylation of SFKs as well as Vav and Bruton's tyrosine kinase (Btk). In our mice with ALI, the pulmonary sections demonstrated considerable amelioration of prominent inflammatory changes, such as haemorrhage, pulmonary oedema, and neutrophil infiltration, after Bletinib treatment. CONCLUSION AND IMPLICATIONS This is the first study to provide evidence that Bletinib regulates neutrophilic inflammation by inhibiting the SFKs–Btk–Vav pathway and that Bletinib ameliorates LPS-induced ALI in mice. Further biochemical optimisation of Bletinib may be a promising strategy for the development of novel therapeutics for inflammatory diseases.

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Short running title

Bletinib mitigates neutrophilic inflammation by inhibiting SFKs

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Conflict of Interests Statement

All authors declare no conflicts of interests.

BACKGROUND AND PURPOSE

Neutrophil overactivation is crucial in the pathogenesis of acute lung injury (ALI). Bletinib (3,3'-dihydroxy-2',6'-bis(*p* -hydroxybenzyl)-5-methoxybibenzyl), a natural bibenzyl first extracted from *Bletilla striata* in 1983, has anti-inflammatory, antibacterial, and antimitotic potential. In this study, we evaluated the therapeutic effects of Bletinib in human neutrophilic inflammation and lipopolysaccharide (LPS)-mediated ALI.

EXPERIMENTAL APPROACH

We assessed integrin expression, superoxide anion production, degranulation, neutrophil extracellular trap (NET) formation, and adhesion in activated human neutrophils through flow cytometry, spectrophotometry, and immunofluorescence microscopy. Moreover, phosphorylation of Src family kinases (SFKs) and downstream proteins was evaluated through immunoblotting. Finally, a murine LPS-induced ALI model was used to investigate the potential therapeutic effects of Bletinib treatment.

KEY RESULTS

In activated human neutrophils, Bletinib reduced degranulation, respiratory burst, NET formation, adhesion, migration, and integrin expression; suppressed the enzymatic activity of SFKs, including Src, Lyn, Fgr, and Hck; and inhibited the phosphorylation of SFKs as well as Vav and Bruton's tyrosine kinase (Btk). In our mice with ALI, the pulmonary sections demonstrated considerable amelioration of prominent inflammatory changes, such as haemorrhage, pulmonary oedema, and neutrophil infiltration, after Bletinib treatment.

CONCLUSION AND IMPLICATIONS

This is the first study to provide evidence that Bletinib regulates neutrophilic inflammation by inhibiting the SFKs–Btk–Vav pathway and that Bletinib ameliorates LPS-induced ALI in mice. Further biochemical opti-

misation of Bletinib may be a promising strategy for the development of novel therapeutics for inflammatory diseases.

Keywords

Bletinib; Src family kinase; Neutrophil; Inflammation; Acute lung injury; Acute respiratory distress syndrome

Abbreviations

ALI, acute lung injury; ARDS, acute respiratory distress syndrome; NET, neutrophil extracellular trap; SFK, Src family kinase; fMLF, N-formyl-$_{\text{L}}$-methionyl-$_{\text{L}}$-leucyl-$_{\text{L}}$-phenylalanine; MPO, myeloperoxidase; NE, neutrophil elastase; FPR, formyl peptide receptor; PMA, Phorbol 12-myristate 13-acetate; NaF, sodium fluoride; DHR 123, dihydrorhodamine 123; LTB4, Leukotriene B4; Macrophage-1 antigen, Mac-1

Bullet point summary

WHAT IS ALREADY KNOWN

Neutrophil overactivation plays a key role in acute lung injury (ALI) pathogenesis.

WHAT THIS STUDY ADDS

Bletinib mitigates neutrophilic inflammation by inhibiting the SFK–Btk–Vav pathway.

CLINICAL SIGNIFICANCE

Bletinib can alleviate lipopolysaccharide-mediated ALI.

Introduction

Neutrophils, the most abundant granulocytes in circulation, are responsible for eliminating pathogens through degranulation, enabling neutrophil elastase (NE) release, respiratory burst with superoxide production, and neutrophil extracellular trap (NET) formation (Brinkmann*et al.,* 2004; Mantovani *et al.,* 2011; Phan *et al.,*2018). Thus, neutrophils are key effectors of both adaptive and innate immune systems (Mantovani *et al.* , 2011). During inflammation, adhesion and migration are both crucial steps of neutrophil recruitment, which is regulated by the conformational change of macrophage-1 antigen (Mac-1; also known as αMβ2 and CD11b-CD18) on neutrophil surfaces (Carrigan *et al* ., 2007; Kolaczkowska & Kubes, 2013; Li *et al.* , 2018; Morisaki *et al.* , 1991). Dysregulated activation and recruitment of neutrophils can cause damage to host tissue through the release of an excessive amount of proteolytic enzymes, reactive oxygen species (ROS), and NETs, resulting in various morbidities, including autoimmune diseases (e.g. systemic lupus erythematosus and rheumatoid arthritis; Nemeth & Mocsai, 2012), infectious diseases (e.g. sepsis; Sonego *et al.* , 2016), inflammatory diseases (e.g. chronic obstructive pulmonary disease; Noguera *et al.* , 2001), atherosclerosis (Soehnlein, 2012), and acute lung injury (ALI; Grommes & Soehnlein, 2011), and other major diseases (e.g. cancers) (Grecian*et al.* , 2018; Jorch & Kubes, 2017).

Acute respiratory distress syndrome (ARDS), the clinical term for ALI, occurs most often in the course of sepsis and severe pneumonia, including coronavirus disease 2019 (COVID-19; Park *et al.* , 2019; Sohrabi *et al.* , 2020). The mortality rate of ARDS remains as high as 30%–40%; moreover, other than lung-protective ventilation, few specific effective therapeutics have been developed (Matthay *et al.* , 2019). ALI is pathologically characterised by diffuse alveolar damage along with neutrophil infiltration and inflammatory oedematous fluid accumulation in the bronchoalveolar space (Grommes & Soehnlein, 2011). Neutrophil activation and recruitment contribute to ALI pathogenesis; moreover, in patients with ALI induced by ischaemia reperfusion or lipopolysaccharides (LPSs), the inhibition of SFK phosphorylation and activity and NET formation, respectively, improve clinical condition and increase survival (Grommes & Soehnlein, 2011; Oyaizu *et al.* , 2012; Pedrazza *et al.* , 2017).

Src family kinases (SFKs) are nonreceptor intracellular protein tyrosine kinases, some of which, including Lyn, Fgr, and Hck, are mainly expressed in human neutrophils (Futosi & Mocsai, 2016). SFKs modulate

multiple functions of neutrophils, such as ROS production, degranulation, adhesion, NET formation, integrin activation, and migration towards inflamed sites (Fumagalli *et al.* , 2013; Mocsai*et al.* , 1999; Nani *et al.* , 2015; Rohwedder *et al.* , 2019; Sarantos *et al.* , 2008). N-formyl-$_{\text{L}}$-methionyl-$_{\text{L}}$-leucyl-$_{\text{L}}$-phenylalanine (fMLF) is a chemotactic peptide recognised by neutrophils through their formyl peptide receptor (FPR), which triggers SFK phosphorylation and thus neutrophil activation (Fumagalli *et al.* , 2007). SFKs regulate the signal transduction in fMLF-activated neutrophils and the phosphorylation of downstream signal proteins such as JNK, ERK, Vav, and Bruton's tyrosine kinase (Btk; El-Hashim *et al.* , 2017; Fumagalli*et al.* , 2013; Tsai *et al.* , 2019).

In traditional Chinese medicine, *Bletilla* tubers have been used to treat pulmonary, gastrointestinal, and dermatological inflammatory and haemorrhagic diseases for thousands of years (He *et al.* , 2016b). Bletinib [3,3'-dihydroxy-2',6'-bis(*p* -hydroxybenzyl)-5-methoxybibenzyl, molecular formula: $C_{29}H_{28}O_5$, molecular weight: 456.538; Figure 1A] is a natural bibenzyl that was first extracted from *Bletilla striata* bulbs by Takagi *et al.* in 1983. Bletinib has antibacterial, antifungal, antiallergic, and antimitotic potential (Matsuda *et al.* , 2004; Morita *et al.* , 2005; Takagi *et al.* , 1983; Yang *et al.* , 2012). In our previous study, although Bletinib demonstrated anti-inflammatory effects on human neutrophils, the underlying mechanism remained unclear (Lin *et al.* , 2016).

