MODULATION OF THE PERMEABILITY-INDUCING FACTOR ANGIOPOIETIN-2 THROUGH BIFONAZOLE IN SYSTEMIC INFLAMMATION

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ABSTRACT—Background: Vascular barrier breakdown in sepsis represents a key component of the maladaptive host response to infection and the release of endothelial Angiopoietin-2 (Angpt-2) is a mechanistic driver of endothelial hyperpermeability. Angpt-2 is associated with morbidity and mortality but a targeted therapeutic approach is not available. We screened for U.S. Food and Drug Administration (FDA) approved drugs that might have off-target effects decreasing Angpt-2 and therefore, ameliorating capillary leakage. Methods: Endothelial cells were isolated from human umbilical veins (HUVECs) and used for in vitro studies at baseline and after stimulation (FDA-library screening, RT-PCR, ELISA, immunocytochemistry, MTT assay). On the functional level, we assessed real-time transendothelial electrical resistance (TER) using an electric cell-substrate impedance sensing device. Results: We found that the anti-fungal Bifonazole (BIFO) reduces spontaneous Angpt-2 release in a time- and dose-dependent manner after 8, 12, and 24 h (24 h: veh: 15.6 ± 0.7 vs. BIFO: 8.6 ± 0.8 ng/mL, P < 0.0001). Furthermore, we observed a reduction in its intra-cellular content by 33% (P < 0.001). Stimulation with tumor necrosis factor α induced a strong release of Angpt-2 that could analogously be blocked by additional treatment with BIFO (veh: 1.58 ± 0.2 vs. BIFO: 1.02 ± 0.1, P < 0.0001). Quantification of endothelial permeability by TER revealed that BIFO was sufficient to reduce Thrombin-induced barrier breakdown (veh: 0.82 ± 0.1 vs. BIFO: 1.01 ± 0.02, P < 0.05). Conclusion: The antifungal BIFO reduces both release and biosynthesis of the endothelial-destabilizing factor Angpt-2 in vitro thereby improving vascular barrier function. Additional studies are needed to further investigate the underlying mechanism and to translate these findings to in vivo models.

KEYWORDS—Ang-2, angpt-2, capillary leakage, endothelial permeability, inflammation, sepsis, tie2

ABBREVIATIONS—Angpt-1—Angiopoietin-1; Angpt-2—Angiopoietin-2; BIFO—Bifonazole; DAPI—4',6-diamidino-2-phenylindole; FDA—U.S. Food and Drug Administration; HRP—Streptavidin-horseradish peroxidase; HUVEC—Human umbilical vein endothelial cells; ICAM-1—Intercellular adhesion molecule-1; L-Name—Nω-Nitro-L-arginine methyl ester hydrochloride; PMN—Phorbol-12-myristate-13-acetate; TNFα—Tumor necrosis factor α; Veh—vehicle; vWF—von-Willebrand factor

INTRODUCTION

The definition of sepsis was updated to the “sepsis-3 definition” in 2016 (1). Sepsis is now defined as a dysregulated host response to infection with life-threatening organ dysfunction (1–3). If residual organ perfusion cannot supply the cellular oxygen demand, it can progress to septic shock and ultimately, death (1, 2). In 2017, 48.9 million sepsis cases were recorded worldwide with striking 11.0 million people dying (4). Sepsis-related deaths therefore, represent almost one fifth of all global deaths (4). Its mortality is up to 55% for severe sepsis (5). Recently, the World Health Organization (WHO) recognized sepsis as a global health priority (6). Nevertheless, sepsis, septic shock, and its often fatal outcome are not very well-known by the broad public in most countries (7).

