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

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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, extracted from the Bletilla plant, exhibits anti-inflammatory, antibacterial, and antimitotic effects. In this study, we evaluated the therapeutic effects of bletinib in human neutrophilic inflammation and LPS-mediated ALI in mice.

Experimental Approach: In human neutrophils activated with the formyl peptide (fMLP), we assessed integrin expression, superoxide anion production, degranulation, neutrophil extracellular trap (NET) formation, and adhesion through flow cytometry, spectrophotometry, and immunofluorescence microscopy. Immunoblotting was used to measure phosphorylation of Src family kinases (SFKs) and downstream proteins. Finally, a LPS-induced ALI model in male BALB/c mice 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

Abbreviations: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; DHR 123, dihydrorhodamine 123; fMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; PFR, formyl peptide receptor; Mac-1, macrophage-1 antigen; MPO, myeloperoxidase; NE, neutrophil elastase; NET, neutrophil extracellular trap; PMA, phorbol 12-myristate 13-acetate; SFK, Src family kinase.

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the enzymic 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 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: Bletinib regulates neutrophilic inflammation by inhibiting the SFK-Btk-Vav pathway. Bletinib ameliorates LPS-induced ALI in mice. Further biochemical optimisation of bletinib may be a promising strategy for the development of novel therapeutic agents for inflammatory diseases.

KEYWORDS
acute lung injury, acute respiratory distress syndrome, bletinib, inflammation, neutrophil, Src family kinase

1 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 the cell surface of neutrophils (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, ROS, and NETs, resulting in various morbidities, including autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis; Nemeth & Mocsai, 2012), infectious diseases, such as sepsis (Sonego et al., 2016), inflammatory diseases, such as chronic obstructive pulmonary disease (Noguera et al., 2001), atherosclerosis (Soehnlein, 2012) and acute lung injury (ALI; Grommes & Soehnlein, 2011) and other major diseases, including 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 therapeutic procedures 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 LPS, the inhibition of the phosphorylation and activity of Src family kinases (SFKs) and NET formation, respectively, improve clinical condition and increase survival (Grommes & Soehnlein, 2011; Oyaizu et al., 2012; Pedrazza et al., 2017).

The SFKs are non-receptor intracellular protein tyrosine kinases, some of which, including Lyn, Fgr, and Hck, are mainly expressed in human neutrophils (Futosi & Mocsai, 2016). SFKs modulate several 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-L-methionyl-L-leucyl-L-phenylalanine (fMLP) is a chemotactic peptide recognised by neutrophils through their formyl peptide receptors (FPRs), which triggers SFK phosphorylation and thus neutrophil activation (Fumagalli et al., 2007). SFKs regulate the signal transduction in fMLP-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, Xiong, et al., 2016). Bletinib [3,3'-dihydroxy-2',6'-bis(p-hydroxybenzyl)-5-methoxybibenzyl, molecular formula: C29H28O5, MW: 456.538; Figure 1a] is a natural bibenzyl compound 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, using a model of ALI induced by LPS in mice.

2 | METHODS

2.1 | Extraction and purification of bletinib

Bletinib was prepared as described by our group (Lin et al., 2016). The air-dried whole plants of B. formosana (10.0 kg) were extracted with ethanol at 60°C and refluxed for 8 h. The extracts were evaporated to provide 818.0 g (dry weight) of extract. The extract samples were fractionated with Diaion HP-20 column chromatography using a stepwise H2O-ethanol gradient elution to obtain five fractions (BF1–BF5). The BF4 (52.4 g) was refractionated on a silica gel column and eluted with a gradient of CHCl3 and MeOH to obtain 11 fractions (BF4-1–BF4-11). Repeated column chromatography of BF4–7 over silica gel with n-hexane-diisopropyl ether (1:4) yielded 18 fractions (BF4–7-1–BF4–7-18). Purification of BF4–7–11 by column chromatography over silica gel eluted with CHCl3-diisopropyl ether (6:1) to

![Figure 1](image-url)
give bletinib (190 mg). The structure of bletinib was determined by NMR and mass spectroscopic methods (Figure 1a). The purity of bletinib was > 97% (Figure S1). Bletinib was dissolved in DMSO for further experimentation. The control concentration of DMSO used in the cell experiments was 0.1%, which did not affect the parameters measured.