In this study, we investigated the effects of Bletinib on neutrophil functions, such as ROS production, degranulation, adhesion, migration, NET formation, and integrin expression, to test the hypothesis that Bletinib regulates the inflammatory condition of activated human neutrophils. Moreover, we elucidated the mechanism and signal transduction pathway underlying the Bletinib-mediated modulation of neutrophilic inflammation by using a murine LPS-induced ALI model.

Methods

Materials

Bletinib was provided by Dr Tian-Shung Wu, Department of Chemistry National Cheng Kung University, Taiwan. The process of Bletinib extraction and purification has been described elsewhere (Lin *et al.* , 2016). In brief, Bletinib was extracted from the rhizomes of*B. formosana* with ethanol at 60 °C and purified through column chromatography. Bletinib (purity >98%) was dissolved in dimethyl sulfoxide (DMSO) for further experimentation. Hanks' balanced salt solution (HBSS) was purchased from Gibco (NY, USA), and Ficoll-Paque was purchased from GE Health-Care (Buckinghamshire, UK). Antibodies against Akt, p-Akt (S473), ERK, p-ERK, JNK, p-JNK, p38 MAPK, phospho-p38 MAPK, Src, p-SFK (Y416), Lyn, Fgr, Hck, Btk, and p-Btk (Y223) were purchased from Cell Signaling Technology (Massachusetts, USA); those against p-Lyn (Y396), p-Hck (Y410), and histone H3 were from Abcam (Cambridge, UK); that against p-Src (Y416) was from Merck Millipore (CA, USA); that against p-Fgr (Y412) was from Thermo Fisher Scientific (MA, USA); and those against Vav and p-Vav (Y174) were from EnoGene Biotech (NY, USA). Moreover, purified anti-mouse Ly6G antibody was obtained from BioLegend (CA, USA).

Animals

All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee of Chang Gung University, Taiwan. Moreover, our animal studies are reported in accordance with the ARRIVE guidelines (McGrath *et al.* , 2010; McGrath & Lilley, 2015). All the experimental procedures complied with *The Guide for the Care and Use of Laboratory Animals* (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory, 2011). Specified pathogen-free (SPF) 8-week-old male BALB/c mice (body weight: 20 ± 3 g) were purchased from BioLASCO (Taiwan). Five mice shared a ventilated cage with standard bedding and were provided with water and standard laboratory chow *ad libitum* , and they were all kept in an SPF animal facility under a 12–12-h light–dark cycle. Mice were acclimatised for at least 1 week before use in experiments.

ALI model

The male BALB/c mice were randomly divided into four groups: vehicle, Bletinib alone, control, and Bletinib

treatment group. The mice were starved overnight and then intraperitoneally injected with 50 μL of Bletinib (25 mg kg^{-1}) or 50 μL of vehicle (10% DMSO). ALI was induced through intratracheal spraying of 50 μL of LPS (from*Escherichia coli* O111:B4; 2 mg kg-body-$weight^{-1}$) or 50 μL of 0.9% saline (in vehicle and Bletinib alone group) after tracheostomy under general anaesthesia with xylazine (6 mg kg^{-1}) and Zoletil 50 (30 mg kg^{-1}). Six hours later, mouse lungs were harvested after sacrifice and were frozen for myeloperoxidase (MPO) activity assay; they were also fixed with 10% formalin for histological sectioning and immunofluorescence staining.

Histological sectioning and immunofluorescence staining

The harvested lung tissues were washed with phosphate-buffered saline (PBS) and fixed with 10% formalin for 24 h. The samples were subsequently dehydrated, embedded with paraffin, sliced into 3-μm-thick sections with a microtome, and placed on glass slides. These sections were stained using haematoxylin and eosin (H&E) as well as anti-MPO or anti-Ly6G antibodies. Then, images were acquired through light microscopy, as described previously (Yuan *et al.* , 2006).

For immunofluorescence staining, tissue sections were incubated with antibodies against H3 (citH3; citrulline R2 + R8 + R17) and Ly6G at dilutions of 1:800 and 1:200, respectively. Anti-IgG secondary antibodies labelled with a fluorescent dye (Alexa Fluor 488 for citH3 or Alexa Fluor 568 for Ly6G) were used at dilutions of 1:1000 and 1:500, respectively. Immunofluorescence images were acquired through confocal microscopy (LSM 510 Meta, Zeiss).

Analysis of MPO activity

The mouse lung tissues were suspended in a 0.5% hexadecyltrimethylammonium bromide buffer (pH 6.0) and then homogenised through sonication. To evaluate MPO activity, the MPO substrate buffer (containing PBS, 0.0005% hydrogen peroxide, and 0.2 mg mL^{-1} *o* -dianisidine hydrochloride) was added to the homogenised tissue, and the light absorbance at 460 nm was detected through spectrophotometry, after which MPO activity was calculated with reference to the standard curve of human MPO activity (Yu *et al.* , 2006).

Human neutrophil isolation

The study was conducted with the approval of the Institutional Review Board of Chang Gung Memorial Hospital (IRB No. 201601111A3) in accordance with the Declaration of Helsinki. After written informed consent was obtained, whole blood samples were drawn from healthy individuals aged 20–30 years who had not taken any medication within the previous 2 weeks. Neutrophils were then isolated using the standard procedures for Ficoll-Hypaque gradient centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes. The isolated neutrophils—containing >98% living cells, confirmed through trypan blue assay—were then suspended in Ca^{2+}-free HBSS (pH 7.4) and stored at 4 °C until use (Chen *et al.* , 2014).

Measurement of extracellular superoxide anion production

Extracellular superoxide anion production in activated neutrophils was assessed through the reduction of ferricytochrome *c* . After incubating them with Ca^{2+} (1 mM) and ferricytochrome*c* (0.5 mg mL^{-1}) at 37 degC, the isolated human neutrophils (6 x 10^5 cells mL^{-1}) were then incubated with 0.1% DMSO or 0.3–10 μM Bletinib for 5 min. The cells were then treated with 1 μg mL^{-1}cytochalasin B (CB) for 3 min and then stimulated with fMLF, MMK-1, sodium fluoride (NaF), or phorbol-12-myristate-13-acetate (PMA). The change in absorbance at 550 nm was detected continuously using a spectrophotometer (U-3010, Hitachi, Tokyo, Japan), and superoxide anion levels were calculated using a method described previously (Hwang*et al.* , 2003).

Measurement of intracellular superoxide anion production

Human neutrophils (2.5×10^6 cells mL^{-1}) were labelled using 2 μM dihydrorhodamine 123 (DHR123) at 37 °C for 10 min and then incubated with DMSO or Bletinib for 5 min and then stimulated with 0.1 μM fMLF/0.5 μg mL^{-1} CB for 15 min. The fluorescence intensity was detected through flow cytometry to evaluate intracellular superoxide anion production of human neutrophils.

Analysis of total ROS production

Total ROS produced by neutrophils was assessed using a luminol-amplified chemiluminescence method described previously (Bedouhene *et al.* , 2017). In brief, human neutrophils (2×10^6 cells mL^{-1}) were preincubated with 6 U mL^{-1} horseradish peroxidase (HRP) and 37.5 μM luminol in a 96-well plate at 37 °C for 5 min. Cells were incubated with DMSO or Bletinib for 5 min, followed by 0.1 μM fMLF stimulation. Chemiluminescence was then detected and analysed in real time on a 96-well chemiluminometer (Tecan Infinite F200 Pro; Männedorf, Switzerland).

