



Biapenem reduces sepsis mortality via barrier protective pathways against HMGB1-mediated septic responses

Jaehong Kim¹ · Samyeol Choo² · Hyunchoe Sim² · Moon-Chang Baek³ · Jong-Sup Bae² 

Received: 13 August 2020 / Revised: 15 December 2020 / Accepted: 16 December 2020
© Maj Institute of Pharmacology Polish Academy of Sciences 2021

Abstract

Background As a late mediator of sepsis, the role of high mobility group box 1 (HMGB1) has been recognized as important, and suppression of HMGB1 release and restoration of vascular barrier integrity are regarded as potentially promising therapeutic strategies for sepsis. For repositioning of previously FDA-approved drugs to develop new therapies for human diseases, screening of chemical compound libraries, biological active, is an efficient method. Our study illustrates an example of drug repositioning of Biapenem (BIPM), a carbapenem antibiotic, for the modulation of HMGB1-induced septic responses.

Methods We tested our hypothesis that BIPM inhibits HMGB1-induced vascular hyperpermeability and thereby increases the survival of septic mouse model from suppression of HMGB1 release upon lipopolysaccharide (LPS)-stimulation. In LPS-activated human umbilical vein endothelial cells (HUVECs) and a cecal ligation and puncture (CLP)-induced sepsis mouse model, antiseptic activity of BIPM was investigated from suppression of vascular permeability, pro-inflammatory proteins, and markers for tissue injury.

Results BIPM significantly suppressed release of HMGB1 both in LPS-activated HUVECs (upto 60%) and the CLP-induced sepsis mouse model (upto 54%). BIPM inhibited hyperpermeability (upto 59%) and reduced HMGB1-mediated vascular disruptions (upto 62%), mortality (upto 50%), and also tissue injury including lung, liver, and kidney in mice.

Conclusion Reduction of HMGB1 release and septic mortality by BIPM (in vitro, from 5 to 15 μ M for 6 h; in vivo, from 0.37 to 1.1 mg/kg, 24 h) indicate a possibility of successful repositioning of BIPM for the treatment of sepsis.

Keywords Biapenem · HMGB1 · Endothelium · Sepsis

Abbreviations

ALT Alanine transaminase
AST Aspartate transaminase
BIPM Biapenem

BUN Blood urea nitrogen
CAM Cell adhesion molecule
CLP Cecal ligation and puncture
ELISA Enzyme-linked immunosorbent assay
ERK Extracellular signal-regulated kinase
HMGB1 High mobility group box
HUVEC Human umbilical vein endothelial cell
IL Interleukin
LDH Lactate dehydrogenase
LPS Lipopolysaccharide
MAPK/SAPK Mitogen-activated protein kinase/stress-activated protein kinase
MOF Multi-organ failure
MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF- κ B Nuclear factor kappa B
TNF Tumor necrosis factor
ZGR Zingerone

Jaehong Kim and Samyeol Choo contributed equally.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s43440-020-00212-0>.

✉ Jong-Sup Bae
baejs@knu.ac.kr

¹ Department of Biochemistry, College of Medicine, Gachon University, Incheon 21999, Republic of Korea

² College of Pharmacy, CMRI, Research Institute of Pharmaceutical Sciences, Kyungpook National University, 80 Daehak-ro, Buk-gu, Daegu 41566, Republic of Korea

³ Department of Molecular Medicine, CMRI, School of Medicine, Kyungpook National University, Daegu 41944, Republic of Korea

Introduction

Despite recent advances in antibiotic therapies and intensive care against severe infections, sepsis still remains as a common cause of morbidity and mortality from overwhelming systemic inflammatory responses [1]. The development of multi-organ failure (MOF) is the cause of sepsis-induced mortality [2, 3]. Notably, most patients die from MOF rather than oxygen deficiencies resulted from profound life-threatening hypoxemia [1]. Although sepsis affects lung and kidney most commonly [2], acute kidney injury results in systemic damages to multiple organs including brain, heart, lung, gut, spleen, and liver, from a systemic inflammatory response [3]. Presently, the pathogenesis of sepsis is still rather complex to understand and intervene easily. Endotoxins are known to be partly responsible for the septic progression from stimulation and induction of macrophages and/or monocytes to serially secrete various pro-inflammatory mediators including early (e.g., interleukin [IL]-1 β and tumor necrosis factor [TNF]- α) and late (e.g., high mobility group box 1 [HMGB1]) ones [4, 5]. For in vitro and in vivo studies with regard to severe vascular inflammation, LPS, an endotoxin, has been used as a useful research tool [6, 7]. HMGB1 protein, released by both damaged cells and activated immune cells, is an important mediator in sepsis [8]. Blood levels of HMGB1 plateau at 24–36 h in animal models of sepsis, differently from early cytokines plateau [8, 9]. HMGB1 has been suggested as a critical late mediator of lethal sepsis from the finding that antibodies neutralizing HMGB1 confer protection against lethal endotoxemia and sepsis even when administered 24 h after the onset of sepsis [8, 10]. Therefore, therapeutic agents inhibiting the release of HMGB1 from damaged cells and activated immune cells may be applicable for the treatment of lethal systemic inflammatory diseases.

To identify HMGB1 modulating compounds, we selected 327 drug candidates associated with pulmonary inflammation from our study of repositioning FDA-approved drugs (1163 in total). Further selection of the compounds was achieved with a high-content screening system (PerkinElmer Operetta, Waltham, Mass) and we found biapenem (BIPM) as a novel HMGB1 modulating compound [11, 12]. BIPM is a carbapenem antibiotic with a broad spectrum of antibacterial activities against many Gram-negative and Gram-positive bacteria, including species producing β -lactamases [13, 14]. Here, we report the protective effects of BIPM on HMGB1-mediated vascular barrier disruption in vitro and in vivo.

Materials and methods

Cell culture and reagents

All of Zingerone (ZGR, a positive control for anti-inflammatory effects [15–17] at 0.7 mg/kg for in vivo assay and 20 μ M for in vitro assay), LPS (L5293), crystal violet, Evans blue, MTT, penicillin G and streptomycin, 2-mercaptoethanol, and DMSO were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Primary human umbilical vein endothelial cells (HUVECs) from Cambrex Bio Science (Charles City, IA, USA) were maintained with a previously established method [18, 19] and used at cell culture passages 3–5 in all experiments. DMSO was used as the vehicle control or for preparation of BIPM or ZGR. BIPM was obtained from Abcam (Cambridge, MA, USA) and human recombinant HMGB1 was purchased from Abnova (Taipei City, Taiwan).