2.2 | Animals

All animal care and the experimental protocols were approved by the Institutional Animal Care and Use Committee of Chang Gung University, Taiwan (IACUC Approval No.: CGU105-060). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020). 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 ± 1 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. Humane endpoints were used to terminate the experiments (by CO₂ asphyxiation) if the weight loss in mice exceeded 20% of body weight, or there was a marked drop in body temperature and heartbeat, no movement in response to handling, or inability to obtain food or water.

2.3 | LPS-induced ALI and mortality model

A total of 24 male BALB/c mice were randomly divided into four groups (6 mice per group): vehicle alone, bletinib alone, LPS control, and bletinib treatment (bletinib + LPS). The mice were starved overnight and then injected i.p. 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⁻¹) or 50 μl of 0.9% saline (in vehicle and bletinib alone group) under general anaesthesia with xylazine (6 mg·kg⁻¹) and Zoletil 50 (tiletamine and zolazepam, 1:1; 30 mg·kg⁻¹). Five hours later, mice were killed (CO₂ asphyxia), the lungs removed and frozen at −80°C or fixed with 10% formalin. The serum levels of glutamic-pyruvic transaminase (GPT), creatinine (CRE), and blood urea nitrogen (BUN) were determined by automated clinical chemistry analyser (Dri-Chem NX500i, Fujifilm®, Japan). The level of blood neutrophils was measured using an automated haematology analyser (XT-1800i, Sysmex Corporation, Kobe, Japan).

For the LPS-induced mortality model, mice were injected i.p. with a single 50-μl dose of LPS (from Escherichia coli O111:B4; 5 mg·kg⁻¹) or 0.9% saline (vehicle alone group). The mice were monitored for 5 days to determine survival.

2.4 | Histological sectioning and immunofluorescence staining

The collected lung tissues were washed with 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) and corresponding antibodies. Then, images were acquired through light microscopy, as described previously (Yuan et al., 2006). The severity of histological lung injury was quantified according to the lung injury scoring system established by the American Thoracic Society in 2011, including (A) neutrophils in the alveolar space, (B) neutrophils in the interstitial space, (C) hyaline membranes, (D) proteinaceous debris filling the airspaces, and (E) alveolar septal thickening (Matute-Bello et al., 2011).

For immunofluorescence staining, tissue sections were incubated with antibodies against H3 (citH3; citrulline R2O17) 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).

2.5 | Analysis of MPO activity and IL-1β levels

The mouse lung tissues were suspended in a 0.5% hexadeoxytrimethylammonium 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).

Total RNA was extracted from mouse lung tissues using homogenization (MagNA Lyser, Roche, Basel, Switzerland) and TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. The mRNA level of IL-1β and GAPDH was determined using iScript cDNA Synthesis Kit, iQ™ SYBR® Green Supermix and quantitative PCR with CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) which was conducted at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 56°C for 30 s. Primers for mouse IL-1β (5′-TGGACCTTTCCAGATGAGCA-3′ and 5′-GTTCATCTCGGAGGCTGTA GTAGT-3′) and mouse GAPDH (5′-AAGGATTGAAGACACCTGGAC-3′ and 5′-GATGGAAATTGTGAG GAGATG-3′) were used.

2.6 | Human neutrophil isolation

The study was conducted with the approval of the Institutional Review Board of Chang Gung Memorial Hospital (IRB No. 201601
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 staining—were then suspended in Ca²⁺-free HBSS (pH 7.4) and stored at 4°C until use (Chen et al., 2014).

### 2.7 Measurement of extracellular superoxide anion production

Extracellular superoxide anion production in activated neutrophils was assessed through the reduction of ferricytochrome c. After incubating the isolated human neutrophils (6 × 10⁵ cells·ml⁻¹) with Ca²⁺ (1 mM) and ferricytochrome c (0.5 mg·ml⁻¹) at 37°C, the cells were then incubated with 0.1% DMSO or 0.3–10 μM bletinib for 5 min. The cells were then pretreated with cytochalasin B (1 or 2 μg·ml⁻¹) for 3 min and then stimulated with fMLP, MMK-1, or sodium fluoride (NaF), or directly activated with 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).

### 2.8 Measurement of intracellular superoxide anion production

Human neutrophils (2.5 × 10⁶ 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 fMLP (0.1 μM) and cytochalasin B (0.5 μg·ml⁻¹) for 15 min. The fluorescence intensity was detected through flow cytometry to evaluate intracellular superoxide anion production of human neutrophils.