Analysis of NE release

Azurophilic degranulation was determined on the basis of NE release from human neutrophils, as reported previously (Sklar *et al.* , 1982). In brief, human neutrophils (6×10^5 cells mL^{-1}) were incubated with DMSO or Bletinib after treatment with 1 mM $CaCl_2$ and 100 μM NE substrate (Methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide) at 37 °C for 5 min. Cells were stimulated with fMLF, leukotriene B4 (LTB4), NaF, or MMK-1 for 10 min before determination of NE release through measuring the change of absorbance at 405 nm on a spectrophotometer.

Analysis of NET formation

Quantification of extracellular DNA. Human neutrophils (10^6 cells mL^{-1}) resuspended in HBSS with 2.5 μM Sytox green were incubated with DMSO or Bletinib for 5 min and stimulated using 10 nM PMA for 3 h. The fluorescence intensity was quantified on a Tecan Infinite 200 reader at 485–535 nm.

NET photography. Neutrophils (3×10^5 cells mL^{-1}) were incubated with DMSO or Bletinib for 5 min before being activated with 10 nM PMA for 2 h. Neutrophils were fixed with 4% paraformaldehyde and treated with 5% goat serum blocking buffer for 1 h and then treated with 5 μg mL^{-1}anti-MPO (Abcam) and 5 μg mL^{-1} anti-NE (Merck Millipore) antibodies for 1 h. These cells were then treated with the Alexa 488 or 568–labelled secondary goat anti-rabbit antibody for another 1 h. Thereafter, the cells were washed with PBS and treated with 1 ng mL^{-1} Hoechst 33342 and ProLong Gold antifade reagent (Invitrogen, CA, USA). Immunofluorescence microscopy and scanning electron microscopy were both used to observe the NET formation of activated neutrophils, as described previously (Hwang *et al.* , 2015; Remijsen *et al.* , 2011).

Evaluation of neutrophil adhesion

Human neutrophils (10^6 cells mL^{-1}) were labelled with Hoechst 33342 and then incubated with DMSO or Bletinib for 5 min. After centrifugation, the cells were resuspended and activated with 0.1 μM fMLF/1 μg mL^{-1} CB for 10 min before incubation with bEnd.3 cells at 37 °C for 30 min. After they were washed with HBSS, the cells were fixed with 4% paraformaldehyde and the neutrophils that adhered to the bEnd.3 cells were detected and quantified on a motorised inverted microscope (Olympus, Japan), as described previously (Chen *et al.* , 2016b).

Analysis of neutrophil migration

A microchemotaxis chamber with 3-μm filters (Millipore) was used to evaluate chemotactic migration of neutrophils. The neutrophils (5×10^6 cells mL^{-1}) treated with Bletinib or DMSO for 5 min were placed in the top chamber, and then 0.1 μM fMLF was added into the bottom chamber. The number of neutrophils that migrated from the top to the bottom chamber after incubation at 5% CO_2 for 1 h was counted on a MoxiZ automatic cell counter (ORFLO).

Evaluation of surface CD18 and CD11b expression

Neutrophils (5×10^5 cells mL^{-1}) were incubated with Bletinib or DMSO for 10 min and then activated using 0.1 μM fMLF/1 μg mL^{-1} CB for 5 min. After centrifugation at 200 *g* for 8 min at 4 °C, the cells were resuspended in 5% bovine serum albumin with FITC-conjugated antibodies against CD18 or CD11b on ice in the dark for 15 min. The fluorescence intensity was then analysed through fluorescence-activated cell sorting (Tsai *et al.* , 2017).

Immunoblotting of neutrophil lysates

The immunoblotting assay was conducted as described previously (Tsai*et al.* , 2019). In brief, neutrophils incubated with Bletinib or DMSO at 37 °C for 5 min were stimulated with 0.1 μM fMLF for 30 s. The proteins were separated from neutrophil lysates through electrophoresis (12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and then transferred to nitrocellulose membranes. The target proteins were identified through immunoblotting with specific antibodies against p38, p-p38, Akt, p-Akt S473, ERK, p-ERK, JNK, p-JNK, Src, p-SFKs Y416, p-Src Y416, Lyn, p-Lyn (Y396), Fgr, p-Fgr (Y412), Hck, p-Hck (Y410), Btk, p-Btk Y223, Vav, and p-Vav (Y174) as well as with HRP-conjugated secondary anti-rabbit antibody (Cell Signaling Technology). The signal intensity was detected and assessed on a UVP BioSpectrum Imaging System (Analytik Jena, USA).

Assessment of enzymatic activity of SFKs

The kinase activity of SFKs was assessed using an ADP-Glo kinase assay kit (Promega, Fitchburg, USA) according to manufacturer instructions and as described previously (Tsai *et al.* , 2019). In brief, the kinase reaction was initiated by adding SFKs (Src, Lyn, Fgr, and Hck), their substrate—125 μM ATP, and 1–10 μM Bletinib or 0.1–3 μM PP2 into the reaction buffer for 1 h. The ADP-Glo reagent was used to end the kinase reaction and remove the remnant ATP; next, the kinase, which converted ADP to ATP, detection reagent was added and incubated for 30 min (Kovacs*et al.* , 2014; Lowell, 2004). Luciferin/luciferase luminescence was determined on an Infinite 200 Pro (Tecan, Switzerland).

Statistical data and analysis

Our statistical data and analysis comply with the recommendations on experimental design and analysis in pharmacology by Curtis *et al.*(2018). All data are presented as means ± standard errors of the means (SEMs). Student's t test was employed for statistical analysis on SigmaPlot (Systat Software, USA). Differences with p values < 0.05 were considered significant statistically.

Results

Bletinib ameliorates superoxide anion production and ROS production in stimulated neutrophils

Superoxide anions produced by neutrophils are responsible for the tissue damage caused during inflammation. To elucidate whether Bletinib modulates inflammatory responses, we examined the effect of Bletinib on superoxide production in human neutrophils stimulated with various chemoattractants. On the basis of the amount of reduction of ferricytochrome c , Bletinib ameliorated superoxide anion production by fMLF-activated human neutrophils in a concentration-dependent manner (IC_{50} = 0.62 ± 0.15 μM; Figure 1B). Similarly, Bletinib attenuated superoxide anion release from neutrophils stimulated by other chemoattractants, such as NaF (a G protein activator), MMK-1 (an FPR2 agonist), and PMA (a PKC activator; Figure 1C, 1D, and 1E, respectively). In addition, Bletinib demonstrated neither cytotoxicity (Figure 1F) nor ROS scavenging activity in the cell-free xanthine/xanthine oxidase system (Figure 1G).

Flow cytometry and chemiluminescence assay were performed to determine if Bletinib affects ROS production in stimulated neutrophils. The results of flow cytometry using DHR123 revealed that Bletinib significantly suppressed intracellular ROS production in fMLF-activated neutrophils (Figure 2A and 2B). Moreover, the results of luminol-amplified chemiluminescence assay in stimulated neutrophils demonstrated that total ROS production (intracellular and extracellular) was significantly attenuated by Bletinib in a concentration-dependent manner (Figure 2C and 2D).

Bletinib inhibits degranulation of activated human neutrophils

Degranulation, an important function of neutrophils during inflammation, was evaluated by measuring the release of NE. Bletinib inhibited the NE release of fMLF-stimulated human neutrophils (IC_{50} = 0.53 ± 0.07 μM) but without affecting that of resting neutrophils (Figure 3A). In addition, Bletinib downregulated NE release from neutrophils stimulated with MMK-1, NaF, and LTB4 in a concentration-dependent manner (Figure 3B, 3C, and 3D, respectively).

Bletinib attenuates NET formation

NET, mainly composed of granular proteins, proteases, and chromatin filaments coated with histones, is crucial in sterile inflammation (Jorch & Kubes, 2017). To elucidate and quantify the effects of Bletinib on NET formation, neutrophils were stained with Sytox green after activation with 10 nM PMA. Fluorescence spectrometry assay results demonstrated that NET formation induced by PMA was significantly mitigated by Bletinib in a concentration-dependent manner (Figure 4A). In addition, scanning electron microscopy of immunofluorescent staining demonstrated the presence of neutrophils co-stained with Hoechst 33342 and antibodies against MPO and NE in the NETs (Figure 4B and 4C).