In vitro cell–cell adhesion assay

First, a monolayer of HUVEC was stimulated with HMGB1 (1 μ g/ml) for 16 h and then incubated with ZGR at 20 μ M or increasing concentrations of BIPM for 6 h. Neutrophils (3×10^5 cells/well) were labeled with Vybrant DiD dye and then added on top of a layer of stimulated HUVECs. The relative levels of labeled cells added were spectrometrically quantified from the fluorescence intensity (total signal) of Vybrant DiD dye with a microplate reader instrument (Tecan, GmbH, Grödig, Austria). After neutrophils and HUVECs were incubated for 1 h, four times gentle washing with PBS was done to remove non-adherent cells and then the residual fluorescence intensity (adherent signal) was reassessed. The percentage of adherent neutrophils to HUVECs was determined with the following formula: % adherence = (adherent signal/total signal) $\times$ 100.

In vitro cell viability assay

The challenged HUVECs were incubated with BIPM for 48 h and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine cell viability [20–22].

In vitro competitive enzyme-linked immunosorbent assay for HMGB1

A competitive enzyme-linked immunosorbent assay (ELISA) determined HMGB1 concentrations in mouse serum or the cell culture media [23].

In vitro ELISAs for total or phosphorylated p38 MAPK/SAPK, NF- κ B, TNF- α , ERK 1/2, IL-1 β , and IL-6

The phosphorylation or total level of p38 mitogen-activated protein kinase/stress-activated protein kinase (MAPK/SAPK), (Cell Signaling Technology, Danvers, MA, USA), representing the activation of p38 MAPK/SAPK pathway, was determined with an ELISA kit. The total and phosphorylation levels of the extracellular signal-regulated kinase (ERK) 1/2 (R&D Systems, Minneapolis, MN, USA), p65 subunit of nuclear factor kappa B (NF- κ B) (Cell Signaling Technology, Danvers, MA, USA) in the nuclear lysates, and the levels of IL-1 β , IL-6, and TNF- α in the cell culture supernatants were analyzed with recommended ELISA kits.

In vitro expression levels of cell adhesion molecules (CAMs) and HMGB1 receptors

With whole-cell ELISAs, the expression levels of intercellular CAM (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin were analyzed as follows [16]. Stimulation with HMGB1 (1 μ g/ml) for either 16 h (to analyze the level of ICAM-1 and VCAM-1) or 22 h (to analyze the level of E-selectin) was given to monolayers of HUVECs grown confluent, and then BIPM or ZGR was administered. The HUVECs were fixed, washed and mixed with mouse anti-human monoclonal antibodies against E-selectin, VCAM-1, and ICAM-1 (Chemicon Temecula, CA, USA), and incubated at 37 °C for 1 h in 5% CO₂. Lastly, the incubated products were washed, mixed with peroxidase-conjugated anti-mouse IgG antibody (Sigma) for 1 h, washed, and then treated with *o*-phenylenediamine substrate for subsequent colorimetric enzyme assay. The expression levels of TLR2, TLR4, and RAGE were determined with specific antibodies (H-80 and A-9), respectively (Santa Cruz Biotechnology Inc., Dallas, TX, USA).

In vitro cell migration assay

Human neutrophils migration potency toward HUVECs was determined as follows [16]. The migration potency was analyzed with Transwell plates, containing filters with a pore size of 8 μ m. To obtain confluent endothelial monolayers, the HUVECs were cultured for 3 days. HUVECs were stimulated with HMGB1 (1 μ g/ml) for 16 h, and then incubated with ZGR at 20 μ M or increasing concentrations of BIPM for 6 h. Before the addition of neutrophils to the upper compartment, the Transwell plates were incubated at 37 °C for 2 h and the floating or non-migrated neutrophils and HUVECs in the upper chamber and on filter membrane were removed. Neutrophils migrated to the other side of the filter were fixed and stained. We counted nine randomly chosen high-power microscopic fields (200X) for quantification. All

the experiments were duplicated *per* well on duplicate wells and the results are presented as migration index.

In vitro permeability assay

For in vitro analysis of endothelial cell permeability with increasing dose of BIPM or ZGR at 20 μ M, the change in flux of Evans blue-bound albumin across functional HUVEC monolayers was spectrophotometrically measured, as described previously [16].

Isolation of cytoplasmic and nuclear extracts and Western blot analyses

The cell pellets were obtained by centrifugation and with their cytoplasmic and nuclear extracts were prepared on ice was conducted, as previously described [24]. As the loading controls for the cytoplasmic and nuclear extracts, the antibodies for lamin B and actin were used, respectively.

Animals and the cecal ligation and puncture (CLP) procedure

6–7-week old male C57BL/6 mice (Body weight: 27 g) were obtained from Orient Bio Co. (Sunngnam, Republic of Korea). 7 days after purchase, the animal models of CLP-induced sepsis were also prepared as previously described [23]. For sham control animals, their ceca were exposed but not punctured or ligated. 24 h after, a BIPM compound with a dose of 0.15, 0.37, 0.73, or 1.1 mg/kg or ZGR of 0.7 mg/kg was intravenously injected and the effects of BIPM on HMGB1 secretion, cell permeability and leukocyte migration were evaluated. Alternatively, a BIPM compound of 0.37 or 1.1 mg/kg or ZGR of 0.7 mg/kg was intravenously injected at 12 and 50 h after CLP and survival rate was assessed (Fig. 5). Our animal protocol was pre-approved by the Animal Care Committee at Kyungpook National University (IRB No. KNU 2017-102).

In vivo H&E staining and histopathological examinations

Male mice ($n=5$) were subjected to CLP and then intravenously given with BIPM (0.37 or 1.1 mg/kg) or ZGR (0.7 mg/kg) at 12 and 50 h after. The mice were sacrificed at day 4. Blinded analyses of lung specimens were done to evaluate pulmonary architecture, infiltration of inflammatory cells, and tissue edema [15]. We classified the results into four grades: Grade 1 for normal histopathology; Grade 2 for minimally infiltrated neutrophils; Grade 3 for moderately infiltrated neutrophils and formation of perivascular edema and partial destruction of pulmonary architecture; and Grade

4 for densely infiltrated neutrophils, formation of abscess, and the complete obliteration of pulmonary architecture.

In vivo leukocyte migration assay

Male mice were anesthetized with 2% isoflurane (Forane; JW Pharmaceutical, Seoul, South Korea) in oxygen that was first administered in a breathing chamber and then via facemask via a small rodent gas anesthesia machine (RC2; Vetequip, Pleasanton, CA, USA). The *in vivo* migration potency of leukocytes was analyzed [15, 25]. The mice were intravenously given with HMGB1 (2 µg/mouse) for 16 h and then given an *i.v.* injection of ZGR (0.7 mg/kg) or BIPM (0.15, 0.37, 0.73, or 1.1 mg/kg). Mice were sacrificed after 6 h and peritoneal fluids were obtained from washing of their peritoneal cavities with normal saline (5 ml). The obtained peritoneal fluids (20 µl) were stained with 0.38 ml of Turk's solution and leukocytes were counted under a light microscope.

In vivo measurement of CXCR1/2, IL-8/CXCL2, CCR2 and CCL2 and tissue injury markers

Neutrophils isolation and expression levels of CXCR1 and CXCR2 were measured as described previously [26]. Monocytes isolation and expression levels of CCR2 were measured as described previously [27] and CCL2 was measured by commercial ELISA kits (R&D Systems). The plasma levels of blood urea nitrogen (BUN), creatinine, alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH) were determined with commercial kits (Pointe Scientific, Lincoln Park, MI, USA).