### 2.9 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 × 10⁶ cells·ml⁻¹) were preincubated with 6 U·ml⁻¹ 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 stimulation with 0.1 μM fMLP. Chemiluminescence was then detected and analysed in real time on a 96-well chemiluminometer (Tecan Infinite F200 Pro; Männedorf, Switzerland).

### 2.10 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 × 10⁵ cells·ml⁻¹) were incubated with DMSO or bletinib after treatment with 1 mM CaCl₂ and 100 μM NE substrate (methoxy succinyl-Ala-Ala-Pro-Val-p-nitroanilide) at 37°C for 5 min. Cells were stimulated with fMLP with cytochalasin B (0.5 μg·ml⁻¹), LTβ₄ with cytochalasin B (2 μg·ml⁻¹), NaF with cytochalasin B (2 μg·ml⁻¹), or MMK-1, with cytochalasin B (0.5 μg·ml⁻¹) for 10 min before determination of NE release through measuring the change of absorbance at 405 nm on a spectrophotometer.

### 2.11 Analysis of NET formation

#### 2.11.1 Quantification of extracellular DNA

Human neutrophils (10⁶ cells·ml⁻¹) resuspended in HBSS with 2.5 μM Sytox green were incubated with DMSO or bletinib for 10 min and stimulated using 10 nM PMA or 10 μg·ml⁻¹ LPS for 3 h. The fluorescence intensity was quantified on a Tecan Infinite 200 reader at 485–535 nm.

#### 2.11.2 NET photography

Neutrophils (3 × 10⁵ cells·ml⁻¹) were incubated with DMSO or bletinib for 10 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).

### 2.12 Evaluation of neutrophil adhesion

Human neutrophils (10⁶ 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 fMLP with cytochalasin B (1 μg·ml⁻¹) for 10 min before incubation with bEnd.3 cells (ATCC Cat# CRL-2299, RRID:CVCL_0170) 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, Wang, et al., 2016).

2.13 | Analysis of neutrophil migration

A microchemotaxis chamber with 3-μm filters (Millipore) was used to evaluate chemotactic migration of neutrophils. Neutrophils (5 × 10⁶ cells·ml⁻¹) treated with bletinib or DMSO for 5 min were placed in the top chamber, and 0.1 μM fMLP or 0.1 μg·ml⁻¹ IL-8 was added into the bottom chamber. The number of neutrophils that migrated from the top to the bottom chamber after incubation at 5% CO₂ for 1 h was counted on a MoxiZ automatic cell counter (ORFLO).

2.14 | Evaluation of surface CD18 and CD11b expression

Neutrophils (5 × 10⁵ cells·ml⁻¹) were incubated with bletinib or DMSO for 5 min and then activated through incubation with 0.1 μM fMLP, with cytochalasin B (1 μg·ml⁻¹) or 0.1 μg·ml⁻¹ IL-8 with cytochalasin B (1 μg·ml⁻¹) for 5 min. After centrifugation at 200× g for 8 min at 4°C, the cells were resuspended in 5% BSA with FITC-conjugated antibodies against CD18 or CD11b on ice in the dark for 15 min. The fluorescent intensity was then analysed through FACS (Tsai et al., 2017).

2.15 | Immunoblotting of neutrophil lysates

The immunoblotting assay was conducted as described previously (Tsai et al., 2019). The immuno-related procedures used comply with the recommendations made by the British Journal of Pharmacology. In brief, neutrophils incubated with bletinib or DMSO at 37°C for 5 min were stimulated with 0.1 μM fMLP for 30 s. The proteins were separated from neutrophil lysates through electrophoresis (12% SDS-PAGE) 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 quantified using the UVP BioSpectrum Imaging System (Analytik Jena, USA).

2.16 | Assessment of enzymic activity of SFKs

The kinase activity of SFKs was assessed using an ADP-Glo kinase assay kit (Promega, Fitchburg, USA) according to the manufacturer’s instructions, 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 remaining 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).

2.17 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018). All data are presented as box-and-whiskers plots (median, min–max). One-way ANOVA and Dunnett’s multiple comparison tests were employed for all experiments. The survival rate of the mice was analysed using the log-rank (Mantel–Cox) test. All statistical calculations were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Differences with P values <0.05 were considered to show statistically significant effects.