Bletinib suppresses ERK, JNK, and SFK phosphorylation in fMLF-activated neutrophils

SFKs and the MAPK/ERK pathway play critical roles in the degranulation, respiratory burst, NET formation, and migration of neutrophils (Hakkim*et al.* , 2011; Minuz *et al.* , 2018; Romero *et al.* , 2010). Therefore, we evaluated the effects of Bletinib on the phosphorylation of Akt, p38 MAPK, ERK, JNK, and SFKs in activated neutrophils. Our immunoblotting results demonstrated that the phosphorylation of p38 MAPK, Akt, ERK, JNK, SFKs, Src (Y416), Lyn (Y396), Fgr (Y412), Hck (Y410), Btk (Y223), and Vav (Y174) was enhanced in fMLF-stimulated neutrophils; however, Bletinib significantly inhibited the phosphorylation of ERK, JNK, SFKs, Src (Y416), Lyn (Y396), Fgr (Y412), Hck (Y410), Btk (Y223), and Vav (Y174) but not that of p38 MAPK and Akt (Figures 5 and 6A and 6B).

Bletinib inhibits SFK activity

SFKs are nonreceptor tyrosine kinases present in neutrophils, with predominant expression of Src, Fgr, Hck, and Lyn (Ear *et al.* , 2017; Kovacs *et al.* , 2014). SFKs are responsible for the generation of the inflammatory environment *in vivo* . Here, our cell-free ADP-Glo kinase assay results demonstrated that Bletinib inhibits kinase activity of Src, Fgr, Hck, and Lyn in a concentration-dependent manner (Figure 6C).

Bletinib reduces adhesion and transmigration of activated human neutrophils

Adhesion and transmigration are both crucial steps in the neutrophil recruitment cascade during inflammation (Kolaczkowska & Kubes, 2013). Here, after incubation with Bletinib (1–10 μM) or DMSO for 5 min and stimulation with fMLF, we incubated the Hoechst 33342-labelled neutrophils (10^6 cells mL^{-1}) with bEnd.3 cells in 37 °C for 30 min. Neutrophils adherent on bEnd.3 cells were detected and counted through fluorescence microscopy, and our results demonstrated that Bletinib inhibited the adhesion function of fMLF-activated neutrophils (Figure 7A and 7B). Moreover, we used a chemotaxis chamber and a cell counter to enumerate the number of migrating neutrophils and found that Bletinib significantly reduced fMLF-induced transwell migration of neutrophils (Figure 7C).

Bletinib reduces Mac-1 expression in activated neutrophils

Mac-1 is a complement receptor composed of CD11b (integrin α_M) and CD18 (integrin β_2) and facilitates leukocyte recruitment during inflammation (Li *et al.* , 2018; Wolf *et al.* , 2018). Here, we determined the surface expression of CD11b and CD18 through flow cytometry and found that Bletinib significantly attenuated the expression of both CD11b and CD18 in fMLF-stimulated human neutrophils (Figure 8).

Bletinib alleviates LPS-induced ALI in mice

Endotoxins such as LPS are the most common causes of ALI in bacterial infection and inflammatory diseases (Dreyfuss & Ricard, 2005; Tsai*et al.* , 2015). To investigate the anti-inflammatory effects of Bletinib *in vivo* , we used BALB/c mice treated with Bletinib (30 mg kg^{-1}) or DMSO administered through intraperitoneal injection followed by intratracheal spraying of LPS after 5 h. The results demonstrated that total protein levels and MPO activity in the mouse lung tissues increased in the control group but were significantly ameliorated in the mice treated with Bletinib (Figure 9A and 9B). In addition, LPS induced the infiltration of cells positive for MPO and Ly6G (specific markers of neutrophils), and the cellular infiltration was significantly suppressed in the Bletinib treatment group. The representative images of mouse lungs in Figure 9C

and 9D indicate that Bletinib treatment considerably alleviated the haemorrhagic condition, erythematous condition, and Ly6G$^+$citH3$^+$ cell accumulation in the LPS control group.

Discussion

B. formosana belongs to the Orchidaceae family and is widely distributed in Taiwan; its tuber is extensively used in traditional Chinese medicine to treat pulmonary, gastrointestinal, and dermatological inflammatory diseases (Lin *et al.* , 2016). Appropriate fermentation processes can be applied to enhance antioxidant activities and total phenolic content of *B. formosana* (Dong*et al.* , 2014). In the present study, Bletinib, a natural compound extracted from *B. formosana* , was found to significantly ameliorate neutrophilic inflammation by inhibiting SFK phosphorylation and activity and the related downstream signal transduction pathway.

Neutrophils have pivotal roles in innate immunity as well as infectious and inflammatory disease progression. For instance, during bacterial infection, neutrophils are chemotactically recruited to inflamed tissues by endotoxin components, such as fMLF and LPS. Upon activation and recruitment, neutrophils produce superoxide anion and release proteolytic enzymes, such as elastase, to attenuate the progression of bacterial infection (Phan *et al.* , 2018). However, excessive superoxide anion production exerts cytotoxicity and damages host tissue (Fridovich, 1986). For instance, although ROS is indispensable in the inflammation process (Tintinger *et al.* , 2009), its overproduction contributes to various acute and chronic diseases, such as ALI (Dreyfuss & Ricard, 2005; Grommes & Soehnlein, 2011; Kellner *et al.* , 2017), coronary artery disease (Belaidi *et al.* , 2016), chronic*Helicobacter pylori* infection (Beceiro *et al.* , 2016), pulmonary hypertension (Chen *et al.* , 2016a), and diabetes mellitus and related complications (He *et al.* , 2016a; Miranda-Diaz *et al.* , 2016; Wu *et al.* , 2016). ARDS/ALI is a major complication of pulmonary oedema and severe pneumonia, such as COVID-19, which can be highly fatal without appropriate treatment (Guan*et al.* , 2020; Sohrabi *et al.* , 2020). Numerous neutrophils are present in the bronchoalveolar lavage fluid of patients with ARDS/ALI, and various murine experimental ALI models have confirmed the therapeutic effects of neutrophil depletion (Nemeth *et al.* , 2020). Kellner *et al.* (2017) revealed that ROS enhances adhesion molecule and proinflammatory cytokine expression, resulting in pulmonary oedema and tissue damage. Researchers worldwide have been searching for compounds with antioxidant and anti-inflammatory effects to serve as potential therapeutics to the life-threatening diseases just mentioned (Boeing *et al.* , 2020; Chen *et al.* , 2015; Hwang *et al.* , 2015; Liao *et al.* , 2015). In the present study, Bletinib significantly mitigated superoxide anion production (Figure 1B–1E), ROS production (Figure 2), and degranulation (Figure 3) in stimulated human neutrophils. However, Bletinib did not exhibit either cytotoxicity (Figure 1F) or superoxide scavenging potential (Figure 1G) in the xanthine/xanthine oxidase cell-free system. These findings indicated that Bletinib reduced oxidative stress and protease release through regulating cellular signalling rather than through cytotoxicity or free radical scavenging activity.

NETs are web-like structures of nuclear chromatin coated with granular proteins of neutrophils, such as NE and MPO, which can trap and eliminate invading pathogens (Brinkmann *et al.* , 2004). However, NETs are involved in noninfectious diseases, such as systemic lupus erythematosus, rheumatoid arthritis, atherosclerosis, chronic obstructive pulmonary disease, and diabetes (Jorch & Kubes, 2017; Uddin*et al.* , 2019). Moreover, NET–microparticle complexes are potent inducers of neutrophil recruitment (Wang *et al.* , 2019). Pedrazza*et al.* (2017) also suggested that excessive NET release causes damage to lung tissues; the authors also reported that the survival rate increased in their LPS-induced ALI model after NET formation was inhibited. NETs may be cytotoxic to pulmonary endothelium and epithelium directly and may have a crucial role in both tumour dissemination and defence (Twaddell *et al.* , 2019). In addition, NET formation and ROS production are both triggered and regulated by SFKs (Nani *et al.* , 2015). In the current study, Bletinib significantly attenuated NET formation and release (Figure 4) and had therapeutic effects in our murine LPS-induced ALI model—it considerably ameliorated haemorrhage, protease release, pulmonary interstitial oedema, and Ly6G$^+$citH3$^+$ neutrophil infiltration*in vivo* (Figure 9). Therefore, we focused on investigating whether Bletinib attenuates oxidative stress, NET formation, and ALI by inhibiting SFK phosphorylation and activity.