In vivo permeability assay

Male mice were first intravenously injected with HMGB1 (2 µg/mouse) for 16 h and then BIPM (0.15, 0.37, 0.73, or 1.1 mg/kg) or ZGR (0.7 mg/kg). Vascular permeability was represented as µg of the Evans blue dye leaked into the peritoneal cavity *per* mouse and determined with a standard curve, as previously described [16].

Statistical analysis

Data are represented as mean ± standard deviation (SD) from three independent experiments, otherwise noted. ANOVA and Tukey's post-hoc tests were performed to make comparisons between the LPS-, CLP-, or HMGB1-only treated group and tested BIPM or ZGR each group. *p* values < 0.05 were considered as statistical significance. For evaluation of the CLP-induced sepsis outcomes, the Kaplan–Meier curve was made to compare differences in survival (Suppl. Table 1). SPSS 22.0 (IBM Corporation, Armonk, NY,

USA) was used for statistical analysis. Data distribution was assessed using Shapiro–Wilk test. If Shapiro–Wilk test results had a *p* value greater than 0.05, the data were assumed to be normally distributed.

Results

BIPM inhibits HMGB1 release in LPS-stimulated HUVECs and CLP-induced sepsis mouse model

We investigated whether LPS-mediated HMGB1 release was regulated by BIPM treatment. After stimulation with LPS, HUVECs were treated with BIPM with increasing concentrations of 1, 2, 5, 10, or 15 µM or ZGR (20 µM) for 16 h, and released amounts of HMGB1 were analyzed with an ELISA. Concordant with a previous finding that LPS induces release of HMGB1 [7], our results showed that secretion of HMGB1 secretion was significantly stimulated with LPS treatment with HUVECs. However, BIPM treatment inhibited the HMGB1 secretion with LPS (Fig. 1a). Zingerone (ZGR) was used as a positive control for anti-inflammatory effects with BIPM administration [15–17]. To analyze the inhibitory effects of BIPM *in vivo*, mice were given *i.v.* injections of BIPM or ZGR and then sacrificed at 12 h and 24 h after CLP, respectively. CLP-induced HMGB1 secretion was significantly reduced with BIPM injection (Fig. 1b). Next, the effects of the BIPM on the expression levels of TLR2, TLR4, and RAGE, well-known HMGB1 receptors, were determined. In HUVECs, BIPM reduced the expression levels of TLR2, TLR4, and RAGE, induced by HMGB1 (Fig. 1c). Unlike the results of this study, a few papers showed that HMGB1 did not affect TLR2/TLR4 expressions [28]. However, the majority of opinions on the effect of HMGB1 on its receptor expression are that HMGB1 increases the expression of TLR2 and TLR4 as well as RAGE [8, 29–32]. To explain the difference between the TLR receptors expressed by HMGB1 or not, it has been proposed that three potential reasons for the lack of a strong correlation between mRNA and protein expression levels are: (1) translational regulation, (2) differences in protein half-lives, and (3) the significant amount of experimental error, including differences with respect to the experimental conditions [33–35]. Cell viability with the administration of BIPM was evaluated from MTT assays with HUVECs. The BIPM did not affect the viability of cells that were treated with increasing concentrations of BIPM (10, 20, or 50 µM) for 48 h, indicating its lack of toxicity (Fig. 1d). Taken together, our data show that BIPM may be effective for early interventions to prevent release of HMGB1 and progression to severe sepsis and resultant septic shock. Detailed statistical information was supplied in the Supplementary Table 1.

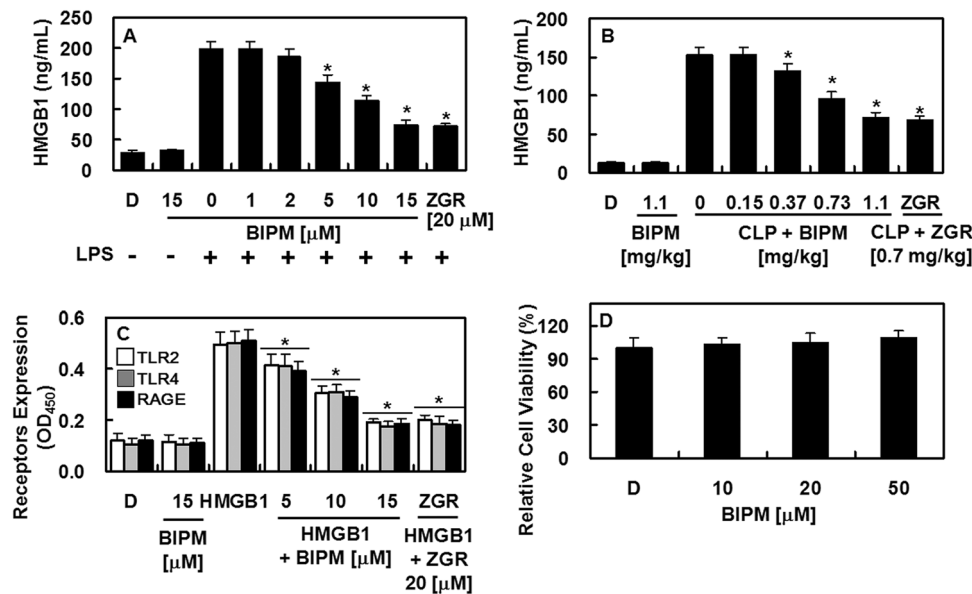


Fig. 1 BIPM on release of HMGB1 and the expression levels of HMGB1 receptors. The effects of BIPM on HMGB1 release by LPS activation (100 ng/mL, 16 h) in HUVECs (**a**), from CLP surgery in mice (**b**, $n=5$) were determined with an ELISA. **c** The effects of BIPM on the expression levels of TLR2 (white bar), TLR4 (gray bar), and RAGE (black bar) were assayed with an ELISA. **d** The effects of BIPM on cellular viability were assayed with MTT assay. Data are

