Remediation of Hemorrhagic Shock-Induced Intestinal Barrier Dysfunction by Treatment with Diphenyldihaloketones EF24 and CLEFMA

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CLEFMA, 4-[3,5-bis(2-chlorobenzylidene)-4-oxo-piperidin-1-yl]-4-oxo-2-butenolic acid

EF24, 3,5-bis(2-fluorobenzylidene)piperidin-4-one

ILBP, Ileal lipid-binding protein

IR, Ischemia-reperfusion

TJ, Tight junction

ZO, zonula occludens

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Abstract

Gut is very sensitive to hypoperfusion and hypoxia, and deranged gastrointestinal barrier is implicated in systemic failure of various organs. We recently demonstrated that diphenyldihalo ketone EF24 improves survival in a rat model of hemorrhagic shock. Here, we tested EF24 and its another analog CLEFMA for their effect on intestinal barrier dysfunction in hypovolemic shock. Hypovolemia was induced in rats by withdrawing 50% of blood. EF24 or CLEFMA (intraperitoneal 0.4 mg/Kg) treatment was provided, without volume resuscitation, after 1 h of hemorrhage. Ileum was collected 5 h after the treatment for investigating the expression of tight junction proteins (zonula occludens, claudin, and occludin) and epithelial injury markers (myeloperoxidase, ileal lipid-binding protein (ILBP), CD163, and plasma citrulline). The ileal permeability for dextran-fluoroisothiocyanate and Evan's blue dye was determined. EF24 and CLEFMA reduced the hypovolemia-induced plasma citrulline levels and the ileal expression of myeloperoxidase, ILBP, and CD163. The drugs also restored the basal expression levels of zonula occludens, claudin, and occludin which were substantially deranged by hypovolemia. In ischemic ileum, the expression of phospho(Tyrosine)-zonula occludens-1 was reduced which was reinstated by EF24 and CLEFMA. On the other hand, the drug treatments maintained the hypovolemia-induced expression of phospho(Threonine)-occludin, but reduced that of phospho(Tyrosine)-occludin. Both EF24 and CLEFMA treatments reduced the intestinal permeability enhanced by hypovolemia. EF24 and CLEFMA attenuate hypovolemic gut pathology and protect barrier function by restoring the status of tight junction proteins. These effects were observed in unresuscitated shock, implying the benefit of EF24 and CLEFMA in pre-hospital care of shock.
Introduction

Moderate to severe blood loss is accompanied by a systemic compensation to maintain cardiac output. This compensation is characterized by increased sympathetic outflow which results in an increase in heart rate and vasoconstriction in non-essential tissues. The resultant hypoperfusion causes a disproportionate decrease in portal blood flow which adversely affects the barrier and absorptive functions of the intestine (Rhodes et al., 1973; Fink and Delude, 2005). The physical boundary of the intestinal barrier is maintained by a columnar epithelium characterized by the presence of adherens junctions (AJ) and tight junctions (TJ) (Groschwitz and Hogan, 2009). Whereas AJs are responsible for maintaining cell-cell contacts, the TJs control the paracellular movement of ions and solutes. The TJs consist of the transmembrane proteins occludin and claudins, and the cytoplasmic scaffolding proteins zonula occludens (ZO) (Hartsock and Nelson, 2008). At steady state, gut barrier is maintained by optimal turnover of these molecular components of TJs. Therapeutic interventions that assist in maintaining or restoring gut homeostasis may be of immense benefit as adjunct treatment in hemorrhagic shock and ischemia-reperfusion (IR) injury. The genesis of barrier dysfunction is not limited to hemorrhagic shock, because the same is also observed in the victims of burn, trauma, sepsis, and radiation injury.

Since gut dysfunction is a trigger in the pathogenesis of multiple organ failure (Moore, 1999; Rotstein, 2000; Senthil et al., 2006; Hauer-Jensen et al., 2007; Groschwitz and Hogan, 2009), there exists a need to address loss of barrier function early after traumatic injury. Apart from prompt and adequate resuscitation (Shi et al., 2002; Vega et al., 2008), no one specific medication alone is known to support circulatory deficit in hemorrhagic shock. Therefore, pharmacologic agents have been investigated as part of a comprehensive resuscitation regimen (Kao and Fink, 2010; Cotton, 2011). For instance, ethyl pyruvate, a free radical scavenger, has been shown to improve survival and/or reduce organ dysfunction in a wide variety of preclinical models of critical illnesses (Fink and Delude, 2005; Fink, 2007). It has been
shown to ameliorate barrier dysfunction in lipopolysaccharide (LPS)-treated Caco-2 monolayers in vitro (Sappington et al., 2003).