The vast majority of sepsis research is currently focusing on highly complex humoral and immune pathways rather than vascular pathophysiology (8, 9). However, in sepsis the endothelial dysfunction seems to play a central role in the pathological host response as virtually all physiological functions of
the endothelium appear to be altered throughout the body (8, 10). Breakdown of the endothelial barrier that clinically leads to the so-called capillary leakage syndrome is a key contributor of multiple organ failure (11, 12). The Angiopoietin (Angpt)-Tie2 system is an important molecular regulator of these pathophysiological processes (9, 13, 14). The transmembrane tyrosine kinase Tie2 is constitutively activated by its protective ligand Angiopoietin-1 (Angpt-1) in quiescence endothelium (9, 13, 14). Angpt-1 ligation leads to Tie2 phosphorylation and activates canonical downstream pathways that maintain endothelial function and integrity (9, 13, 14). Its antagonist Angiopoietin-2 (Angpt-2) is pre-stored within Weibel-Palade bodies of endothelial cells and can be released upon an injurious stimuli, for example, systemic inflammation (14). Tie2 deactivation by excess Angpt-2 leads to endothelial-mediated inflammation and increases endothelial permeability (9, 15–17). Of note, circulating levels of Angpt-2 in septic patients are closely associated with morbidity and mortality (15–17). Therapeutic strategies that target Angpt-2 have been shown to be effective in animal models of sepsis (18, 19). However, so far, no approved treatment targeting this endothelium destabilizing system is available.

In order to overcome difficulties with drug development, we analyzed if approved drugs with well-known safety profiles might have beneficial off-target effects on the release of Angpt-2 in endothelial cells. We therefore screened an U.S. Food and Drug Administration (FDA)-drug library and identified the antifungal Bifonazole (BIFO) as a potential negative modulator of Angpt-2 in vivo. BIFO is a substituted imidazole antifungal that is mainly used for topical application on local mycosis (20). It has a broad activity against different fungal species and even some gram-positive bacteria (20). BIFO can inhibit the ergosterol synthesis by inhibition of the cytochrome P450 depending 14α demethylation and inhibition of HMG-CoA reductase which ultimately leads to malformation of fungal cell membrane (20–23).

We hypothesized that BIFO might decrease endothelial Angpt-2 release thereby improving vascular barrier function.

**MATERIALS AND METHODS**

**Cell culture studies**

Primary endothelial cell isolation was performed according to institutional and governmental guidelines. In brief, human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical veins. HUVECs were isolated with heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA), phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA) and Collagenase (Biochrom, Berlin, Germany). Then, they were grown to confluency in endothelial growth medium (EBM-2) containing 2% FBS according to manufacturer’s instructions (Lonza, Basel, Switzerland). For HUVEC donation, informed consent was obtained and the protocol was determined regarding manufacturer’s recommendation by electrical criteria (1303 ± 2012).

HUVECs were used in passages 3 to 5 and split with Trypsin/EDTA (Sigma–Aldrich, St. Louis, MO). 10 ng/mL recombinant human TNF-α (R&D Systems, Minneapolis, MN), 50 ng/mL Phorbol 12-myristate-13-acetate (PMA) (Merck Millipore, Darmstadt, Germany), 10 μM DMSO for molecular biology (Sigma–Aldrich, St. Louis, MO), 10 μM Mibefradil dibydrochloride hydrate (Sigma–Aldrich, St. Louis, MO), 50 μM TTX-A2 (Sigma–Aldrich, St. Louis, MO), 1 mM Nitro-L-arginine methyl ester hydrochloride (L-Name) (Sigma–Aldrich, St. Louis, MO), 1 μM Wortmannin (Sigma–Aldrich, St. Louis, MO) and 11 μM Thrombin (Merck Millipore, Darmstadt, Germany). Specific Tie2 siRNA (Silence Therapeutics, Berlin, Germany) or control siRNA (oligonucleotide without homology to any known mammalian gene) was transfected with lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific, Waltham, MA) in serum-free medium. Each condition was reproduced for n = 4 to 10 times.

**FDA-approved drug screening**

We analyzed an FDA-approved drug-screening library (Cayman chemicals, Ann Arbor, MI) that contains 875 compounds on their individual effect on the release of Angpt-2 from endothelial cells into the supernatant according to the manufacturer’s instructions. Among others, the library contains anti-diabetic, antiseizure, antipsychotic, anti-infective agents, NSAIDS, and chemotherapeutics.