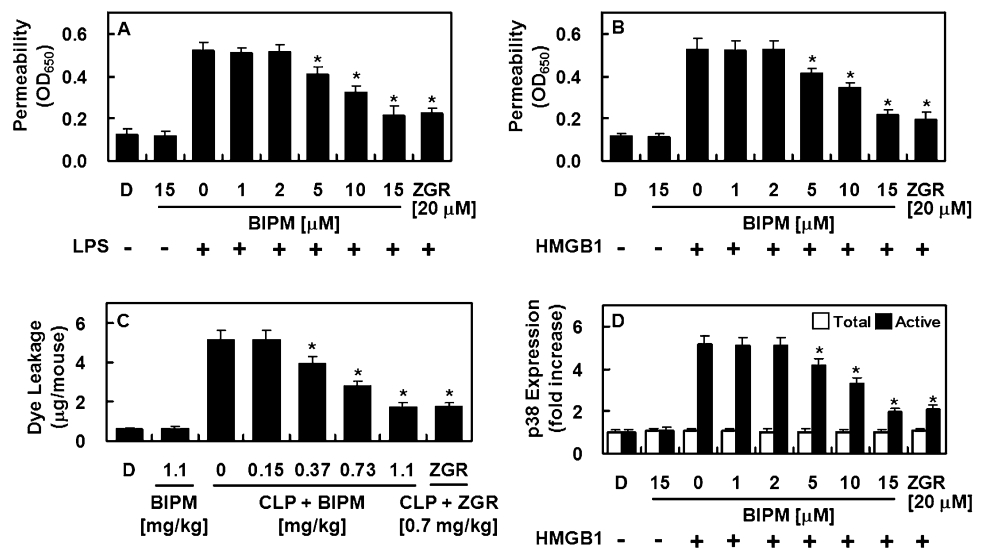
shown as mean $\pm$ SD values from three independent experiments conducted in triplicate on different days. 0.2% DMSO (**d**) was used as the vehicle control. Data distribution was assessed using Shapiro–Wilk test. One-way ANOVA and Tukey’s post-hoc tests were performed, except **c**. Two-way ANOVA and Turkey’s post-hoc tests were performed for **c**. * $p < 0.05$ versus treated with LPS alone (**a**), CLP alone (**b**), or HMGB1 alone (**c**)

BIPM inhibits HMGB1-mediated disruption of vascular barrier

LPS and HMGB1 disrupt the integrity of vascular barriers [36]. We determined to analyze vascular permeability to evaluate the protective potencies of BIPM to maintain the integrity of vascular barrier in HUVECs. HUVECs were treated with either LPS (100 ng/ml) for 4 h (Fig. 2a) or HMGB1 (1 μg/ml) for 16 h (Fig. 2b), and then treatment

of BIPM (1, 2, 5, 10, 15 μM) or ZGR (20 μM) for 16 h was given. The changes in barrier integrity were assessed from monitoring of the flux of Evans blue-bound albumin across the monolayer of HUVECs. BIPM suppressed both of LPS and HMGB1-mediated hyperpermeability represented by increased flux of Evan blue-bound albumin (Fig. 2a and b). The effects of BIPM were also confirmed in vivo. Male mice were intravenously given BIPM or ZGR at 12 h after CLP and were then sacrificed after additional 12 h and

Fig. 2 Effects of BIPM on HMGB1-induced permeability in vitro and in vivo. The effects of BIPM on LPS- (**a**), HMGB1- (**b**) or CLP- (**c**) induced permeability in HUVECs (**a**, **b**) or mice (**c**, $n=5$) were determined. **d** The effects of BIPM on HMGB1-mediated total or phosphorylation levels of p38 were determined with an ELISA. Data are expressed as mean $\pm$ SD values of three independent experiments on different days. One-way ANOVA and Tukey’s post-hoc tests were performed. * $p < 0.05$ versus treated with LPS alone (**a**), HMGB1 alone (**b**, **d**) or CLP alone (**c**)



vascular permeability were assayed by measuring amounts of Evans blue dye in peritoneal washings. CLP-induced hyperpermeability was reduced by the administration of BIPM (Fig. 2c). Noting that the vascular disruptive response caused by HMGB1 is mediated by the phosphorylation of p38 [37, 38], HUVECs were stimulated with HMGB1, treated with BIPM (1, 2, 5, 10, 15 M) or ZGR (20 μ M) for 16 h and then the effects of BIPM or ZGR on the phosphorylation level of p38 were assayed with an ELISA. HMGB1 increased the phosphorylation of p38, which was suppressed by BIPM (Fig. 2d). The reductions in HMGB1-mediated barrier disruption, increased permeability, and phosphorylation of p38 suggest an anti-septic potential of BIPM. Detailed statistical information was supplied in the Supplementary Table 1.

Effects of BIPM on expression of CAMs, adhesion of neutrophils and migration of leukocytes mediated by HMGB1

HMGB1 increases surface expression levels of various endothelial CAMs, including E-selectin, ICAM-1, and VCAM-1, required for the migration of leukocytes to the site of inflammation across the vascular endothelium. Thus, we analyzed the regulation of CAMs in HUVECs, adherence of human neutrophils to HUVECs, and transmigration of neutrophils through the HUVEC monolayers, mediated by HMGB1. Stimulation with HMGB1 (1 μ g/ml) for 16 h and subsequent treatment with BIPM or ZGR for 6 h were given to HUVECs. BIPM (Fig. 3a) dose-dependently reduced the expression levels of CAMs, upregulated with HMGB1. BIPM also reduced the adherence of human neutrophils to, and their subsequent transmigration into HUVECs (Fig. 3b, c, and i). Our *in vivo* experiment that inhibition of the HMGB1-induced peritoneal migration of leukocytes with BIPM confirmed these results (Fig. 3d). To confirm the potential inhibitory effects of BIPM on CLP-induced migration of leukocytes, we next determined the effects of BIPM on the expressions of the corresponding chemokines and chemokine receptors *in vivo*. Data showed that chemokines for neutrophils, such as IL-8 and CXCL2, were up-regulated by CLP, which was reversed by BIPM (Fig. 3e) and their chemokine receptors, such as CXCR1 and CXCR2, were down-regulated by CLP, which was reversed by BIPM (Fig. 3f). In addition, chemokine for monocytes, such as CCL2 (Fig. 3g) and its chemokine receptor, such as CCR2 (Fig. 3h), were up-regulated by CLP, which was reversed by BIPM. Thus, our data shown that BIPM reduced the adhesion and migration of neutrophils/leukocytes, enhanced by treatment of HMGB1. Detailed statistical information was supplied in the Supplementary Table 1.