Recently, we reported that a diphenyldihaloketone, 3,5-bis(2-fluorobenzylidene)piperidin-4-one or EF24 (Fig. 1A), suppresses inflammatory phenotype in lung of rats hemorrhaged by 50% of circulating blood (Yadav et al., 2013). In the process, EF24 also significantly improved the survival in this preclinical model of hemorrhagic shock (Yadav et al., 2013). We have also shown that EF24 inhibits LPS-induced nuclear factor κB (NF-κB) and reduces secretion of pro-inflammatory cytokines in LPS-stimulated dendritic cells (DCs) used as an in vitro model of sterile inflammation (Vilekar et al., 2012). Another potent analog of EF24 is 4-[3,5-bis(2-chlorobenzylidene)-4-oxo-piperidine-1-yl]-4-oxo-2-butenoic acid or CLEFMA (Fig. 1A), which has bis-2-chloro in place of bis-2-fluoro functional groups and carries an additional maleic acid chain at the piperidonyl nitrogen (Lagisetty et al., 2010). Here, we hypothesized that the salutary effects of diphenyldihaloketones in unresuscitated hemorrhagic shock would involve remediation of intestinal barrier function loss. The putative mechanism of EF24 action is based on its ability to suppress NF-κB activation by inhibiting the catalytic activity of IkappaB kinase (Kasinski et al., 2008). The results unraveled for the first time that EF24 and CLEFMA administration dramatically improved histologic, functional, and molecular signatures of intestinal barrier in a rat model of fixed (50%) volume hemorrhagic shock. Significantly, these effects were observed without any accompanying volume resuscitation.

**Materials and Methods**

EF24 and CLEFMA were synthesized in-house by the procedures published elsewhere (Lagisetty et al., 2009; Lagisetty et al., 2010). Sterile solutions of these drugs were prepared in normal saline using poly(ethylene glycol)-400 as a co-solvent (3 parts saline + 1 part PEG400). The primary rabbit antibodies against rat antigens were obtained from Santa Cruz Biotech (Dallas, TX). Horse radish peroxidase (HRP)-
conjugated secondary goat anti-rabbit IgG was from Sigma (St. Louis, MO). All other chemicals were obtained from diverse vendors represented by VWR (Radnor, PA).

**Rat model of hypovolemic shock.** The animal experiments were performed according to the NIH Animal Use and Care Guidelines and were approved by the Institutional Animal Care and use Committee of the University of Oklahoma Health Sciences Center. Male Sprague Dawley rats (250-300 g) were purchased from Harlan laboratories (Indianapolis, IN). The rats were housed in regular light/dark cycles of 12/12. Before initiating the experiment, we allowed the rats to acclimatize for at least 5 days. The method of femoral artery catheterization in rats has been described elsewhere (Awasthi et al., 2007). Briefly, left femoral artery was cannulated with a Teflon-tipped catheter and the catheter was subcutaneously tunneled and secured to the nape; the rats were allowed 2 days to recover from surgery. The cannulated rats were clustered a priori in four groups (n = 4 - 6/group): control (CTRL), hemorrhagic shock + vehicle (HS+V), hemorrhagic shock+EF24 (HS+EF), and hemorrhagic shock+CLEFMA (HS+CL). On the day of the experiment, the rats were handled under isoflurane (2-3%) anesthesia in medical air stream (2 l per min). The rats were heparinized with 100 units of heparin to prevent catheter blockade. Hemorrhagic shock was induced by withdrawing approximately 50% of circulating blood at the rate of 1.0 ml/min. The total volume of blood was estimated approximately 6% of the total body weight (Weiss et al., 2000). The hypovolemic rats were allowed to wake up and freely compensate for 1 h, before drug solution was administered intraperitoneally. The drug treatment consisted of approximately 0.4 mg/Kg bodyweight, whereas HS+V and CTRL groups received equivalent amounts of vehicle (25% PEG400 in saline) or 0.9% saline, respectively, in an identical fashion. The treatment volume was approximately 100 µl for all groups, irrespective of small differences in body weight. Blood pressure was digitally monitored by instrumenting the rats to an iWorx data acquisition system (Dover, NH). After 6 h of hemorrhage, the surviving rats were euthanized with an overdose of SOMNASOL, Euthanasia-III Solution (Butler Schein
Animal Health, Dublin, OH). Small intestine was immediately isolated and cleaned of luminal debris with ice-cold saline.

**Real time polymerase chain reaction (RT-PCR).** The total RNA was extracted using RNA-STAT60 (TEL-TEST Inc, Friendswood, TX) and quantified by absorbance values at 260 nm. The reverse transcriptase reaction was performed for 1 h at 42°C using 2 μg of total RNA, 1 μg of oligo(dT), 200 U of M-MLV reverse transcriptase enzyme, 500 μM dNTP mix, and 25 U of RNase inhibitor (Promega, Madison, WI). The resultant cDNA was used to carry out 40 PCR cycles consisting of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C on an ABIPrism 7000 sequence detection system (Applied biosystems, Foster City, CA). The reactions were performed using SybrGreen II (Qiagen, Valencia, CA) and Go Taq Colorless master mix (Promega, Madison, WI). Each PCR reaction was set up in triplicate wells in a total volume of 25 μl containing cDNA equivalent of 20 ng total RNA. The quantitative values of the genes of interest were normalized using β-actin as the endogenous reference, and fold-increase over control values was calculated using the relative quantification method of 2-ΔΔCt. All the primers were of 20 base pairs (Table S1, supplemental information).