**Antibodies and reagents**

All reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise specified. Antibodies against Angpt-2 (AF623) (R&D Systems, Minneapolis, MN), vWF (A008202) (Agilent Dako, Santa Clara, CA), VE-Cadherin (556611) (BD Pharmingen, San Jose, CA), Phallloidin/F-Actin (A22833) (Invitrogen, Carlsbad, CA) were utilized. As secondary antibodies goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, CA) and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, CA) were used.

**Enzyme-linked immunosorbent assay**

Enzyme-linked immunosorbent assay (ELISA) was performed for Angpt-2 from endothelial cell culture supernatants and endothelial cell lysates with the commercial human Angpt-2 DuoSet kit (DY623, R&D Systems, Minneapolis, MN). All additionally needed reagents were purchased from R&D Systems except for Normal Mouse Serum (NMS) (Jackson ImmunoResearch Laboratories, Westgrove, PA) and Bovine Serum Albumine (BSA) (Sigma–Aldrich, St. Louis, MO).

**Fluorescent immunocytochemistry**

Coverslips were coated with collagen (Sigma–Aldrich, St. Louis, MO) and HUVECs were grown to confluency. Then, coverslips were fixed with a 1:1 solution of Aceton (Thermo Fisher Scientific, Waltham, MA) and Methanol (Th. Geyer Hamburg, Hamburg) or 2% Paraformaldehyde (Th. Geyer Hamburg, Hamburg). All coverslips were then blocked with 10% donkey serum (Jackson ImmunoResearch, West Grove, PA) and those fixed with 2% Paraformaldehyde were additionally permeabilized with 0.1% Triton X-100 in PBS (Sigma–Aldrich, St. Louis, MO). The primary antibody was incubated for 1 h at room temperature, followed by washing with PBS (Thermo Fisher Scientific, Waltham, MA). The secondary antibody was incubated for 1 h at room temperature. Alexa Fluor 488 donkey anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA), Alexa Fluor 555 donkey anti-goat IgG (Thermo Fisher Scientific, Waltham, MA) and Alexa Fluor 555 donkey anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA) were used as secondary antibodies. Pictures were taken with a Leica DMI 6000B microscope and obtained with the same light exposure conditions and gain.

**RNA – Isolation and quantitative PCR**

RNA was isolated from cultured HUVECs using the RNeasy MicroKit (Qiagen, Hilden, Germany) following manufacturer’s instructions. With the transcriptor First Strand cDNA Synthesis (Roche Diagnostics, Rotkreuz, Switzerland), 0.5 μg of total extracted RNA was reverse transcribed to cDNA. After that, SYBR Green real-time-PCR using a LightCycler 480II (Roche, Basel, Switzerland) was performed. The following primers were used: human β-Actin (fw: CTG GAA CGG TGA AGG TGA CA, rev: AGT CCG CGG CCA CAT TGT G), human Angpt-2 (fw: GCC GCT CGA ATA CGA TGA CT, rev: ACT GAC GCT TCA TTA GCC ACT GAG TGT TGT), human Actin (fw: CTG GAA CGG TGA AGG TGA CA, rev: AGT CCT CGG CCA CAT TGT G). Human Tie2 (fw: CAG TAC GTG A008202 (Agilent Dako, Santa Clara, CA), VE-Cadherin (556611) (BD Pharmingen, San Jose, CA), Phallloidin/F-Actin (A22833) (Invitrogen, Carlsbad, CA) and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, CA) were used.

**Transendothelial electrical resistance**

Special cell culture plates (ibidi, 8W10E) were coated with collagen for 1 h at 37°C and HUVECs were grown at 37°C. Monolayer confluence was determined regarding manufacturer’s recommendation by electrical criteria.