2.18 | Materials

Hanks’ balanced salt solution (HBSS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA), and Ficol-Paque was purchased from GE Health-Care (Little Chalfont, Buckinghamshire, UK). Antibodies against Akt (Cat# 4691, RRID:AB_915783), p-Akt (S473; Cat# 4060, RRID:AB_2315049), ERK (Cat# 4695, RRID:AB_390779), p-ERK (Cat# 4370, RRID:AB_2315112), JNK (Cat# 9258, RRID:AB_2141027), p-JNK (Cat# 9251, RRID:AB_331659), p38 MAPK (Cat# 8690, RRID:AB_10999090), phospho-p38 MAPK (Cat# 4511, RRID:AB_2139682), Src (Cat# 2109, RRID:AB_2106059), p-SFK (Y416; Cat# 2101, RRID:AB_331697), Lyn (Cat# 2732, RRID:AB_10694080), Fgr (Cat# 2755, RRID:AB_2246957), Hck (Cat# 14643, RRID:AB_2687496), Btk (Cat# 8547, RRID:AB_10950506), and p-Btk (Y223; Cat# 5082, RRID:AB_10561017) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against 4-hydroxynonenal (4-HNE; Cat# ab66545, RRID:AB_722490), MPO (Cat# ab9535, RRID:AB_307322), IL-1β (Cat# ab9722, RRID:AB_308765), NE (Cat# ab68672, RRID:AB_1658868), occludin (Cat# ab216327, RRID:AB_2378295), p-Lyn (Y396; Cat# ab226778), p-Hck (Y410; Cat# ab61055, RRID:AB_942255), and histone H3 (citrulline 2 + 8 + 17; Cat# ab5103, RRID:AB_304752) were purchased from Abcam (Cambridge, UK). Antibodies against p-Src (Y416; Cat# 05-677, RRID:AB_309898) were purchased from Merck Millipore (Burlington, MA, USA). Anti-p-Fgr antibodies (Y412; Cat# PAS-64583, RRID:AB_2662495) and FITC-conjugated antibodies against CD11b (Cat# 11-0113-42, RRID:AB_2572437) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies against Vav (Cat# E20-74862) and p-Vav (Y174;
Cat# E011142) were purchased from Enogene Biotech (New York, NY, USA). Moreover, purified anti-mouse Ly6G antibody (Cat# 127602, RRID:AB_1089180) and FITC anti-human CD18 antibody (Cat# 302106, RRID:AB_314224) were obtained from BioLegend (San Diego, CA, USA). Tocris Bioscience (Ellisville, MO, USA) supplied MMK-1 and LTβR. Sigma-Aldrich (St. Louis, MO, USA) supplied cytochalasin B, fMLP and PP2; IL-8 was supplied by ProSpec (Rohovot, Israel). Xylazine was supplied by KVP Pharma und Veterinärprodukte GmbH (Kiel, Germany) and Zoletil 50 by Virbac (Carros, France).

2.19 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (http://www.guidetopharmacology.org) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos et al., 2019; Alexander, Fabbro et al., 2019a, 2019b).

3 | RESULTS

3.1 | Bletinib decreases 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 decreased superoxide anion production by fMLP-activated human neutrophils in a concentration-dependent manner (IC₅₀ = 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 S2A-C). Furthermore, bletinib did not exhibit ROS scavenging activity in the cell-free xanthine oxidase system (Figure S2D) or cytotoxicity (Figure S3).

**FIGURE 2** Bletinib reduces NET formation in PMA-stimulated neutrophils. Human neutrophils were pretreated with 0.1% DMSO or 1–10 μM bletinib for 10 min and then incubated with or without 10 nM PMA. (a) NET formation was quantified using Sytox green, a nucleic acid stain.
(b) Neutrophils were stained with antibodies against NE (red) or MPO (green) and then analysed with confocal microscopy. DNA was detected using Hoechst 33342 (blue).
(c) Scanning electron microscopy images of neutrophils. Representative images are shown. All data are shown as box (interquartile range) and whiskers (min, max) plots, with medians; n = 5. *P < 0.05, significantly different from the DMSO + fMLP group.
Chemiluminescence and flow cytometry assay were performed to determine if bletinib affects ROS production in stimulated neutrophils. 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 1c). Moreover, the results of flow cytometry, using DHR123, revealed that bletinib significantly suppressed intracellular ROS production in fMLP-activated neutrophils (Figures 1d and S2E).

3.2 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 release of NE by fMLP-stimulated human neutrophils (IC50 = 0.53 ± 0.07 μM) without affecting resting neutrophils (Figures 1e and S4A). In addition, bletinib down-regulated NE release from neutrophils stimulated with MMK-1, NaF or LTB4 in a concentration-dependent manner (Figures S4 and S5, respectively).