**Immunohistochemistry (IHC).** The protein expression levels of ZO-1, ZO-2, claudin, and occludin were assessed in formalin-fixed ileal tissues by using an IHC kit from DakoCytomation (Carpinteria, CA). In brief, the tissue samples were fixed with paraformaldehyde and embedded in paraffin. The tissues were sectioned and processed in the imaging core facility of the Oklahoma Medical Research Foundation (Oklahoma City, OK). Briefly, the slides were blocked with a protein-block solution for 20 min and incubated overnight with primary antibody dilutions (Table S2, supplemental information). The slides were washed and incubated with biotinylated link universal antiserum, followed by horseradish peroxidase (HRP)-streptavidin conjugate. The slides were rinsed and the color was developed using 3,3-diaminobenzidine hydrochloride as a chromogen. Finally, the sections were rinsed in distilled water.
counterstained with Mayer's hematoxylin solution and mounted with DPX-mounting medium for evaluation. For histopathological examination, the paraffin-embedded tissues were sectioned, stained with Hematoxylin and Eosin (H & E) stain, observed with Olympus microscope IX701, and digitally recorded using an Olympus DP70 camera.

**Immunoblotting.** The isolated frozen tissues were minced and incubated on ice for 30 min in ice-cold whole-cell lysate buffer consisting of 10% NP-40, 5 M NaCl, 1 M HEPES, 0.1 M ethyleneglycoltetraacetic acid, 0.5 M ethylenediaminetetraacetic acid (EDTA), 0.1 M phenylmethylsulfonyl fluoride (PMSF), 0.2 M sodium orthovanadate (NaOV), 1 M NaF, 2 μg/mL aprotinin, and 2 μg/mL leupeptin. The protein was extracted by homogenization using a dounce homogenizer and centrifugation at 14,000 rpm for 10 min. The proteins were fractionated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), electro-transferred on to nitrocellulose membranes, blotted with primary antibodies followed by HRP-conjugated secondary antibody. The primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX) and used at a dilution of 1:1,000 (Table S2, supplemental information). The immunoreactive bands were detected by SuperSignal West Femto detection reagent (Thermo Fischer Scientific, Rockford, IL). The blots were imaged using Ultraquant image acquisition machine (Claremont, CA) and the densitometric readings for proteins were normalized with those of actin.

**Immunoprecipitation.** Ileal tissues were lysed in ice-cold homogenization buffer containing 25 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM PMSF, 0.2 mM NaOV, 50 mM NaF, 150 mM NaCl, and 1 μg/ml each of leupeptin and pepstatin. After a 10 min spin at 20,000 g, 4°C the supernatant was collected and protein concentration was determined (Bio-Rad Bradford protein assay). Homogenate volumes corresponding to 0.2 mg total protein were incubated overnight with 2 μg of monoclonal anti-phospho-Tyr or anti-phospho-Thr antibodies (Table S2, supplemental information) at 4°C and then with 20 μl bead-immobilized protein A (GBiosciences, St. Louis, MO) for 2 h to collect the immunocomplex. The beads
were washed three times and the bound complex was eluted from the agarose beads with SDS-PAGE sample buffer for electrophoresis and immunoblotting with rabbit anti-ZO-1 and anti-occludin antibodies at 1:1,000 dilution in TBST.

**Intestinal permeability.** We assessed intestinal permeability by two methods— one in situ and the other in vitro. For in situ assessment of intestinal permeability in hypovolemic rats, the rats were injected with 50 µl solution of Evan’s blue dye (EBD) in saline (30 mg/Kg via the arterial catheter) one hour prior to euthanasia. The accumulation of dye in intestinal tissue was quantitated by determining the absorbance of tissue lysate at 620 nm. Briefly, approximately 100 mg ileal tissue was extracted in dimethylformamide (500 µl). The extracts were centrifuged at 12,000 x g, and the absorbance values of supernatants were determined. The amount of EBD accumulated in intestinal tissue was normalized with the concentration of EBD in plasma for each individual rat. The EBD concentration in plasma was estimated by comparing the absorbance (620 nm) of appropriate dilutions of plasma with a standard curve.

The in situ method was accompanied by a determination of permeability in isolated ileal segments following a method described elsewhere (Cruz et al., 2011). Briefly, the harvested small intestine was carefully cleaned of luminal debris with ice-cold saline and inverted over a plastic pipette. Approximately 5 cm segments were cut and tied on one end to create a pouch. After filling the pouch with 1 ml saline, the other end was also tied and the segments were immersed in a saline bath containing 0.1 mg/ml fluorescein isothiocyanate (FITC)-Dextran (mol. wt. 4,000). The bath was kept at 37°C, with constant bubbling of oxygen and gentle mixing. After allowing 45 min of incubation, the external surface of intestinal pouches was thoroughly washed with saline, and the internal content was collected for fluorescence reading. The fluorescence values were calculated on per unit volume per hour basis and expressed as
percent of that in the external bath. At least three intestinal segments from each rat (n = 4 per group) were assayed to determine the mean ± sem values.