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was able to induce Angpt-2 release about 1.5 fold of its baseline level, whereas additional BIFO treatment significantly reduced Angpt-2 in the supernatants of these stimulated cells to a level known from unstimulated endothelial cells (veh: ELISA from protein lysate after 24 h—was reduced in HUVECs treated with BIFO for 24 h by 33% ($P < 0.0005$) (Fig. 2D). Fluorescent immunocytochemistry for Angpt-2 and von-Willebrand factor (vWF) confirmed these results. In BIFO treated HUVECs, Angpt-2 was significantly decreased, whereas vWF (stored within the same intracellular vesicles) was unchanged—indicating a specific inhibitory mechanism of BIFO on Angpt-2 (Fig. 2E). Given the observed reduction of Angpt-2 in the endothelial lysate, we hypothesized that Angpt-2 transcription might be affected by BIFO. However, this hypothesis could not be confirmed (Fig. S1A, http://links.lww.com/SHK/B293). Interestingly, we saw an upregulation of Tie2 transcription, Angpt-2’s membrane bound target receptor, in BIFO treated cells (Fig. S2A, http://links.lww.com/SHK/B294) and postulated that an earlier described negative feedback loop via PI3K/AKT signaling would negatively affect Angpt-2 biosynthesis. However, experiments with Tie2 RNAi and pharmacological inhibition of PI3K/AKT (Wortmannin) did not support this hypothesis (Fig. S2BC, http://links.lww.com/SHK/B294). Together, BIFO reduces both Angpt-2 release and biosynthesis in a time- and dose-dependent manner but independently from its transcription and Tie2 signaling.

BIFO Modulates Angpt-2 in Systemic Inflammation

**RESULTS**

**Identification of Bifonazole as a possible Angpt-2 suppressor**

We used an FDA-approved drug library for unbiased screening of potential Angpt-2 suppressors and identified the antifungal Bifonazole (BIFO) as one of few drugs that was able to suppress Angpt-2 up to ten times (Fig. 1) making it a suitable candidate for further investigation.

**Bifonazole reduces baseline Angpt-2**

In order to confirm the Angpt-2 suppressing effect of BIFO, we performed experiments on HUVECs. A dose course revealed that 10 μM BIFO was sufficient to reduce the spontaneous release of Angpt-2 from HUVECs into the supernatant (Fig. 2A). A time course with 10 μM BIFO showed that Angpt-2 release in the supernatant was already reduced after 8 h of treatment with the most prominent effect at the latest tested time-point (i.e., 24 h) (veh: 15.6 ± 0.7 vs. BIFO: 8.6 ± 0.8 ng/mL, $P < 0.0001$) (Fig. 2B). HUVEC toxicity upon 10 μM BIFO stimulation was excluded using an MTT cell viability assay (Fig. 2C). Additionally, intracellular Angpt-2—assessed by ELISA quantified by ELISA. The results are shown as a fold change of the mean value and sorted from strongest inhibitor (left) to strongest inducer (right). Angpt-2 indicates Angiopoietin-2; ELISA, Enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; HUVECs, human umbilical vein endothelial cells.

**Transwell assay**

HUVECs were grown to confluency in 24-well plates corning 6.5 mm Transwell inserts with 0.4 μm polycarbonate membranes in the upper chambers (inserts) (1831M002) (Corning Incorporated, Corning, NY). Inserts were prepared with medium and flow-through was collected in order to check membranes. If no flow-through was found, inserts were put onto a new 24-well plate and HUVECs were treated with BIFO for 1 h. Then HUVECs were treated with 1 U/mL Thrombin for 24 h. Streptavidin-horseradish peroxidase (HRP) was added to the upper chamber and flow-through was collected from the lower chamber at indicated time points. Leakage of cell monolayers was quantified by the concentration of HRP in the lower chamber by photometric reading at 450 nm. Further details on measuring leakage with a Transwell-Assay are described elsewhere (26).

**Statistical analysis**

We used GraphPad Prism5 (La Colla, CA) for data analysis and graph generation. Data was tested for Gaussian distribution with Kolmogorov–Smirnov test. When data showed Gaussian distribution unpaired t test with Welch’s correction was used for comparison of two independent groups or One-Way-ANOVA with Bonferroni post test was used for comparison of more than two groups. When data did not show Gaussian distribution Mann–Whitney U test was used for comparison of two independent groups or One-Way-ANOVA with Dunn’s post test was used for statistical comparison of more than two independent groups. Results were seen as significant for $P < 0.05$. Columns are presented as mean ± SD.