SFKs are nonreceptor tyrosine kinases initially known for their role in malignant tumour progression; their

potential in immune system regulation has been discovered in the past decade (Kovacs *et al.* , 2014; Parsons & Parsons, 2004). The phosphorylation of cellular proteins by SFKs, including Src, Fgr, Hck, and Lyn, occurs immediately after neutrophil activation. SFKs also play a critical role in modulating several effector functions of neutrophils, such as ROS production, degranulation, and NET formation (Ear *et al.* , 2017). In particular, during cerebral ischaemia, Src is responsible for inducing the upregulation of the activity of ERK and its target transcription factors (Hu *et al.* , 2009). In the current study, Bletinib inhibited the phosphorylation of ERK, JNK, SFKs, Src, Lyn, Hck, and Fgr (Figures 5 and 6A) and directly suppressed their enzymatic activities (Figure 6C) in fMLF-activated human neutrophils.

Btk is a Tec family nonreceptor tyrosine kinase; by contrast, Vav is a Rho family guanine nucleotide exchange factor that regulates NADPH oxidase activation. Chen *et al.* (2015) reported that Btk is one of the regulatory proteins in the downstream signalling pathway of SFKs and Vav activation is modulated by both SFKs and Btk. Our current results indicated that Bletinib abolished Btk and Vav phosphorylation (Figure 6B), indicating that Bletinib regulates the downstream cellular functions through the SFKs–Btk–Vav signalling pathway.

SFKs, which may regulate neutrophil responses to fMLF stimulation, are essential for β_2 integrin–mediated neutrophil adhesion and transmigration (Baruzzi *et al.* , 2008; Evangelista *et al.* , 2007; Sarantos *et al.* , 2008). In the present study, Bletinib abrogated human neutrophil adhesion to bEnd.3 cells and fMLF-induced transmigration of neutrophils (Figure 7).

Mac-1 integrin is crucial in mediating neutrophil recruitment, including adhesion and transmigration (Lee *et al.* , 2019; Li *et al.* , 2018; Sule *et al.* , 2019; Wolf *et al.* , 2018). Therefore, we investigated whether Bletinib affects integrin expression in neutrophils. Our results demonstrated that Bletinib significantly reduced surface expression of CD11b and CD18 in neutrophil (Figure 8), indicating that Bletinib regulates neutrophil adhesion and transmigration by inhibiting SFK phosphorylation and activity and Mac-1 surface expression.

In conclusion, to our knowledge, this is the first study to demonstrate Bletinib, a natural compound extracted from *B. formosana* , as a SFK inhibitor: Bletinib strongly inhibits superoxide anion production, ROS production, degranulation, NET formation, adhesion, transmigration, and CD11b/CD18 integrin expression in activated human neutrophils, all mediated by inhibiting the phosphorylation and enzymatic activity of SFKs. In addition, in our murine LPS-induced ALI model, Bletinib demonstrated a therapeutic effect, suggesting the potential of Bletinib to be a novel therapeutic of choice for ARDS.

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Figure 1

Bletinib suppresses superoxide anion production in stimulated human neutrophils. (A) Chemical structure of Bletinib. Neutrophils (6×10^5 cells mL^{-1}) were incubated with 0.1% DMSO (control) or 0.3–10 μM Bletinib for 5 min before activation with chemoattractants. Superoxide anion production was induced by (B) fMLF, (C) MMK-1, (D) NaF, or (E) PMA in the presence of CB and then measured using ferricytochrome c reduction method. (F) Cytotoxicity was assessed using the percentage of total LDH released. (G) Superoxide scavenging capacity of Bletinib was assessed in a xanthine/xanthine oxidase cell-free system. WST-1 reduction was measured through spectrophotometry at 450 nm, with 20 U mL^{-1}superoxide dismutase as the positive control. All data are shown as mean ± SEM (n = 6). $^*p < 0.05$ versus the control group.

Figure 2

Bletinib reduces ROS production in fMLF-stimulated neutrophils. (A) Human neutrophils labelled with DHR123 were pretreated with 0.1% DMSO (control) or 0.1–3 μM Bletinib, activated with 0.1 μM fMLF, and then monitored through flow cytometry. (B) Intracellular ROS levels in neutrophils represented as mean ± SEM fluorescence intensity of DHR123. (C) Neutrophils were incubated with 0.1–3 μM Bletinib or 0.1% DMSO for 5 min with or without 0.1 μM fMLF. The change in chemiluminescence was monitored on

an ELISA reader in real time. (D) Peak chemiluminescence is shown as mean ± SEM (n = 5). *$p < 0.05$ versus the control group.

Figure 3

Bletinib attenuates NE release in activated neutrophils. Neutrophils were treated with 0.1–10 μM Bletinib or DMSO for 5 min, and NE release was induced by (A) fMLF, (B) MMK-1, (C) NaF, or (D) LTB4 along with the NE substrate and then evaluated spectrophotometrically. Data are expressed as mean ± SEM (n = 6). *$p < 0.05$ versus the control group.

Figure 4

Bletinib reduces NET formation in PMA-stimulated neutrophils. Human neutrophils were pretreated with DMSO or 1–10 μM Bletinib for 10 min and then incubated with or without 10 nM PMA for 3 h. (A) NET formation was quantified using Sytox green, a nucleic acid stain. Neutrophils were stained with antibodies against NE (red) or MPO (green) and then analysed through (B) confocal microscopy and (C) scanning electron microscopy. DNA was detected using Hoechst 33342 (blue). Representative images are shown. Data are expressed as mean ± SEM (n = 5). *$p< 0.05$ versus the control group.

Figure 5

Bletinib inhibits ERK, JNK, and SFK (Y416) phosphorylation in fMLF-stimulated neutrophils. Neutrophils were preincubated with DMSO or 10 μM Bletinib and then stimulated with 0.1 μM fMLF. All the immunoblotting experiments were performed under consistent conditions. Immunoblots for (A) SFKs, (B) Akt, (C) ERK, (D) JNK, and (E) p38 and the related quantifications are presented (mean ± SEM, n = 5). *$p< 0.05$ versus the control group.

Figure 6

Bletinib inhibits SFK phosphorylation and enzymatic activity. Phosphorylation of (A) SFKs, namely Src, Lyn Fgr, and Hck, and (B) downstream proteins, Btk and Vav, was determined through immunoblotting. (C) The ADP-Glo kinase assay kit was used to evaluate the enzymatic activity. Src, Lyn, Fgr, or Hck (1.5 ng mL^{-1}) was incubated with DMSO, 1–10 μM Bletinib, or 0.1–3 μM PP2, and then 125 μM ATP (substrate) was added to the reaction mixture for 60 min, which was followed by enzymatic activity detection. Data are expressed as mean ± SEM (n = 5). *$p < 0.05$ versus the control group.

Figure 7

Bletinib inhibits adhesion and transmigration of fMLF-activated human neutrophils. Hoechst 33342-labelled neutrophils (10^6cells mL^{-1}) were treated with DMSO or 1–10 μM Bletinib for 5 min, followed by no stimulation or stimulation with 0.1 μM fMLF/1 μg mL^{-1} CB for another 5 min. Neutrophils were then incubated with bEnd.3 cells at 37 °C for 30 min. The neutrophils adherent on bEnd.3 cells were detected and enumerated through fluorescent microscopy. (A) Representative histograms of fluorescent microscopy. (B) Adherent neutrophils: enumeration and quantification. (C) Human neutrophils were treated with DMSO or 1–10 μM Bletinib for 5 min in the top of the chemotaxis chamber and then were either not activated or activated with 0.1 μM fMLF for another 90 min. Migrated neutrophils were measured using a cell counter. Data are expressed as mean ± SEM (n = 5). *$p < 0.05$ versus the control group.