**MPO assay.** The frozen ileal tissue samples were thawed, diced with a razor blade, and homogenized in 50 mM potassium phosphate buffer (pH 6.0). The homogenate was centrifuged at 10,000 rpm for 15 minutes and the pellet was resuspended in 0.3 ml of phosphate buffer containing 50 mM hexadecyltrimethylammonium bromide (HTAB). The mixture was subjected to three cycles of bath sonication (20 s), snap-freezing in liquid nitrogen, and thawing to room temperature. The supernatants were collected by centrifuging the mixture at 10,000 rpm for 10 min. The aliquots of supernatants were diluted 1:2 and 1:10 with the HTAB-phosphate buffer and added with 30 times the volume of phosphate buffer containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The absorbance was monitored at 460 nm. At least 4 ileal tissues per group were assayed, each in triplicate.

**Plasma citrulline enzyme-linked immunosorbent assay (ELISA).** Concentration of citrulline in plasma samples was determined by using a commercial sandwich ELISA test kit (MyBioSource, San Diego, CA). The manufacturer-recommended method was followed.

**Data analysis.** The results were analyzed by one-way analysis of variance (ANOVA) applying the Bonferroni post-test using Prism 6 software (GraphPad, San Diego, CA, USA). A p < 0.05 was considered statistically significant. The densitometry of immunoreactive bands was performed from three replicates using Image J 1.46r freeware (NIH, USA).

**Results**

The gut barrier dysfunction stimulates the activation of ‘gut-liver-lung’ axis, and it often precedes multiple organ dysfunction in severe hemorrhagic shock. In our previous study, we had found that administration of a diphenyldihaloketone EF24 improved the survival rate in a rat fixed-volume
hemorrhagic shock model (Yadav et al., 2013). CLEFMA, an analog of EF24, was not tested in the same manner. In order to substantiate the therapeutic effect of these diphenyldihaloketones, we now present the histological, molecular, and functional evidences demonstrating the improvement of gut barrier function in fixed-volume hemorrhagic shock. The structure of EF24 and CLEFMA work are provided in Fig. 1A, whereas the experimental design is depicted in Fig. 1B. The characteristics of this rat model are summarized in Fig. 1C-E. The blood withdrawal caused a drop in mean arterial pressure (MAP, mm Hg) from 97.5±6.9 to 43±4.1. After 6 h of the treatment with EF24, CLEFMA, or vehicle control (saline), MAP was significantly increased, but remained well below the basal level (Fig. 1D). The corresponding mean hematocrit value at 6 h after hemorrhage was 26±0.9 as compared to 45±3.7 of control rats (Fig. 1E).

**EF24 and CLEFMA treatments ameliorate hemorrhage-induced ileal injury.** Hematoxylin & eosin-stained pictures of ileum at low magnification (100X) showing global injury induced by hemorrhagic shock are provided in supplemental section (Fig. S1), whereas high magnification pictures (400X) are shown in Fig. 2A. The control rats showed a normal ileal structure, characterized by intact villi and a normal epithelial cell lining. Rats sacrificed 6 h after shock showed substantial extension of the subepithelial space, edema, and denudation as well as massive lifting and sloughing of the villi. The villi heights were considerably shorter in the case of untreated rats as compared to the control rats and the columnar architecture of ileal barrier was completely lost in untreated rats. There appeared to be increased number and activity of goblet cells in ileal epithelium of hemorrhaged rats. Treatments with EF24 and CLEFMA considerably reduced this injury, with ileal epithelium showing close to normal architecture.

**EF24 and CLEFMA treatments reduce myeloperoxidase activity and CD163 expression in ileal tissue.** The influx of neutrophils in the ileal epithelium was determined by estimating the activity of myeloperoxidase (MPO), an enzyme released during degranulation of neutrophils and monocytes.
Treatments with EF24 and CLEFMA markedly ameliorated the hemorrhage-induced increase in ileal MPO activity (Fig. 2B). We also estimated cytokine-induced neutrophil chemoattractant-1 (CINC-1), the rat equivalent of IL-8, which is a powerful neutrophil chemoattractant expressed by macrophages in inflamed intestinal tissue (MacDermott, 1999). There was no change in ileal IL-8 levels after hemorrhage or drug treatment (Fig. S2, supplemental information), suggesting a possibility of some other chemoattractant for neutrophil accumulation in ileal tissue. CD163 is a hemoglobin-heptaglobin scavenger receptor which marks the presence of monocytes/macrophages in the subepithelial lamina propria; it is highly expressed on resident tissue macrophages and to a lesser extent on monocytes (Fabriek et al., 2005). Figure 2C shows that the expression of CD163 in ileal tissue was increased in rats subjected to hemorrhagic shock. This hemorrhagic shock-induced expression of CD163 was suppressed by EF24 and CLEFMA treatments.

**EF24 and CLEFMA treatments restore the expression of TJ and antimicrobial proteins in ileal tissue.**

One of the early events in the failure of gut wall integrity is the TJ loss. We found that the mRNA expression of ZO-1, occludin, and claudin-4 was increased, but that of ZO-2, ZO-3, claudin-1, claudin-2, and claudin-3 was suppressed (Fig. 3A). In general, the mRNAs that were reduced by hemorrhagic shock showed large increase by EF24 and CLEFMA treatments, whereas the effect of these drugs was moderately suppressive on the mRNAs that were increased by hemorrhage.