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**FIG. 1. Identification of Bifonazole as a possible suppressor of Angiopoietin-2.** HUVECs were grown in 96-well-plates and stimulated for 24 h with different FDA-approved drugs. Angpt-2 release in the supernatants was quantified by ELISA. The results are shown as a fold change of the mean value and sorted from strongest inhibitor (left) to strongest inducer (right). Angpt-2 indicates Angiopoietin-2; ELISA, Enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; HUVECs, human umbilical vein endothelial cells.

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Together, BIFO reduces both Angpt-2 release and biosynthesis in a time- and dose-dependent manner but independently from its transcription and Tie2 signaling.

**Bifonazole reduces Angpt-2 after stimulation**

As BIFO was able to reduce spontaneous Angpt-2 release and biosynthesis, we analyzed its effect on stimulated cells. Therefore, HUVECs were stimulated with three different mediators known to be involved in sepsis pathophysiology such as the cytokine tumor necrosis factor α (TNFα) (27). TNFα was able to induce Angpt-2 release about 1.5 fold of its baseline level, whereas additional BIFO treatment significantly reduced Angpt-2 in the supernatants of these stimulated cells to a level known from unstimulated endothelial cells (veh:
Fig. 2. Bifonazole reduces baseline Angiopoietin-2. (A) HUVECs were treated with different concentrations of BIFO for 24 h. Angpt-2 was measured in the supernatant by ELISA (n = 4–8). (B) HUVECs were treated with 10 μM BIFO or veh for the indicated amount of time. Angpt-2 in the supernatant was quantified by ELISA (n = 5–6). (C) HUVECs were treated with veh, 10 μM BIFO or 10% DMSO for 24 h. Cell viability was measured (n = 4). (D) Angpt-2 was measured in cell lysates by ELISA after 24 h treatment with 10 μM BIFO or veh (n = 5–6). (E) HUVECs were grown to confluency and stimulated with either 10 μM BIFO or veh for 24 h. Fluorescent immunocytochemistry for Angpt-2 (red), vWF (green) and DAPI (blue) was performed (n = 5). Representative pictures were taken with a 63× objective. Columns are presented as mean ± SD. Angpt-2 indicates Angiopoietin-2; BIFO, Bifonazole; DAPI, 4',6-diamidino-2-phenylindole; ELISA, Enzyme-linked immunosorbert assay; HUVECs, human umbilical vein endothelial cells; veh, vehicle.
1.6 ± 0.2 vs. BIFO: 1.0 ± 0.1 ng/mL, P < 0.0001) (Fig. 3A and B). Although Angpt-2 could be decreased by BIFO treatment in the supernatants, mRNA transcription was again not involved in this regulation (Fig. S1 B and C, http://links.lww.com/SHK/B293). Phorbol-12-myristate-13-acetate (PMA) is a strong inductor of exocytosis of Weibel-Palade bodies (28, 29). Consistently, we observed an increase of Angpt-2 in the supernatants up to five times. Even under these extreme conditions, BIFO was still sufficient to reduce Angpt-2 levels in the supernatant by almost 50% after 12 and 24 h (P < 0.0001) (Fig. 3A and B). This decrease let to the hypothesis that BIFO might mechanistically inhibit the exocytosis of Angpt-2 from Weibel-Palade bodies. As intracellular calcium plays an important role in exocytosis regulation, we analyzed the effects of the t-type calcium channel blockers Mibefradil and TTA-A2 (30, 31) (Fig. S3AB, http://links.lww.com/SHK/B295). Additionally, we co-stimulated HUVECs with the nitric oxygen synthase (NOS) inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-Name) (32) (Fig. S3C, http://links.lww.com/SHK/B295). These studies, however, did not confirm our assumption. Lastly, BIFO also reduced the Angpt-2 releasing effect of thrombin (veh: 1.2 ± 0.04 vs. BIFO: 0.5 ± 0.06 ng/mL, P < 0.0001) (Fig. 3A and B). Together, the data indicate that BIFO counteracts the induction of Angpt-2 release independently from the endothelium mediator.