3.3 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 and 10 μg/ml 1 LPS. Fluorescent spectrometry assay results demonstrated that NET formation, induced by PMA and LPS, was significantly reduced by bletinib (Figures 2a and S6). In addition, scanning electron microscopy and immunofluorescent staining demonstrated the presence of NETs and neutrophils co-stained with Hoechst 33342 and antibodies against MPO and NE in the NETs (Figure 2b,c).

3.4 Bletinib suppresses ERK and SFK phosphorylation in fMLP-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 SFKs, Akt, ERK, JNK, and p38 in activated neutrophils. Our immunoblotting results demonstrated that the phosphorylation of SFKs, Akt (S473), ERK, JNK, and p38 is enhanced in fMLP-stimulated neutrophils. However, bletinib significantly inhibited the phosphorylation of ERK, SFKs, Src (Y416), Lyn (Y396), Fgr (Y412), Hck (Y410), Btk (Y223), and Vav (Y174) but not that of Akt, JNK, and p38 (Figures 3 and 4a–f).

3.5 Bletinib inhibits SFK activity

SFKs are non-receptor 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. The cell-free ADP-Glo kinase assay results demonstrated that both bletinib and PP2, a selective inhibitor of SFKs (Hanke et al., 1996), inhibited the kinase activity of Src, Fgr, Hck, and Lyn, in a concentration-dependent manner (Figure 4g–j).
3.6 | 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 fMLP, we incubated the Hoechst 33342-labelled neutrophils (10⁶ cells·ml⁻¹) with bEnd.3 cells in 37°C for 30 min. Neutrophils adherent to bEnd.3 cells were detected and counted by fluorescence microscopy and our results demonstrated that bletinib inhibited the adhesion function of fMLP-activated neutrophils (Figure 5a,b). We also used a chemotaxis chamber and a cell counter to quantitate the number of migrating neutrophils and found that bletinib significantly reduced fMLP-induced transwell migration of neutrophils (Figure 5c). Furthermore, IL-8 serves as a chemotactic factor for the attraction of neutrophils. IL-8-induced neutrophil migration was also inhibited by bletinib (Figure S7A).

3.7 | Bletinib reduces Mac-1 expression in activated neutrophils

Mac-1 is a complement receptor composed of CD11b (integrin αM) and CD18 (integrin β2) 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 fMLP-stimulated human neutrophils (Figure 6). Furthermore, the CD11b expression induced by IL-8 was decreased by bletinib in human neutrophils (Figure S7B).

### 3.8 Bletinib alleviates LPS-induced ALI and mortality in mice

Endotoxins such as LPS are the most common causes of ALI in bacterial infection and inflammatory diseases (Chen et al., 2019; 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 (25 mg·kg⁻¹) or DMSO administered i.p., followed by intratracheal spraying of LPS for 5 h. The exterior photos and HE-stained histopathological features of lungs revealed that LPS induced haemorrhage and erythema, interalveolar septal thickening, and pulmonary interstitial oedema formation. Infiltration of inflammatory MPO- and Ly6G-positive cells infiltration (specific markers of neutrophils), protease release (NE), cytokine production (IL-1β), oxidative stress–induced lipid peroxidation (4-HNE), vascular permeability (occludin), MPO activity, and total protein levels were observed after LPS administration. Distortion of the pulmonary architecture was significantly suppressed in the bletinib treatment group (Figures 7, S8, and S9A). Furthermore, LPS-activated phosphorylation of Vav (p-Vav) was also attenuated by bletinib (Figure 7). Furthermore, LPS-induced NET formation (Ly6G⁺citH3⁺ cell accumulation) was considerably reduced after bletinib treatment (Figure 8a). Bletinib did not alter the serum level of GPT, GOT, CRE, and BUN in mice (Figure S9B), suggesting that bletinib treatment did not cause toxicity to liver or kidney. The therapeutic ability of bletinib to increase survival was further observed in LPS-primed mice. BALB/c mice were injected with LPS (5 mg·kg⁻¹), and their survival rate was monitored for 5 days. All mice in the LPS group died within 2 days, whereas the bletinib-treated mice (25 mg·kg⁻¹) exhibited significantly prolonged survival after LPS injection (log-rank test; Figure 8b).