Among the major defenses against potential post-hemorrhage infectious insult, the secretions of Paneth cells play an important role (Gunther et al., 2013). They contribute to the intestinal antibacterial defense by releasing antibacterial factors stored in their cytoplasmic granules. Lysozyme is an early marker of Paneth cells health. We estimated the status of the rat homologs of defensins lysozyme (LYZ-2), α-defensin-related sequence-1 (Defa-1), angiopoetin-4 (Angpt-4), and defensin-5 (Defa-5) by RT-PCR, and found that although hemorrhage did not significantly alter the mRNA levels of antimicrobial
proteins, CLEFMA treatment increased the expression levels of all four antimicrobial proteins in a significant manner; the effect of EF24 treatment on their mRNA levels was not significant (Fig. 3B).

The protein expression of ZO-1, ZO-2, occludin, and claudin-1 was first assessed by IHC (Fig. 4). At protein levels also, the expression of ZO-1 and occludin was enhanced, whereas that of ZO-2 and claudin-1 was reduced by hypovolemia. These effects of blood loss on the expression of various TJ proteins were reversed when the hypovolemic rats were treated with EF24 or CLEFMA (Fig. 4). The results from IHC analyses of tissues were confirmed by immunoblotting of ileal lysates (Fig. 5A and 5B). The expression-inducing effect of hypovolemic shock on ZO-1 and occludin was contrary to what was expected in a deranged ileal epithelium. Since ZO-1 is post-transcriptionally regulated by tyrosine (Tyr) phosphorylation, we investigated whether hypovolemia and EF24 treatment have any effect on the expression of phosphorylated form of ZO-1. To this end, we immunoprecipitated the tissue lysate with anti-phosphotyrosine antibody and probed the blot with anti-ZO-1 antibody. We found that hypovolemia significantly reduced the Tyr-phosphorylated ZO-1. EF24 as well as CLEFMA treatment recovered the phospho(Tyr)-ZO-1 to its basal levels (Fig. 5C). In case of occludin, the Tyr-phosphorylation was not much affected by hemorrhagic shock, but it was substantially reduced by EF24 and CLEFMA treatments. At the same time, threonine (Thr) phosphorylation of occludin was found to be increased by hemorrhagic shock. Treatments with EF24 and CLEFMA appeared to have maintained the hemorrhage-induced Thr phosphorylation of occludin (Fig. 5C).

**EF24 and CLEFMA treatments diminish epithelial cell injury.** Injury to intestinal epithelia is marked by an increased shedding of ileal-lipid binding protein (ILBP), resulting in lowered detection of cell-associated ILBP. ILBP is a small cytosolic protein (15 KDa) belonging to a family of fatty acid binding proteins. We examined ILBP levels in ileal tissue by IHC and immunoblotting. As shown in Fig. 6A-B,
hemorrhagic shock substantially decreased the expression of ILBP in ileum, but EF24 and CLEFMA treatments recovered the basal level of ILBP expression.

Systemic citrulline almost exclusively originates from the intestine, where enterocytes synthesize it from either arginine or glutamine. As such, the estimation of plasma citrulline levels is being debated as a clinical marker of acute intestinal failure in critically ill patients (Piton et al., 2011). It is also a surrogate marker for estimating NO production by nitric oxide synthase (NOS) activity. As shown in Fig. 6D, hemorrhagic shock significantly increased the plasma citrulline levels. Both EF24 and CLEFMA treatments reduced this hemorrhage-induced citrulline concentration in plasma, but only the EF24-effect was statistically significant.

**EF24 and CLEFMA treatments reinstate intestinal barrier.** To assess the functional changes in intestinal barrier function, we performed two separate evaluations- in vitro transmembrane permeability of FITC-dextran and in situ EBD method. The results of both the permeability tests showed a significant increase in permeability after hemorrhagic shock (Fig. 7). The flux of FITC-dextran in isolated inverted sacs of ileal segments (Fig. 7A) was significantly increased in hemorrhaged rats. Both EF24 and CLEFMA reduced the hemorrhage-induced flux of FITC-dextran. The in situ accumulation of EBD also demonstrated the same scenario. Both EF24 and CLEFMA treatments reduced the levels of transmembrane localization of EBD induced by hypovolemia (Fig. 7B).
Discussion

Shock is a multi-factorial syndrome characterized by an inadequate tissue perfusion and cellular hypoxia affecting multiple organ systems. Gut is one of the major target organs of the systemic inflammatory response syndrome (SIRS) after hemorrhagic shock, and from a prognostic viewpoint the status of intestinal barrier function appears to be the most important determinant of clinical outcome (Moore, 1999). One of the early events in severe blood loss is the disintegration of gut wall integrity characterized by marked TJ loss and increased permeability, allowing luminal bacteria, toxins, and other macromolecules to become systemic. The present study focused on the health of intestinal barrier after hemorrhagic shock and treatment with two diphenyldihaloketones, EF24 and CLEFMA. Earlier, we have shown that EF24 treatment suppresses hemorrhage-induced pulmonary markers of inflammation, namely NF-kB, toll-like receptor 4 (TLR4), cyclooxygenase (COX)-2, and interleukin receptor (IL-1R1), as well as systemic pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α) and IL-6 (Yadav et al., 2013; Yadav et al., 2014). We also reported that EF24 potently suppressed NF-kB activation, modulates DC phenotype and reduces secretion of pro-inflammatory cytokines (Vilekar et al., 2012). These initial observations lead us to hypothesize that EF24 and CLEFMA will protect ileum from barrier function loss in hemorrhagic shock.