**Functional barrier improvement**

As BIFO was able to reduce Angpt-2 levels in the supernatant in stimulated HUVECs, its effect on permeability was tested in functional settings. First, TER was measured at different time points in order to objectively quantify endothelial permeability. As BIFO showed its strongest Angpt-2 inhibitory effect after 24 h, HUVECs were pre-incubated with BIFO for 24 h and then stimulated with thrombin. Thrombin temporarily induced capillary leakage, which lead to a decrease in impedance measured over cell membranes. BIFO partially protected this hyperpermeability shown by higher impedences throughout the whole experiment (Fig. 4A). Greatest differences were shown after 2.5 h (veh: 0.82 ± 0.10 vs. BIFO: 1.01 ± 0.02, P < 0.05) (Fig. 4B).

Additionally, a classical Transwell-Assay was performed to confirm BIFO’s protective functional anti-permeability effect (Fig. 4C). Within the Transwell-Assay, hyperpermeability can be measured by the collected flow-through through an endothelial monolayer that directly correlates with the endothelial permeability. HUVECs were co-stimulated with BIFO and Thrombin and flow-through was analyzed. As expected, permeability was clearly increased upon Thrombin stimulation and BIFO could antagonize this effect significantly after 8, 12 (veh: 2.21 ± 0.51 vs. BIFO: 1.35 ± 0.23, P < 0.001), and 24 h (Fig. 4C).

After quantifying BIFO’s protective effect on the endothelium, endothelial cellular architecture was visualized. HUVECs were again treated with BIFO and Thrombin. Fluorescent immunocytochemistry was performed for VE-Cadherin, F-Actin and 4,6-diamidino-2-phenylindole (DAPI) (Fig. 4D). In healthy HUVECs structural proteins like F-Actin were ordered appropriately in a cortical configuration and intercellular junctional proteins, represented by VE-Cadherin, were clearly visible and continuously expressed surrounding every cell. In Thrombin treated HUVECs, however, the cytoskeleton was massively stressed and intercellular interaction disconnected indicating the morphological correlate of the former functional permeability experiments. To better visualize the locations where leakiness occurs most distinctively, we further schematically mapped the disconnected cells. Leakage was still

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**Fig. 3.** **Bifonazole reduces Angiopoietin-2 after stimulation.** 10 ng/mL TNF-α, 1 U/mL Thrombin, 50 ng/mL PMA or control were applied to HUVECs for (A) 12 h or (B) 24 h after pretreatment with 10 μM BIFO or veh for 1 h (n = 5–12). Angpt-2 was measured in the supernatants by ELISA. Columns are presented as mean ± SD. Angpt-2 indicates Angiopoietin-2; BIFO, Bifonazole; HUVECs, human umbilical vein endothelial cells; PMA, phorbol-12-myristate-13-acetate; veh, vehicle.
visible in BIFO and Thrombin-treated cells; however, it was strongly reduced (Fig. 4D).

In summary, our observations indicate that BIFO can effectively counteract mediator induced endothelial permeability (most likely via Angpt-2 reduction).

**DISCUSSION**

Here, we systematically screened an FDA drug library for potential candidates that might reduce endothelial Angpt-2 in a drug-repurposing context. Using this unbiased approach, the
antifungal Bifonazole (BIFO) was found as a possible suppressor of Angpt-2. A series of in vitro experiments confirmed BIFO’s off-target effect on Angpt-2 biosynthesis and exocytosis in a dose- and time-dependent manner. Moreover, functional analysis demonstrated that BIFO was sufficient to ameliorate mediator induced endothelial permeability.