BIPM suppresses NF- κ B/ERK signaling and TNF- α , IL-1 β , and IL-6 production

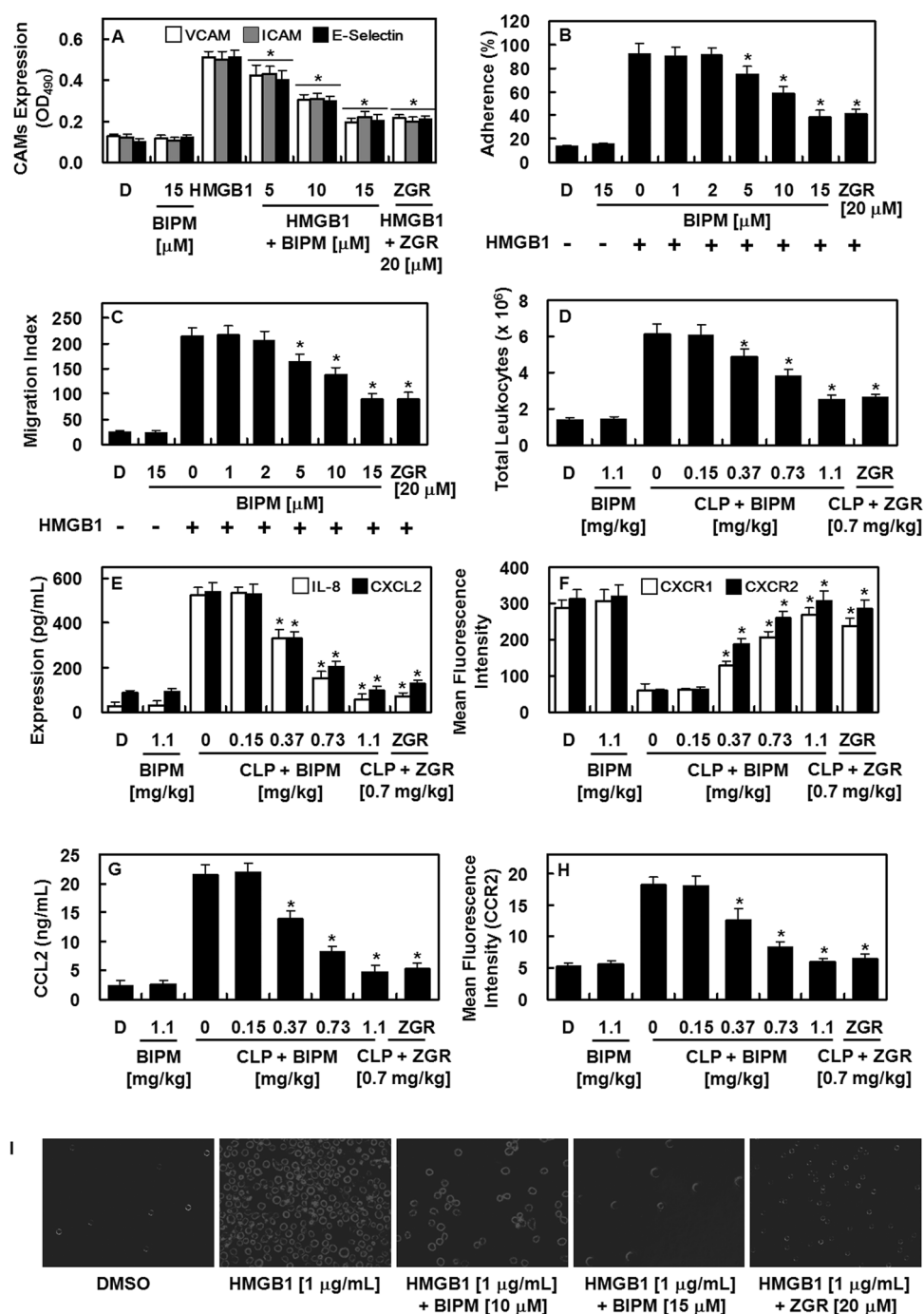
HMGB1 aggravates sepsis by inducing the production of inflammatory cytokines: TNF- α , IL-1 β , and IL-6, through various signaling pathways including NF- κ B and ERK 1/2 [23, 39, 40]. Thus, we analyzed the suppressive effects of BIPM on the production of inflammatory cytokines: TNF- α , IL-1 β , and IL-6, induced by HMGB1, and also on the activation of the NF- κ B and ERK 1/2 signaling. HUVECs were stimulated with HMGB1 (1 μ g/ml, 16 h) and then treated with BIPM for 16 h. We show that both the production of inflammatory cytokines and activation of NF- κ B and ERK 1/2 signaling, enhanced by HMGB1 in HUVECs, were inhibited by treatment with BIPM (Fig. 4a–f). Additionally, HMGB1 induced the nuclear localization of p65 NF- κ B in HUVECs, which was suppressed by treatment with BIPM (Fig. 4g). Detailed statistical information was supplied in the Supplementary Table 1.

Effects of BIPM on the survival rate and tissue injury in sepsis model mice

Next, we treated mice with BIPM following CLP surgery to assess the protective effects of BIPM against mortality from CLP-induced sepsis. We injected BIPM (0.37 or 1.1 mg/kg) or ZGR (0.7 mg/kg) at 12 and 50 h after CLP and, for 132 h after CLP, the survival rates of the animals were evaluated every 12 h. This double administration regimen for BIPM significantly improved the survival rates of mice after CLP ($p < 0.00001$; Fig. 5a). Our data show that single injection BIPM at 12 h after CLP significantly reduced HMGB1 levels (Fig. 1b) and that double administration of BIPM was required to inhibit HMGB1-mediated inflammatory responses to increase survival rate of mice. Our data show that BIPM may be applicable for the control of sepsis.

The systemic inflammation during septic progression can induce MOF, with the liver and kidney being the major target organs [41]. The potentially protective effects of BIPM against CLP-induced lung injury were also analyzed. BIPM ameliorated the CLP-induced lung edema as well as severe impairment of lung tissue (Fig. 5b and c). CLP increased both of the plasma levels of ALT and AST (Fig. 5d), markers for hepatic injury, and of BUN and creatinine (Fig. 5e and f), markers for renal injury. However, BIPM suppressed all of these increases and also the increase of LDH, an important marker of tissue injury (Fig. 5g). Noting that AST-to-ALT ratio (AST/ALT ratio) is elevated in sepsis or septic shock and concerning the massive elevation of the AST/ALT ratio indicated a predominantly damage of liver tissue [42–44], we next determined the effects of BIPM on elevated AST/ALT ratio by CLP. As shown in Fig. 5d (insert number), elevated AST/ALT ratio (3.38) by CLP was reduced by

Fig. 3 BIPM on HMGB1-mediated pro-inflammatory responses. The effects of BIPM on the HMGB1-induced **a** expression levels of E-selectin (black bar), VCAM-1 (white bar), and ICAM-1 (gray bar) in HUVECs, **b**, **e** adherence of neutrophils to HUVEC monolayers, and **c** transmigration of neutrophils through HUVEC monolayers were analyzed. **d** The effects of BIPM on CLP-induced migration of leukocytes into the peritoneal cavities of mice were measured from counting of leukocytes in peritoneal fluids. **e**, **f** Expressions of IL-8, CXCL2, CXCR1, or CXCR2 in purified neutrophils were measured as described in method sections. **g**, **h** Expressions of CCL2 or CCR2 in purified monocytes were measured as described in method sections. **i** Shown are representative images from three independent experiments on different days with similar results in **c**. Data are expressed as mean $\pm$ SD values of three independent experiments on different days. One-way ANOVA and Tukey's post-hoc tests were performed. * $p < 0.05$ versus treated with HMGB1 alone (**a–c**) or CLP alone (**d–h**)



BIPM (2.74, 0.37 mg/kg; 1.67, 1.1 mg/kg). Detailed statistical information was supplied in the Supplementary Table 1.