The TJ proteins belonging to the ZO family not only provide scaffolding for the assembly of other tight junction proteins via their PDZ domains, but also act as membrane-associated guanylate kinase-like signaling proteins in cellular growth pathways (Bauer et al., 2010). We found that hemorrhage affected the expression of ZO-1 and ZO-2 differently—whereas ZO-1 was upregulated, ZO-2 showed downregulation after hemorrhagic shock; EF24 and CLEFMA, both recovered ZO-1 and ZO-2 expressions to their basal levels. The reasons for differential response of ZO-1 and ZO-2 to hemorrhagic shock are not clear, but literature provides some clues about the behavior of ZO-1. It turns out that appropriate
localization of these PDZ-containing proteins close to the plasma membrane in the enterocytes is more important than mere cellular expression levels for effective barrier function. This restricted localization is dependent on post-translational modification, such as tyrosine phosphorylation (Rao et al., 2002). Even when the expression levels of ZO-1 remain unaltered, the tyrosine phosphorylation and re-localization from apical membrane of intestinal villi could occur in breached intestinal barrier (Hamada et al., 2010). Phosphorylation also affects mutual interaction among TJ proteins. For instance, the interaction between ZO-1 and occludin is phosphorylation-dependent (Tash et al., 2012).

Dephosphorylated ZO-1 has been shown to cause absence of membranous localization of occludin in active celiac disease (Ciccocioppo et al., 2006). Our observation that hemorrhage induces the expression of ZO-1, but decreases the phosphorylated form of ZO-1 also points to the importance of phospho-ZO-1 in the formation of intestinal barrier.

The claudins and occludins are two major transmembrane proteins that interact with ZO proteins and directly determine the paracellular permeability to different ions and large molecules (Hu et al., 2013). The function of claudins in epithelial barrier is also subject to modulation by Ser/Thr phosphorylation and interaction with PDZ-binding domains (Groschwitz and Hogan, 2009). We found that hemorrhage downregulated the expression of claudins 1, 2, and 3, but upregulated the expression of claudin 4. Even though such differential expression of various claudin proteins in response to ischemia/reperfusion (I/R) injury of the intestine has also been noted by others (Takizawa et al., 2012), the reasons are not clearly understood. Among 27 known members of claudin family, claudins 1 and 3 have sealing functions, claudin 2 forms a channel providing selectivity to the cations, whereas the role of claudin 4 in barrier function is not known (Gunzel and Fromm, 2012). Recent reports have associated claudin 4 expression with epithelial malignancies and premalignant precursor lesions (Neesse et al., 2012). Interestingly, Transforming growth factor (TGF)-β has been reported to transcriptionally upregulate claudin-4 expression via a Smad-4-dependent pathway (Kotler et al., 2013). Since TGF-β is increased in
hemorrhagic shock (Ayala et al., 1993), its role in hypovolemia-induced claudin-4 expression could not be ruled out.

Unlike claudins, occludin protein has the greatest effect on the flux of large macromolecules (Al-Sadi et al., 2011). The expression of occludin is markedly decreased in intestinal permeability disorders, such as Crohn’s disease and ulcerative colitis (Gassler et al., 2001), suggesting that decreased occludin expression is associated with an increase in intestinal permeability. Our finding is contrary to this conjecture, and we found that hemorrhagic shock increased occludin expression at both mRNA and protein levels. The effect of phosphorylation status of occludin in maintaining ileal homeostasis has been elegantly investigated by Rao’s group (Seth et al., 2007; Rao, 2009). The phosphorylation of occludin at Ser/Thr helps whereas phosphorylation at Tyr residue deters its interaction with ZO-1 (Seth et al., 2007; Rao, 2009). Our results from phospho-Tyr co-immunoprecipitation show that the ileum-preserving EF24 and CLEFMA treatments reduce ileal phospho(Tyr)-occludin in hypovolemic rats support these in vitro findings in Caco-2 monolayers. We also found that hemorrhagic shock induced Thr-phosphorylation of occludin, and treatments with EF24 and CLEFMA preserved this modification. The occludin-specific effect of EF24 and CLEFMA may be explained by their ability to reduce Tyr phosphorylation and maintain Thr phosphorylation of occludin, thereby aiding its interaction with ZO-1. Earlier, Seth et al (Seth et al., 2007) predicted that the net role of occludin is determined by the relative levels of Tyr and Thr phosphorylation. It is noteworthy that the Tyr/Thr phosphorylation ratio of occludin is regulated by protein phosphatases 2A and 1 (Seth et al., 2007; Sheth et al., 2009). Overall, these results imply that mere increased expression of occludin may not be sufficient, but phosphorylation at appropriate amino acid residue may be important in regulating its interaction with other TJ proteins.