### 4 DISCUSSION

The plant *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...
The 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, and in the progression of infectious and inflammatory conditions. For instance, during bacterial infection, neutrophils are recruited by chemotaxis, to inflamed tissues by endotoxin components, such as fMLP 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 is cytotoxic and damages host tissue (Fridovich, 1986). For instance, although ROS is an essential component of the inflammatory 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, Li, et al., 2016), and diabetes mellitus and related complications (He, Fang, et al., 2016; 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 have a high mortality 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 a number of experimental ALI models in mice 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 therapeutic agents to treat the life-threatening diseases listed above (Boeing et al., 2020; Chen et al., 2015; Hwang et al., 2015; Liao et al., 2015).

In the present study, bletinib significantly decreased superoxide anion production (Figures 1b and S2), ROS production (Figure 1c,d), and degranulation (Figures 1e and S5) in stimulated human neutrophils. However, bletinib did not exhibit either superoxide scavenging potential (Figure S2D) in the xanthine/xanthine oxidase cell-free system or cytotoxicity (Figure S3). 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 also involved in non-infectious conditions, 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, as inhibition of NET formation increased survival in their LPS-induced ALI model. 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). Bletinib significantly reduced NET formation and release (Figures 2 and S6) and had therapeutic effects in our LPS-induced ALI model in mice. Haemorrhage, protease release, pulmonary interstitial oedema formation, cytokine production, oxidative stress accumulation, tight junction destruction, SFKs signalling activation, NET formation, and neutrophil infiltration were all significantly decreased in vivo (Figures 7, 8, and S8). Therefore, we focused on investigating whether bletinib attenuates oxidative stress, NET formation, and ALI by inhibiting SFK phosphorylation and activity.

SFKs are non-receptor tyrosine kinases initially known for their role in malignant tumour progression and their potential in regulation of the immune system has emerged 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 up-regulation of the activity of ERK and its target transcription factors (Hu et al., 2009). In the current study, bletinib inhibited the phosphorylation of SFKs, including Src, Lyn, Hck, and Fgr, and directly suppressed their enzymic activities in fMLP-activated human neutrophils (Figures 3 and 4).

Btk is a Tec family non-receptor TK and, by contrast, Vav is a Rho family guanine nucleotide exchange factor that regulates activation of
NADPH oxidase. 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 4e,f), indicating that bletinib regulates the downstream cellular functions through the SFKs–Btk–Vav signalling pathway. Bletinib was particularly inhibitory on the responses induced by fMLP than those induced by other activators (Figures 1, S2, and S5). Therefore, our data suggest that SFKs may make a greater contribution to the neutrophil activation mediated by agonists of FPR1. However, we cannot rule out the possibility that bletinib interacts with other FPR1-mediated signal pathways.

SFKs, which may regulate neutrophil responses to fMLP stimulation, are essential for β2 integrin–mediated neutrophil adhesion and transmigration (Baruzzi et al., 2008; Evangelista et al., 2007; Sarantos et al., 2008). In the present study, bletinib abolished human neutrophil adhesion to bEnd.3 cells and fMLP-induced transmigration of neutrophils (Figure 5). 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 neutrophils (Figure 6) and neutrophil infiltration in mice (Figure 7), indicating that bletinib regulates neutrophil adhesion and transmigration by inhibiting SFK phosphorylation and activity and Mac-1 surface expression. Additionally, the number of circulating neutrophils is increased by LPS in mice, and this effect was suppressed by bletinib treatment (Figure S9C), suggesting that bletinib may prevent the release of neutrophils from bone marrow.

In conclusion, to our knowledge, this is the first study to demonstrate bletinib, a natural compound extracted from B. formosana, is 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 the inhibition of the phosphorylation and enzymic activity of SFKs. In addition, in our murine LPS-induced ALI model, bletinib exhibited therapeutic effects, suggesting the potential of bletinib as a novel therapeutic agent of choice for ARDS.

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AUTHOR CONTRIBUTIONS

K.T.I., P.J.C., Y.H.W., H.H.T., and S.H.C. designed/performed experiments and analysed the data. S.H.Y. and Y.T.L. helped to analyse the data. T.S.W. provided the bletinib. K.T.I., P.J.C., and T.L.H. wrote and completed the manuscript. T.L.H. supervised the entire study.

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 and Analysis, Immunoblotting and Immunohistochemistry, Natural Products Research and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

CONFLICT OF INTERESTS

All authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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SUPPORTING INFORMATION

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