So far, neither this drug nor any other antifungal have been implicated in angiopoietin processing. First, we analyzed BIFO’s effect on endothelial Angpt-2 release under baseline and mediator-stimulated conditions. Given that sepsis-mediators can enhance the release of Angpt-2, it is of no surprise that BIFO’s effect was most potent in the context of this situation. To experimentally induce the release of Angpt-2 into the supernatant, TNFα (an essential contributor to sepsis pathology in humans), thrombin (an inducer of vascular permeability) (33, 34) and PMA were used (28, 29). PMA is not involved in sepsis pathophysiology per se but is a well-known inducer of Weibel-Palade-body exocytosis, the primary source of Angpt-2. All three stimuli showed a strong and reproducible phenotype of vascular leakage. Most importantly, BIFO co-stimulation was sufficient to reduce this pathologically driven Angpt-2 release in all three conditions. BIFO also reduced the intracellular Angpt-2 content indicating a potential additional effect on its biosynthesis.

In clinical practice, BIFO has only been used as a topical antifungal (20) and systemic concentrations studied under experimental conditions were—from a PK/PD viewpoint—very low in order to assess potential systemic side effects associated with its local application (35–37). It is important to mention that we have applied a rather high dose of BIFO in our in vivo studies. Nevertheless, the ED50 is unknown and we have excluded any cytotoxic effects using our 10 μM dose in different cell types.

Mechanistically, we aimed to differentiate if reduced Angpt-2 concentration in the supernatant was a direct effect on exocytosis or an indirect effect via reduction of de novo protein synthesis. Indeed, the observed decrease in intracellular Angpt-2 suggested a role of protein synthesis although Angpt-2 transcription—as it has been earlier shown for the anti-migraine drug Flunarizine (27)—was unaffected upon BIFO stimulation. Interestingly, the angiopoietin receptor Tie2 was upregulated by over 50% upon BIFO stimulation indicating that an earlier described negative feedback loop on Angpt-2 as it has been suggested by Daly et al. (38, 39) might be involved (Fig. S2A, http://links.lww.com/SHK/B294). However, neither Tie2 RNAi nor pharmacological blockade of its major downstream pathway (PI3K/AKT) did abolish BIFOs effect on Angpt-2 (Fig. S2 B and C, http://links.lww.com/SHK/B294). Aside from transcriptional mechanisms, we looked into potential posttranslational modifications and analyzed degradation processes via the canonical ubiquitin-pathway but did not find any involvement (data not shown). Finally, we analyzed BIFO’s effect on exocytosis. As BIFO’s Angpt-2 lowering effect is not affected by intracellular calcium, nor nitric oxygen concentration, typical mechanisms of exocytosis do not seem to be involved in BIFO’s Angpt-2 regulating mechanism (Fig. S3A–C, http://links.lww.com/SHK/B295). Experiments that help to decipher the exact underlying mechanism are highly desirable.

CONCLUSIONS

A potential off-target effect of the anti-fungal BIFO on Angpt-2 release and synthesis was confirmed in a series of in vivo experiments after initial detection in an unbiased FDA drug library screening. Given the protective effect on vascular permeability, BIFO might hold promise as a modulator of the injurious host response to an infection.

Further in vivo studies are needed in order to confirm these findings in a living organism and to further translate them into clinical use.

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Recently, anti-inflammatory effects of BIFO via decreasing intercellular adhesion molecule (ICAM)-1 expression have been shown in human skin model systems (22). Given that it has been shown that ICAM-1 is regulated downstream of Tie2 (e.g., via Angpt-2) and that ICAM-1 is substantially increased during systemic inflammation (40, 41) it could be very well that the described effect of BIFO on ICAM-1 is an Angpt-2 dependent mechanism. We have not investigated this interesting question in this project.

To test functional effects of BIFO on endothelial permeability two distinct methods were used. In both assays (TER and transwell), BIFO co-stimulation of endothelial cells was sufficient to reduce spontaneous and mediator induced vascular barrier breakdown. Together, BIFO not only reduces Angpt-2, but also stabilizes the endothelial barrier function in an experimental setting in vivo.

This study has limitations. First of all, it is hypothesis generating in nature and the in vivo findings require further in vivo confirmation. Secondly, the underlying mode of action of BIFO’s effect on Angpt-2 in the endothelium remains unknown and lastly, it is unclear if BIFO might hold promise as a potential therapeutic strategy against injurious Angpt-2 release in sepsis.

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