The remarkable restoration of TJ proteins and accompanying histology by treatments with EF24 and CLEFMA was manifested in the resurrection of ileal barrier function. The treatments significantly
reduced hemorrhagic shock-induced increase in the intestinal permeability. We also found that only CLEFMA treatment upregulated mRNA levels of antimicrobial proteins; neither hypovolemia nor EF24 significantly altered the antimicrobial protein mRNA levels. The hypothesis that the variation in chemical structure of EF24 and CLEFMA results in these differences is a subject of further investigation. Not much is known about the changes in antimicrobial proteins secreted by Paneth cells in hemorrhagic shock, but in one report rat enteric α-defensin gene was found to be upregulated immediately after termination of shock (Condon et al., 1999). Since no long-term assessment was performed in the published study (Condon et al., 1999), our results could be reconciled by speculating that the defense mechanisms originating from Paneth cells are progressively salvaged over time. Paneth cells develop from epithelial progenitor cells and are restricted to crypt base in the gut epithelium (Gunther et al., 2013).

The above described salutary effects of EF24 and their time-course pointed towards a rather rapid localization in the intestinal tissue. We examined the biodisposition of EF24 by labeling it with imageable radionuclide technetium-99m (Tc-99m, 140 KeV γ ray, 6 h decay half-life) for gamma camera imaging. For this we synthesized an N-HYNIC conjugate of EF24 by employing a procedure described elsewhere (Lagisetty et al., 2012). As shown in Fig. S3 (supplemental information), intravenously injected Tc-99m-EF24 rapidly accumulated in rat liver and intestine. The first sign of EF24 localization in gut was observed within 5 min of injection. The accumulation increased over time and EF24 appeared to be retained in intestinal tissue up to 6 h after injection. No other organ except liver and intestines accumulated significant radioactivity, suggesting that its clearance depended on hepatobiliary route. The image-derived knowledge of drug accumulation suggests that EF24 is naturally cleared from circulation into the intestine, which might explain the remarkable effects of EF24 and CLEFMA on intestinal integrity in hemorrhagic shock. What molecular pathways are involved in these actions remains a subject of continued investigation in our laboratory.
Conclusions

Besides hemorrhagic shock, dysregulated barrier function is a hallmark in many other disorders such as inflammatory bowel diseases, food allergy, celiac disease, Type I diabetes, etc. (Groschwitz and Hogan, 2009; Salim and Soderholm, 2011). The intestinal pathology of hemorrhagic shock is also similar to that observed in victims of burn, sepsis, radiation exposure (MacNaughton, 2000; Hauer-Jensen et al., 2007) or drug toxicities (Meng et al., 2013; Russo et al., 2013). Despite such widespread impact, the current therapies for the management of barrier function loss remain inadequate. The results of this study show that the administration of EF24 or CLEFMA significantly improved the intestinal integrity of rats subjected to hemorrhage. These encouraging observations lead us to speculate about the additive benefits of EF24 or CLEFMA when administered in combination with conventional resuscitation fluids used to treat hemorrhagic shock.

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Authorship contributions

Participated in research design: Awasthi.

Conducted experiments: Yadav, Hussain, Sahoo, and Awasthi.

Contributed new reagents or analytic tools: Awasthi.

Performed data analysis: Yadav and Awasthi.

Wrote or contributed to the writing of the manuscript: Yadav, Hussain, and Awasthi.
REFERENCES


Footnote

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Figure captions

**Figure 1:** Structures of (A) EF24, 3,5-bis(2-fluorobenzylidene) piperidin-4-one, and CLEFMA, 4-(3, 5-bis(2-chlorobenzylidene)-4-oxo-piperidine-1-yl)-4-oxo-2-butenoic acid. (B) Experimental design:

Hemorrhagic shock was induced by withdrawing 50% of blood through indwelling femoral artery catheter. The hemorrhaged rats were treated with EF24 or CLEFMA (abbreviated as EF and CL) after 1 h (0.4 mg/Kg bodyweight, i.p.). The untreated hemorrhaged rats (HS+V group) received equal volume of vehicle (100 µl). The control group consisted of normal rats subjected to catheter implantation, but no hemorrhage or drug treatment was provided. At 6 h, the rats were euthanized and ileum was collected for evaluations described in this article. (C) A representative blood pressure profile at baseline and after hemorrhagic shock. (D) Mean arterial pressure (MAP) in various groups of rats. Blood pressure was recorded at baseline (BL, n = 12), immediately after hemorrhage (HS, n = 12) and after 5 h of treatment with EF24 (n = 4), CLEFMA (n = 4), or vehicle (V, n = 4). (E) Mean hematocrit values at baseline (BL, n = 12), immediately after shock (HS, n = 12), and after 5 h of treatment with EF24, CLEFMA, or vehicle (n = 12). The Hematocrit values of rats belonging to the three treatment groups were combined to obtain a composite value for HS+EF/CL/V group (* p < 0.05 vs. BL, # p <0.05 vs. HS).