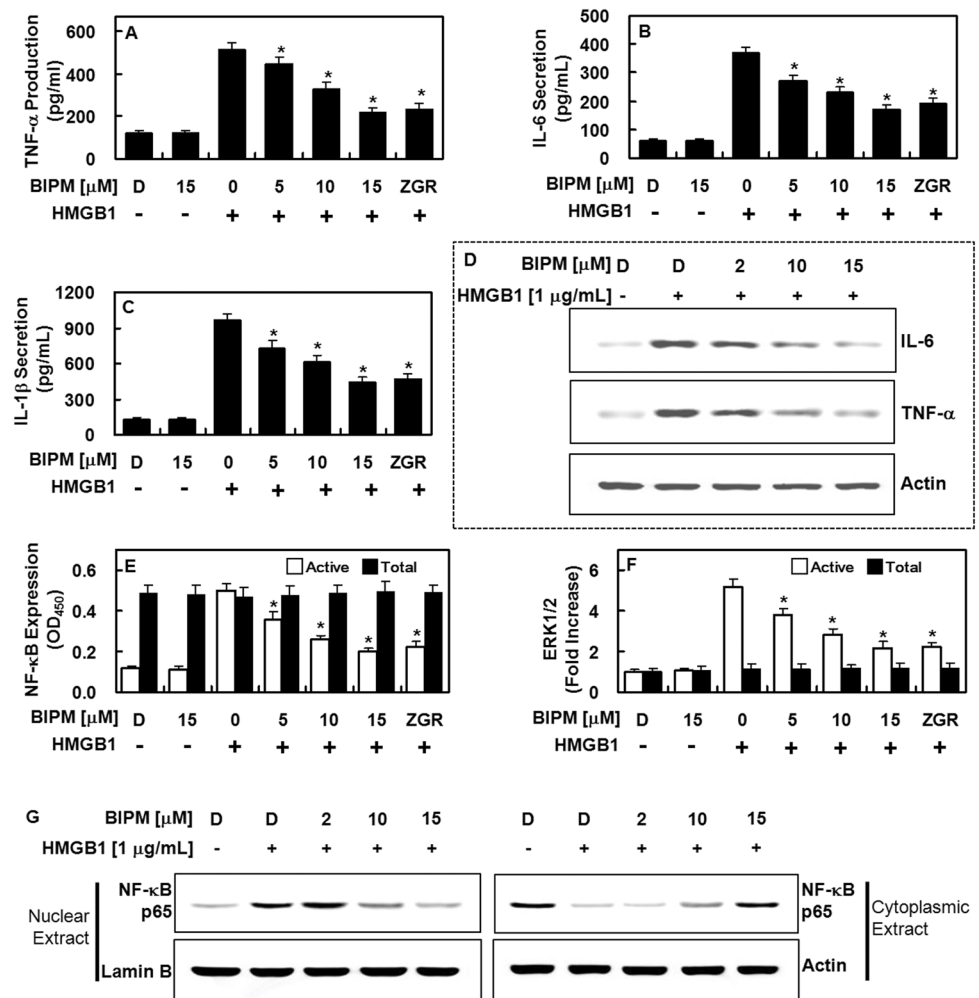
Discussion

We evaluated the protective effects of BIPM against the vascular barrier-disruptive responses notable in septic conditions. The disruption of vascular barrier integrity, in septic responses, is a primary and important process. It can result

in abnormal, serious systemic edema and vascular hyperpermeability, notably found in septic patients [45]. Thus, the maintenance of vascular homeostasis and the restoration of vascular barrier integrity after the destructive responses are important therapeutic goals for sepsis.

Suppression of HMGB1 release (Fig. 1a and b) and expression level of HMGB1 receptors, such as TLR2, TLR4, and RAGE (Fig. 1c), and HMGB1-mediated hyperpermeability (Fig. 2a–c), via the inhibition of p38 signaling activation (Fig. 2d) may be, at least partly, responsible for the

Fig. 4 BIPM on the production of TNF- α , IL-6, and IL-1 β and the activation of the NF- κ B/ERK1/2 pathways mediated by HMGB1. The effects of BIPM on the production of TNF- α (a, d), IL-6 (b, d), and IL-1 β (c) in the HUVECs mediated by HMGB1 were shown with ELISAs (a–c) or Western blot analyses (d, e, f). The effects of BIPM on the HMGB1-mediated activation of e NF- κ B p65 (white bar for phosphor-p65 and black bar for total p65) and of f ERK1/2 (white bar for phosphor-ERK1/2 and black bar for total ERK1/2) in HUVECs was analyzed. g Subcellular levels of NF- κ B in nuclear and cytoplasmic fractions were evaluated with Western blot analyses; actin and lamin B were used as loading controls for the cytoplasmic and nuclear extracts, respectively. Data are expressed as mean $\pm$ SD values of three independent experiments on different days. One-way ANOVA and Tukey's post-hoc tests were performed. * p < 0.05 versus treated with HMGB1 alone



molecular mechanism of the anti-inflammatory effects of BIPM against HMGB1-mediated septic responses. Additionally, BIPM inhibited the interaction between leukocytes and endothelial cells from the suppression of the surface levels of CAMs, such as E-Selectin, VCAM, and ICAM (Fig. 3).

The response of leukocytes to chemokines is an important event in various inflammatory responses [26]. Our data indicate that both migration of leukocytes into peritoneal cavity (Fig. 3d and i) and levels of chemokines involved in the recruitment of leukocytes to inflammatory sites were significantly reduced with BIPM or ZGR treatment (Fig. 3e, g, and h). Bacterial products, such as LPS, inhibit the expression of leukocyte chemokine receptors during sepsis and down-regulation of chemokine receptors of leukocytes may be relevant to the impairment of antibacterial functions of leukocytes during sepsis [26]. The expression levels of chemokine receptors, CXCR1 and CXCR2, were reported to be down-regulated by activation of Toll-like receptors 2 and 4 with LPS treatment [46]. Indeed, CXCR1 and CXCR2 were down-regulated in our CLP animal model and treatment of BIPM or ZGR

restored the levels of CXCR1 and CXCR2 (Fig. 3f). Our findings indicate that our BIPM treatment alleviated the inflammatory process.

From suppression of HMGB1 release, BIPM effectively downregulated the production of inflammatory cytokines TNF- α , IL-6, IL-1 β (Fig. 4a–c), the activation of the inflammatory transcriptional factors NF- κ B and ERK1/2 (Fig. 4e and f) and the translocation of NF- κ B from the cytosol to the nucleus (Fig. 4g), all induced by HMGB1 and this suppression of HMGB1 release and resultant downregulation of multiple inflammatory cascades appears to be the underlying mechanisms of the anti-inflammatory effects of BIPM.