Figure 8

Bletinib decreases CD11b (integrin α_M) and CD18 (integrin β_2) expression in fMLF-activated neutrophils. Neutrophils were incubated with DMSO or 1–10 μM Bletinib for 10 min and then either not stimulated or stimulated with 0.1 μM fMLF/1 μg mL^{-1} CB for another 5 min. The fluorescence intensity of FITC-labelled antibodies against (B) CD11b and (D) CD18 was detected through flow cytometry. Data are shown as mean ± SEM (n = 6). *$p< 0.05$ versus the control group.

Figure 9

Bletinib mitigates LPS-induced ALI in mice. BALB/c mice were treated with the vehicle (DMSO) or 25 mg kg^{-1} Bletinib through intraperitoneal injection followed by intratracheal spraying of LPS after 5 h. (A) MPO activity and (B) total protein concentration in lung tissues were measured 6 h after the LPS spraying. (C) Light microscopy images of H&E-stained, MPO-positive, and Ly6G-positive lung sections. (D) Immunofluorescence images of DAPI-, Ly6G-, and citH3-positive lung sections (n = 5). $*p < 0.05$ versus the control group, $\#p < 0.05$ versus the vehicle group.

Figure 1

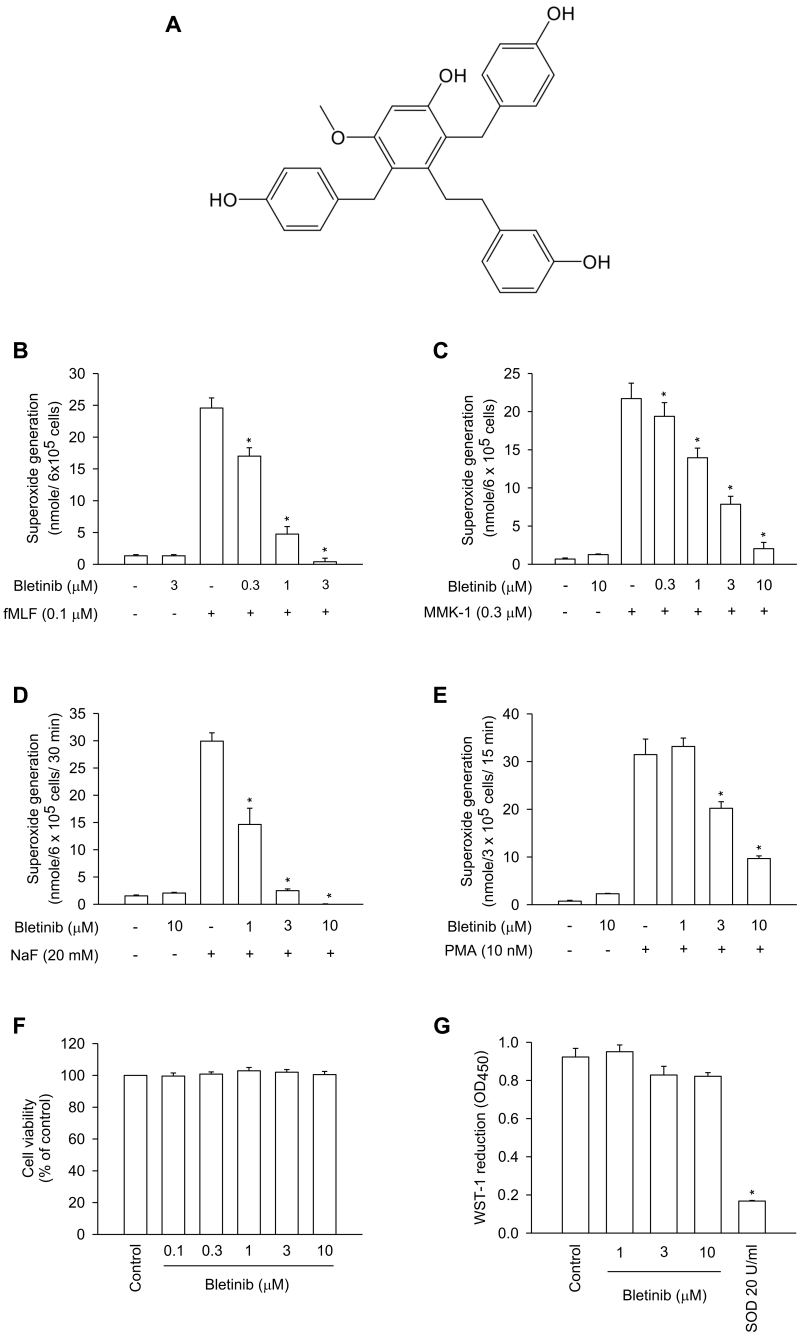
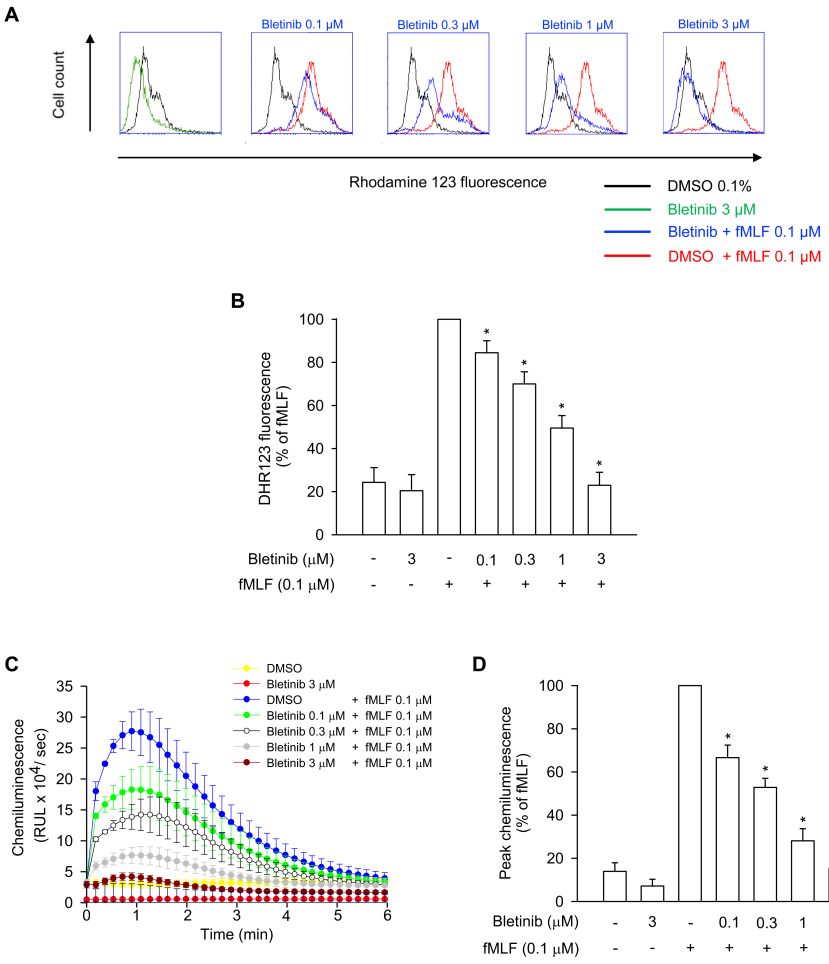