**Figure 2:** (A) A representative set of H & E stained ileal tissues of rats subjected to hemorrhagic shock. The arrows point to the disruption in integrity of epithelial integrity. (B) Effect of EF24 and CLEFMA on HS-induced myeloperoxidase (MPO) activity in ileal tissue (* p < 0.05 vs. Ctrl, # p <0.05 vs. HS, n = 4/group). The groups are defined in the caption to figure 1. (C) CD163 expression in ileal tissue of rats subjected to hemorrhagic shock (upper panel). CD163 expression was quantitated by densitometry (lower panel; p = 0.24 CTRL vs HS+V, p = 0.13 HS+V vs HS+EF, and p = 0.07 HS+V vs HS+CL, n = 3/group). HS = Hemorrhagic shock, V = Vehicle, CL = CLEFMA.
Figure 3: Messenger RNA levels of (A) tight junction proteins (ZO-1, ZO-2, ZO-3, Occludin, Claudin-1, Claudin-2, Claudin-3, and Claudin-4) and (B) antimicrobial proteins Defa-1 (defensin α-1), Defa-5 (defensin α-5), angiopoietin 4 (Angpt-4), and lysozyme-2 (Lyz-2) in ileal tissues. At least 3 tissues per group were assayed, each in triplicate (* p < 0.05 vs. Ctrl, # p < 0.05 vs. HS).

Figure 4: Immunohistochemical staining (400x) for the expression of tight junction proteins, ZO-1, ZO-2, claudin-1, and occludin, in ileal tissue from hemorrhaged rats treated with EF24 or CLEFMA. The pictures are representative of at least 3 tissues stained from each group. ZO-1, ZO-2, and occludin expression are indicated by brown stain associated with membranous structures (arrowheads), whereas the claudin-1 is stained blue (arrowheads). HS = Hemorrhagic shock.

Figure 5: (A) Expression of tight junction proteins ZO-1, ZO-2, claudin, and occludin in ileal tissue from various groups (n ≥ 3 per group). (B) The immunoblots were analyzed by densitometry. Actin expression was probed to ensure equal loading of total protein in each well and to normalize the densitometry values. (C) The effect of hemorrhagic shock and drug treatments on the phosphorylation status of ZO-1 and occludin proteins. Tyrosine phosphorylation was probed for both ZO-1 and occludin, whereas threonine phosphorylation was examined only for occludin. The ileal tissue homogenate was immunoprecipitated by anti-phospho-tyrosine or anti-phospho-threonine antibodies and the blots were probed with anti-ZO-1 and anti-occludin antibodies. The immunoprecipitation was performed on two separate tissues from each group. HS = Hemorrhagic shock, V = Vehicle, and CL = CLEFMA.

Figure 6: (A) A representative immunohistochemical staining of ileal lipid-binding protein (ILBP) expression (blue stain, arrowheads) in rat ileum. (B) The ileal tissue lysates were immunoblotted with anti-rat ILBP antibody. (C) The immunoblots were analyzed by densitometry. At least 3 randomly selected ileal tissues per group were assayed. Actin expression was probed to ensure equal loading of total protein in each well and to normalize the ILBP densitometry values. (D) Plasma levels of citrulline (n
= 4 per group). HS = Hemorrhagic shock, V = Vehicle, EF = EF24, and CL = CLEFMA (* p < 0.05 vs. Ctrl, # p < 0.05 vs. HS).

**Figure 7:** Effect of EF24 and CLEFMA on hemorrhagic shock-induced increase in intestinal permeability for dextran-FITC4000 and Evan's blue dye (EBD). (A) Approximately 5 cm saline-filled inverted sacs of ileum (n = 4 segments per rat, 4 rats per group) were allowed to equilibrate with dextran-FITC solution at 37°C. After 45 min, the inner contents of the segments were assayed for accumulation of fluorescent dextran. Permeability index was expressed as fluorescence units/cm/h. (B) EBD was injected 1 h before euthanasia and its accumulation in the ileal tissues was estimated by colorimetry (n = 3 per group). The EBD accumulation in ileal tissue was quantitated with respect to the plasma EBD concentration. HS = Hemorrhagic shock, V = Vehicle, and CL = CLEFMA (* p < 0.05 vs. Ctrl, # p < 0.05 vs. HS).
Figure 1

A: Chemical structures of EF24 and CLEFMA.

B: Timeline of catheter implantation, shock, drug treatment, and euthanasia.

C: Graph showing blood pressure (mmHg) with baseline and post-hemorrhage measurements.

D: Bar graph showing mean arterial pressure (MAP) with BL, HS, HS+V, HS+EF, and HS+CL groups at 5 h post-hemorrhage.

E: Graph showing hematocrit (%) with BL, HS, HS+V, EF, and CL groups at 5 h post-hemorrhage.
Figure 2

Control | HS+Vehicle | HS+EF24 | HS+CLEFMA

B

MPO activity (A.U.)

CTRL | HS+V | HS+EF24 | HS+CL

C

CD163 densitometric units

CTRL | HS+V | HS+EF24 | HS+CL

β-actin
Figure 3

A. Tight junction proteins

- ZO-1
- ZO-2
- ZO-3
- Occludin

B. Antimicrobial proteins

- Defa1
- Defa5
- Angpt4
- Lys2
Figure 7
A. FITC-dextran permeability in vitro

B. EBD accumulation in vivo