We demonstrated that BIPM reduced HMGB1 release in LPS-activated HUVECs, suppressed the CLP-mediated release of HMGB1, and alleviated HMGB1-mediated vascular barrier disruption by increasing the integrity of barrier. Furthermore, the barrier-protective effects of BIPM were confirmed using an in vivo mouse model of sepsis, in which treatment with BIPM reduced CLP-induced mortality and multiple organ injuries. Our findings indicate that BIPM might be a potential candidate for the treatment of severe

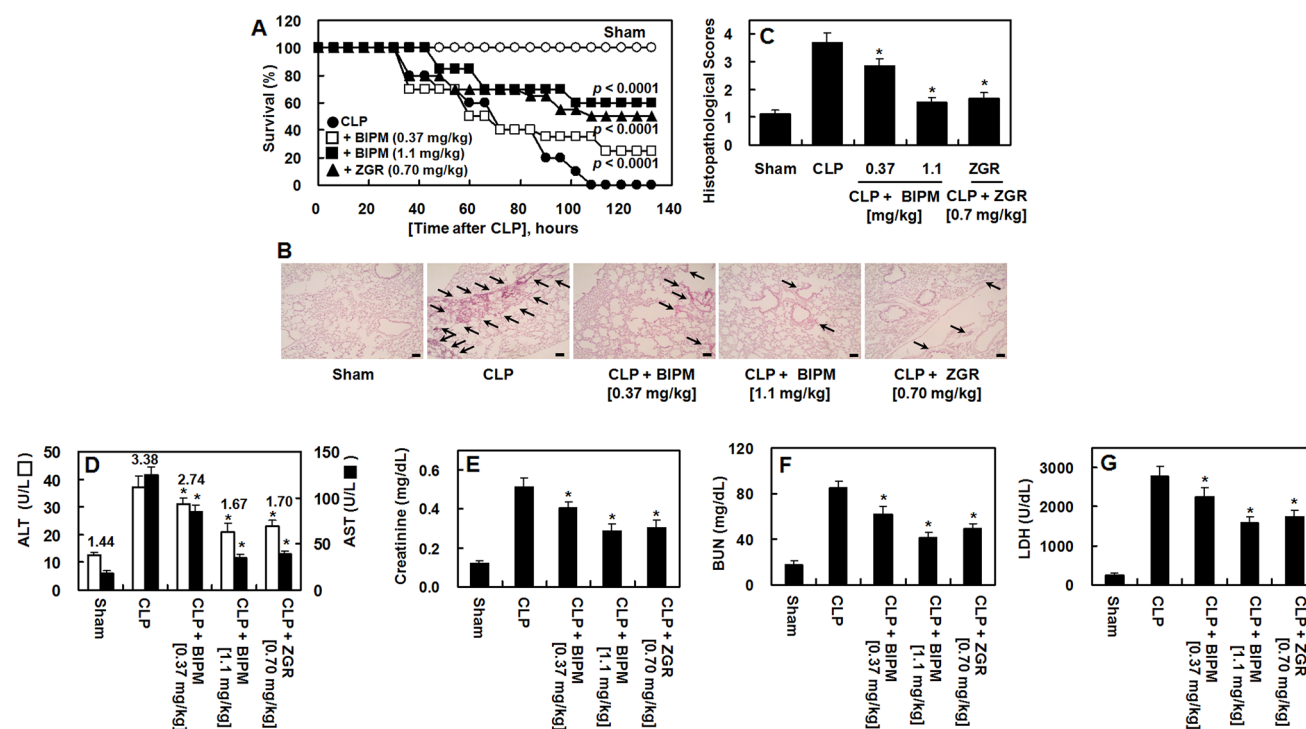


Fig. 5 BIPM on lethality and tissue injury on mice after CLP. **a** The suppressive effects of BIPM on mice lethality induced by CLP were monitored every 12 h for 132 h. Sterile saline was administered to sham-operated mice (open circle) and control CLP mice (filled circle) ($n=20$). **b** Representative photographs of H&E stained lung tissues in **c** ($\times 200$) from three independent experiments performed on different days with similar results. The arrows indicate leukocyte infiltration. **c** The histological analysis of BIPM effects on lung injury induced by CLP ($n=5$); the histopathological scores of the injured lung tis-

sues were counted and evaluated as described. **d–g** The suppressive effects of BIPM on the plasma levels of various organ injury markers; **d** alanine transaminase (ALT) and aspartate transaminase (AST), **e** creatinine, **f** blood urea nitrogen (BUN), and **g** lactate dehydrogenase (LDH), were analyzed ($n=20$). Data are shown as mean $\pm$ SD values of three independent experiments performed on different days with similar results. Logrank test was performed. $*p<0.05$ versus CLP alone

vascular inflammatory diseases, including sepsis and septic shock.

Acknowledgements This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (Grant number: HI15C0001) and by the National Research Foundation of Korea (NRF) grant funded by the Korean government (2017M3A9G8083382, 2020R1A2C1004131 and 2020R1F1A1071980).

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

References

- Hotchkiss RS, Moldawer LL, Opal SM, Reinhart K, Turnbull IR, Vincent JL. Sepsis and septic shock. *Nat Rev Dis Primers*. 2016;2:16045.
- Ziesmann MT, Marshall JC. Multiple organ dysfunction: the defining syndrome of sepsis. *Surg Infect (Larchmt)*. 2018;19:184–90.
- Ologunde R, Zhao H, Lu K, Ma D. Organ cross talk and remote organ damage following acute kidney injury. *Int Urol Nephrol*. 2014;46:2337–45.
- Riedemann NC, Guo RF, Ward PA. Novel strategies for the treatment of sepsis. *Nat Med*. 2003;9:517–24.
- Abraham E, Arcaroli J, Carmody A, Wang H, Tracey KJ. HMG-1 as a mediator of acute lung inflammation. *J Immunol*. 2000;165:2950–4.
- Buras JA, Holzmann B, Sitkovsky M. Animal models of sepsis: setting the stage. *Nat Rev Drug Discov*. 2005;4:854–65.
- Wang H, Li W, Li J, Rendon-Mitchell B, Ochani M, Ashok M, et al. The aqueous extract of a popular herbal nutrient supplement, *Angelica sinensis*, protects mice against lethal endotoxemia and sepsis. *J Nutr*. 2006;136:360–5.
- Bae JS. Role of high mobility group box 1 in inflammatory disease: focus on sepsis. *Arch Pharm Res*. 2012;35:1511–23.
- Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a late mediator of endotoxin lethality in mice. *Science*. 1999;285:248–51.
- Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, et al. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proc Natl Acad Sci USA*. 2004;101:296–301.
- Baek MC, Jung B, Kang H, Lee HS, Bae JS. Novel insight into drug repositioning: methylthiouracil as a case in point. *Pharmacol Res*. 2015;99:185–93.