Figure 2



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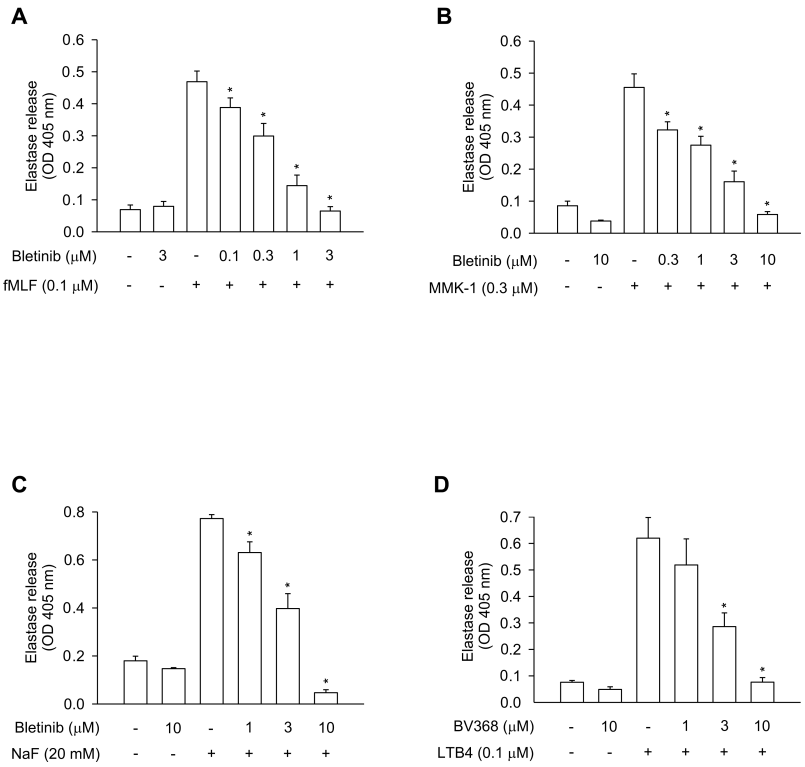

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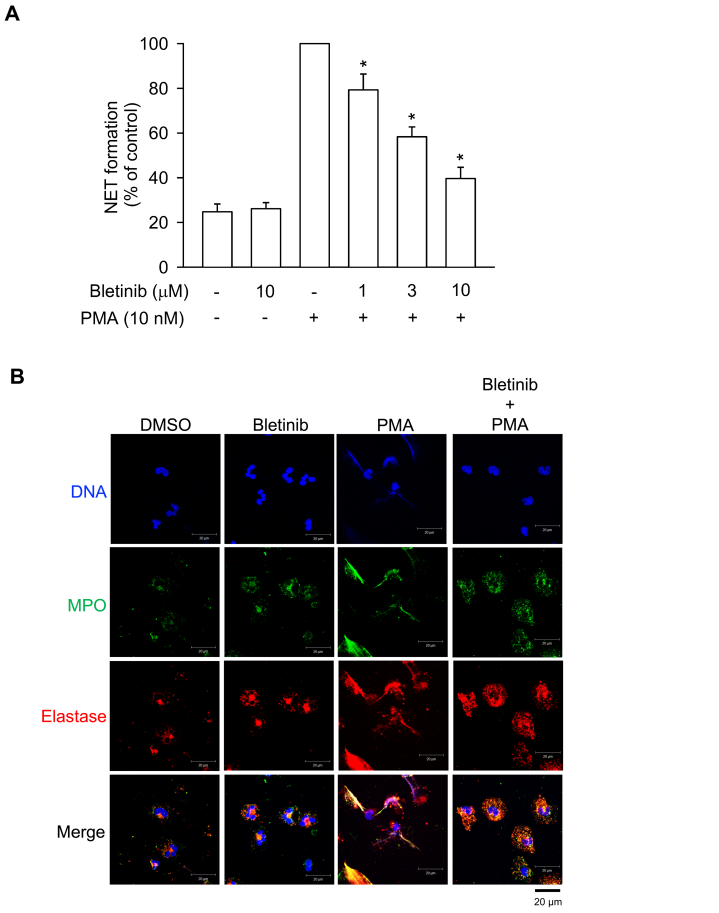

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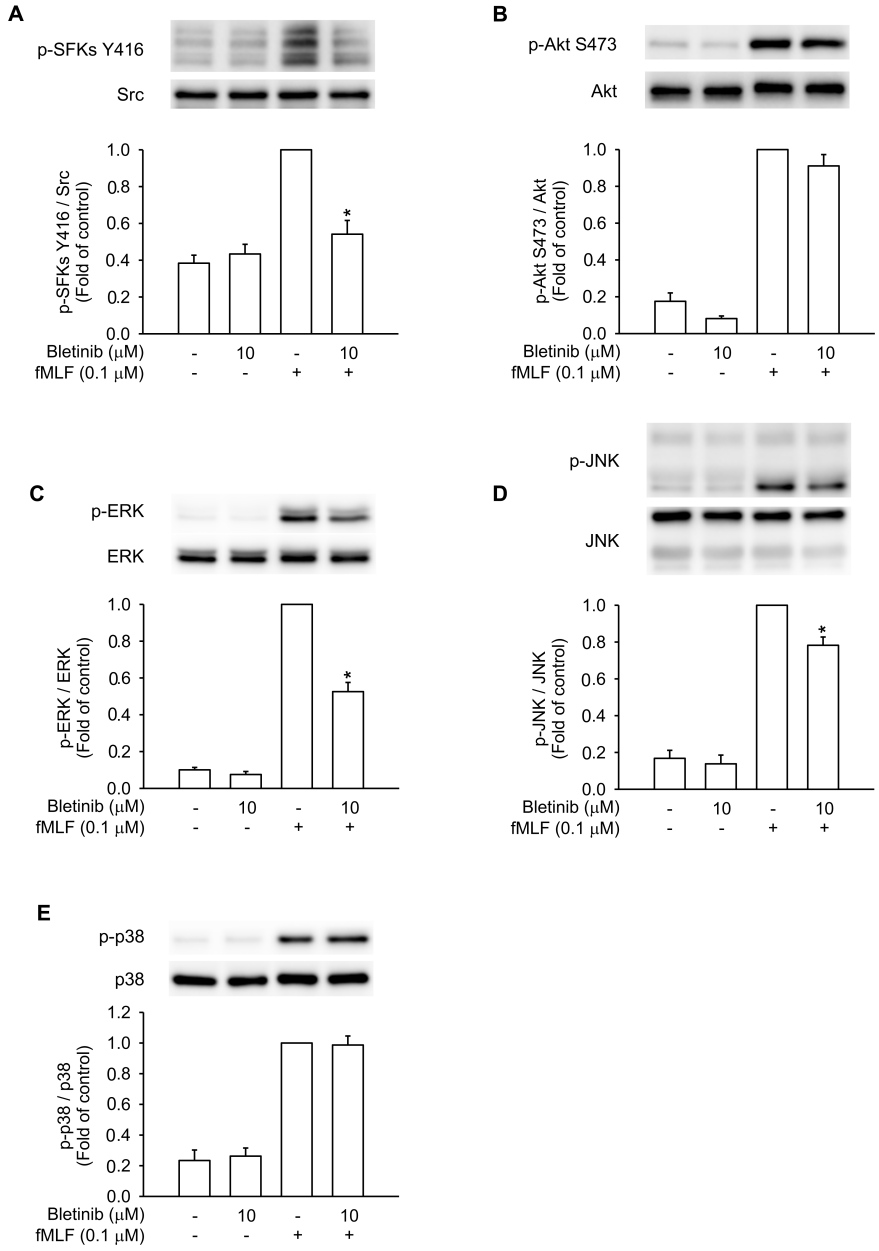