12. Jung B, Kim J, Bae JS. Dabrafenib, as a novel insight into drug repositioning against secretory group IIa phospholipase A2. *Int J Pharmacol.* 2016;12:415–21.
13. Wang X, Zhang X, Zong Z, Yu R, Lv X, Xin J, et al. Biapenem versus meropenem in the treatment of bacterial infections: a multicenter, randomized, controlled clinical trial. *Indian J Med Res.* 2013;138:995–1002.
14. Pei G, Yin W, Zhang Y, Wang T, Mao Y, Sun Y. Efficacy and safety of biapenem in treatment of infectious disease: a meta-analysis of randomized controlled trials. *J Chemother.* 2016;28:28–36.
15. Lee W, Ku SK, Bae JS. Zingerone reduces HMGB1-mediated septic responses and improves survival in septic mice. *Toxicol Appl Pharmacol.* 2017;329:202–11.
16. Lee BS, Lee C, Yang S, Ku SK, Bae JS. Renal protective effects of zingerone in a mouse model of sepsis. *BMB Rep.* 2019;52:271–6.
17. Lee W, Hwang MH, Lee Y, Bae JS. Protective effects of zingerone on lipopolysaccharide-induced hepatic failure through the modulation of inflammatory pathways. *Chem Biol Interact.* 2018;281:106–10.
18. Kim JE, Lee W, Yang S, Cho SH, Baek MC, Song GY, et al. Suppressive effects of rare ginsenosides, Rk1 and Rg5, on HMGB1-mediated septic responses. *Food Chem Toxicol.* 2019;124:45–53.
19. Lee IC, Bae JS. Pelargonidin protects against renal injury in a mouse model of sepsis. *J Med Food.* 2019;22:57–61.
20. Jeong SY, Kim M, Park EK, Kim J-S, Hahn D, Bae J-S. Inhibitory functions of novel compounds from *dioscorea batatas* decne peel on HMGB1-mediated septic responses. *Biotechnol Bioproc Eng.* 2020;25:1–8.
21. Lee W, Ku SK, Kim JE, Cho GE, Song GY, Bae JS. Pulmonary protective functions of rare ginsenoside Rg4 on particulate matter-induced inflammatory responses. *Biotechnol Bioproc Eng.* 2019;24:445–53.
22. Lee I-C, Ryu C-W, Bae J-S. Novel herbal medicine C-KOK suppresses the inflammatory gene iNOS via the inhibition of p-STAT-1 and NF- κ B. *Biotechnol Bioproc Eng.* 2020;25:536–42.
23. Jung B, Kang H, Lee W, Noh HJ, Kim YS, Han MS, et al. Anti-septic effects of dabrafenib on HMGB1-mediated inflammatory responses. *BMB Rep.* 2016;49:214–9.
24. Lee W, Lee H, Lee T, Park EK, Bae JS. Inhibitory functions of maslinic acid, a natural triterpene, on HMGB1-mediated septic responses. *Phytomedicine.* 2020;69:153200.
25. Lee IC, Kim DY, Bae JS. Sulforaphane reduces HMGB1-mediated septic responses and improves survival rate in septic mice. *Am J Chin Med.* 2017;45:1253–71.
26. Tikhonov I, Doroshenko T, Chaly Y, Smolnikova V, Pauza CD, Voitenok N. Down-regulation of CXCR1 and CXCR2 expression on human neutrophils upon activation of whole blood by *S. aureus* is mediated by TNF- α . *Clin Exp Immunol.* 2001;125:414–22.
27. Mack M, Cihak J, Simonis C, Luckow B, Proudfoot AE, Plachy J, et al. Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. *J Immunol.* 2001;166:4697–704.
28. Zhong H, Li X, Zhou S, Jiang P, Liu X, Ouyang M, et al. Interplay between RAGE and TLR4 regulates HMGB1-induced inflammation by promoting cell surface expression of RAGE and TLR4. *J Immunol.* 2020;205:767–75.
29. Tafani M, Schito L, Pellegrini L, Villanova L, Marfe G, Anwar T, et al. Hypoxia-increased RAGE and P2X7R expression regulates tumor cell invasion through phosphorylation of Erk1/2 and Akt and nuclear translocation of NF- κ B. *Carcinogenesis.* 2011;32:1167–75.
30. Cohen MJ, Carles M, Brohi K, Calfee CS, Rahn P, Call MS, et al. Early release of soluble receptor for advanced glycation endproducts after severe trauma in humans. *J Trauma.* 2010;68:1273–8.
31. Klune JR, Dhupar R, Cardinal J, Billiar TR, Tsung A. HMGB1: endogenous danger signaling. *Mol Med.* 2008;14:476–84.
32. Bae JS, Rezaie AR. Activated protein C inhibits high mobility group box 1 signaling in endothelial cells. *Blood.* 2011;118:3952–9.
33. Greenbaum D, Colangelo C, Williams K, Gerstein M. Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol.* 2003;4:117.
34. Beyer A, Hollunder J, Nasheuer HP, Wilhelm T. Post-transcriptional expression regulation in the yeast *Saccharomyces cerevisiae* on a genomic scale. *Mol Cell Proteom.* 2004;3:1083–92.
35. Liu Y, Beyer A, Aebersold R. On the dependency of cellular protein levels on mRNA abundance. *Cell.* 2016;165:535–50.
36. Lee W, Ku SK, Lee YM, Bae JS. Anti-septic effects of glyceollins in HMGB1-induced inflammatory responses in vitro and in vivo. *Food Chem Toxicol.* 2014;63:1–8.
37. Qin YH, Dai SM, Tang GS, Zhang J, Ren D, Wang ZW, et al. HMGB1 enhances the proinflammatory activity of lipopolysaccharide by promoting the phosphorylation of MAPK p38 through receptor for advanced glycation end products. *J Immunol.* 2009;183:6244–50.
38. Palumbo R, Galvez BG, Pusterla T, De Marchis F, Cossu G, Marcu KB, et al. Cells migrating to sites of tissue damage in response to the danger signal HMGB1 require NF- κ B activation. *J Cell Biol.* 2007;179:33–40.
39. Erlandsson Harris H, Andersson U. Mini-review: the nuclear protein HMGB1 as a proinflammatory mediator. *Eur J Immunol.* 2004;34:1503–12.
40. Min G, Ku SK, Park MS, Park TJ, Lee HS, Bae JS. Anti-septic effects of pelargonidin on HMGB1-induced responses in vitro and in vivo. *Arch Pharm Res.* 2016;39:1726–38.
41. Astiz ME, Rackow EC. Septic shock. *Lancet.* 1998;351:1501–5.
42. Gonnert FA, Kunisch E, Gajda M, Lambeck S, Weber M, Claus RA, et al. Hepatic fibrosis in a long-term murine model of sepsis. *Shock.* 2012;37:399–407.
43. Spec A, Barrios CR, Ahmad U, Proia LA. AST to ALT ratio is elevated in disseminated histoplasmosis as compared to localized pulmonary disease and other endemic mycoses. *Med Mycol.* 2017;55:541–5.
44. Ainosah RH, Hagraas MM, Alharthi SE, Saadah OI. The effects of ursodeoxycholic acid on sepsis-induced cholestasis management in an animal model. *J Taibah Univ Med Sci.* 2020;15:312–20.
45. Bogatcheva NV, Verin AD. The role of cytoskeleton in the regulation of vascular endothelial barrier function. *Microvasc Res.* 2008;76:202–7.
46. Sabroe I, Jones EC, Whyte MK, Dower SK. Regulation of human neutrophil chemokine receptor expression and function by activation of Toll-like receptors 2 and 4. *Immunology.* 2005;115:90–8.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.