Figure 6

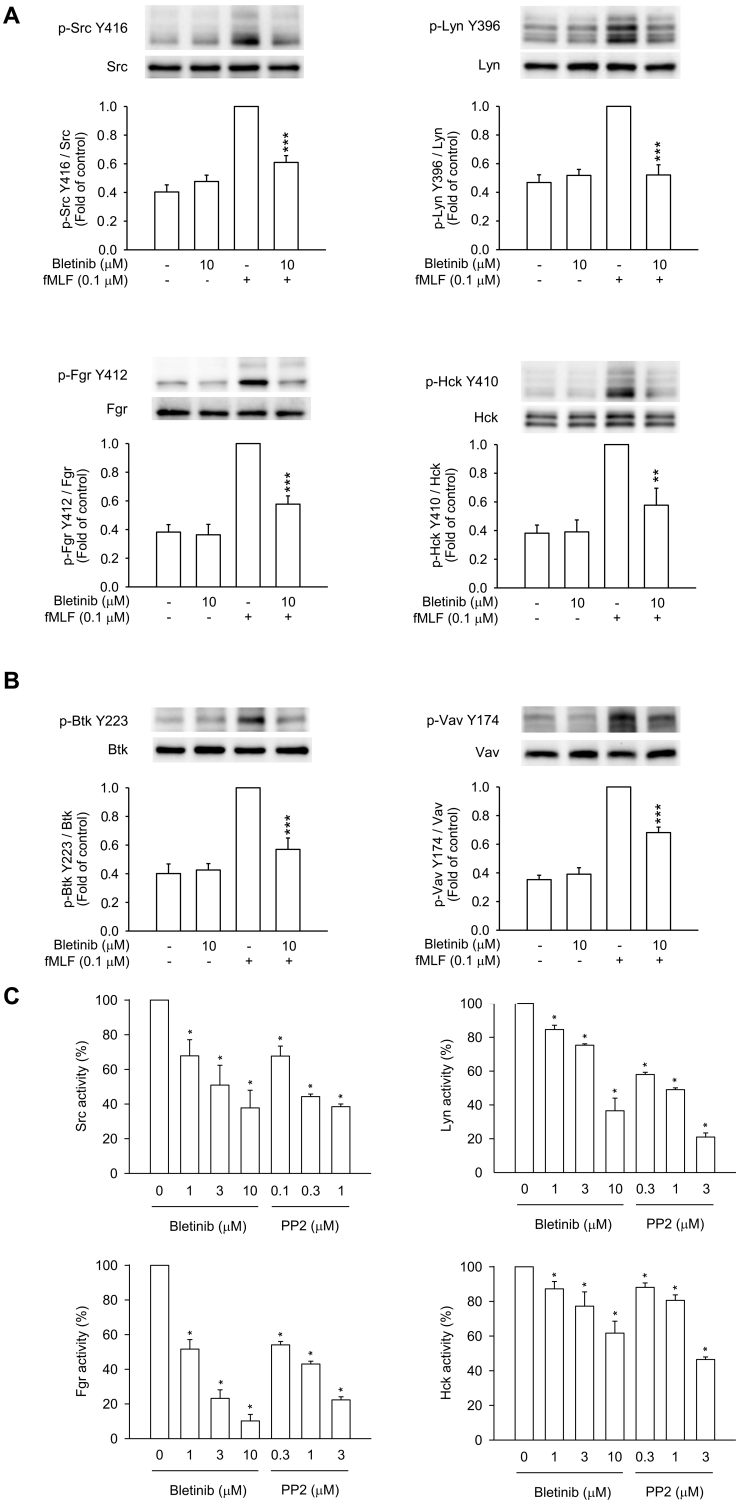

Figure 7

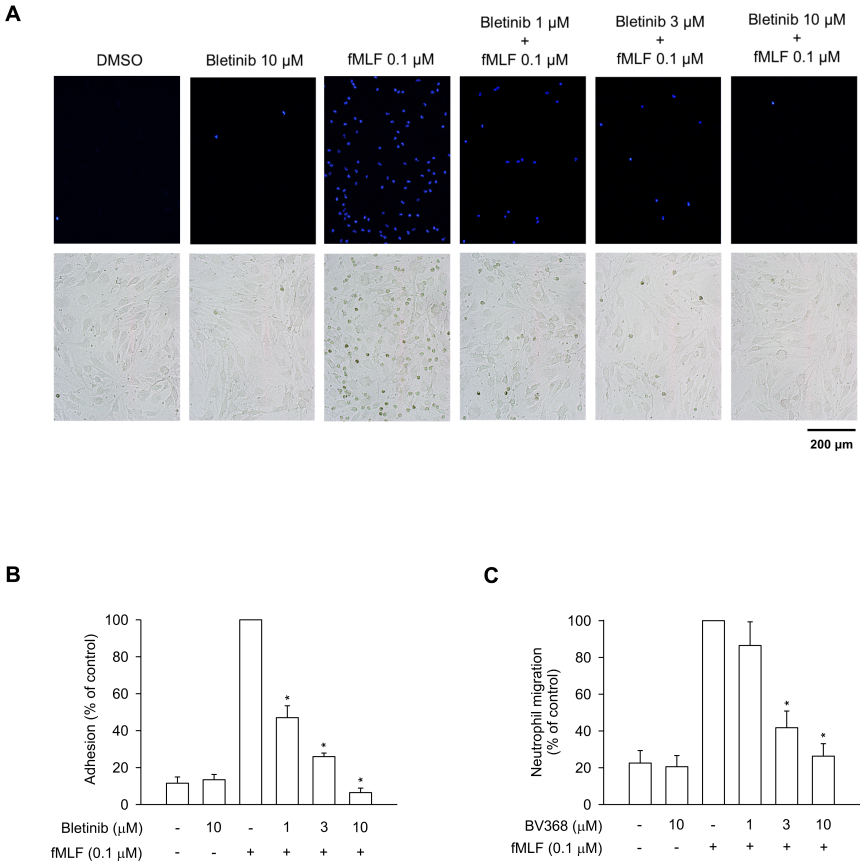

Figure 8

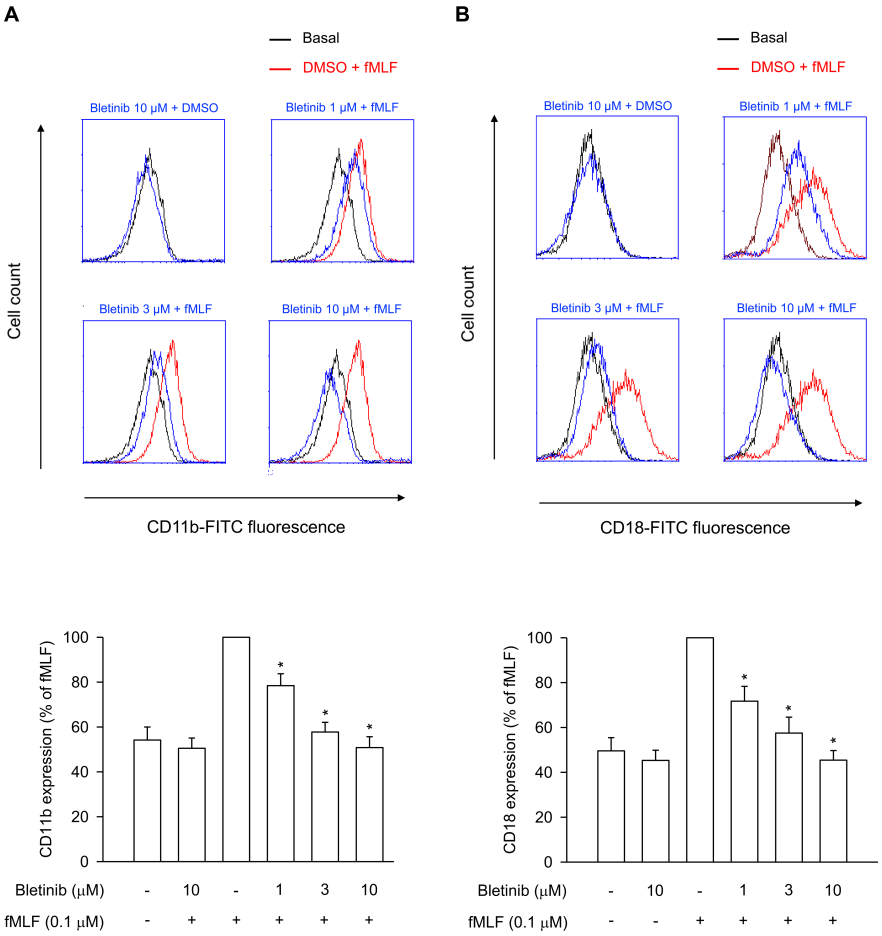

Figure 9