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## Late intervention with the small molecule BB3 mitigates postischemic kidney injury

[Prakash Narayan](#)<sup>✉1</sup>, [Bin Duan](#)<sup>1</sup>, [Kai Jiang](#)<sup>1</sup>, [Jingsong Li](#)<sup>1</sup>, [Latha Paka](#)<sup>1</sup>, [Michael A. Yamin](#)<sup>1</sup>, [Scott L. Friedman](#)<sup>2</sup>, [Matthew R. Weir](#)<sup>3</sup>, and [Itzhak D. Goldberg](#)<sup>1</sup>

<sup>1</sup>Department of Preclinical Research, Angion Biomedica Corporation, New York, New York;

<sup>2</sup>Division of Liver Diseases, Icahn School of Medicine at Mount Sinai, New York, New York; and

<sup>3</sup>Division of Nephrology, Department of Medicine, University of Maryland Medical Center, Baltimore, Maryland

<sup>✉</sup>Corresponding author.

Address for reprint requests and other correspondence: P. Narayan, Angion Biomedica Corporation, 51 Charles Lindbergh Blvd., Uniondale, NY 11553 (e-mail: [pnarayan@angion.com](mailto:pnarayan@angion.com)).

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### Abstract

Ischemia-reperfusion-mediated acute kidney injury can necessitate renal replacement therapy and is a major cause of morbidity and mortality. We have identified BB3, a small molecule, which when first administered at 24 h after renal ischemia in rats, improved survival, augmented urine output, and reduced the increase in serum creatinine and blood urea nitrogen. Compared with control kidneys, the kidneys of BB3-treated animals exhibited reduced levels of kidney injury molecule-1, neutrophil gelatinase-associated lipocalin, and reduced tubular apoptosis and acute tubular necrosis but enhanced tubular regeneration. Consistent with its hepatocyte growth factor-like mode of action, BB3 treatment promoted phosphorylation of renal cMet and Akt and upregulated renal expression of the survival protein Bcl-2. These data suggest that the kidney is amenable to pharmacotherapy even 24 h after ischemia-reperfusion and that activation of the hepatocyte growth factor signaling pathway with the small molecule BB3 confers interventional benefits late into ischemia-reperfusion injury. These data formed, in part, the basis for the use of BB3 in a clinical trial in kidney recipients presenting with delayed graft function.

**Keywords:** kidney, small molecule, ischemia-reperfusion, therapy

ISCHEMIA-REPERFUSION (IR)-mediated acute kidney injury (AKI), often manifested during cardiopulmonary bypass, renal transplantation, and volume restitution after hemorrhagic shock, is a major cause of morbidity and mortality ([3](#), [5](#), [7](#), [16](#), [34](#), [40](#)). Extensive studies have suggested that renal IR triggers a proinflammatory cascade that culminates in a tubular epithelial cell response including tubular apoptosis, tubular flattening or dilation, and acute tubular necrosis ([6](#), [15](#), [30](#)). Identifying an effective strategy to promote tubular survival and repair is essential for minimizing damage and accelerating functional recovery after AKI, keeping patients off dialysis, and preserving long-term kidney function.

Hepatocyte growth factor (HGF) is a pleiotropic growth factor with roles in diverse biological processes including organ development, tissue homeostasis, and injury repair ([26](#), [33](#), [45](#)). In models of AKI secondary to various insults including IR, cold preservation/transplantation, or toxin exposure, administration of recombinant human HGF attenuates injury and augments renal function ([9](#), [14](#), [27](#), [31](#),

37). These biological actions of HGF are mediated by the transmembrane receptor cMet, a receptor tyrosine kinase. In vivo, HGF expression is restricted to cells of mesenchymal origin, whereas cMet is more ubiquitously expressed, although more preferentially, in epithelial cells, including the tubular epithelium (28, 33). HGF administration in the setting of AKI promotes tubular regeneration, mitigates acute tubular necrosis, and reduces renal dysfunction via activation of the cMet-Akt axis and upregulation of cell survival proteins, including Bcl-2 (20, 27, 28, 32, 49). A salient feature of this receptor is its upregulation after injury in at-risk tissues such as the ischemic penumbra (28, 32, 39). In preclinical models of AKI and acute tissue injury, renal cMet upregulation has been observed at 6 h after insult with peak expression at ~24 h, whereas HGF release from stores and de novo synthesis are early and transient (27, 31, 39). Delayed receptor upregulation thus provides a unique and expanded opportunity for therapeutic interventions via activation of the HGF/cMet pathway. In fact, electroporation-mediated HGF gene transfection after insult has been reported to be therapeutic, promoting repair of peritubular capillaries and tubular epithelial cells (25). While HGF administration and subsequent activation of the HGF/cMet-Akt axis has enormous potential for the treatment of AKI, translation of protein- or gene-based therapies into the clinic remains fraught with obstacles including the short-half-life of the protein and the challenges of gene therapy approaches.

BB3, a small molecule identified by reductive distillation of HGF-like peptides via a search engine combining phage display and molecular modeling, emulates the biological activity of HGF and is therapeutic in the setting of ischemic and IR tissue injury (4). In the present study, we evaluated the interventional effects of BB3 in IR-related AKI.

## MATERIALS AND METHODS

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### Kidney IR

The study protocol was submitted to and approved by the Institutional Animal Care and Use and Committee of Angion Biomedica. Animals were allowed to acclimatize for a minimum of 5 days before use and had free access to water and standard rodent chow. Humane end points were used in the animal survival experiment and throughout the in-life protocols.

Briefly, ~10-wk-old male Sprague-Dawley rats (275–300 g body wt) were anesthetized with ketamine (25 mg/kg ip) and xylazine (5 mg/kg ip) and placed on a heating pad table to maintain a core body temperature of ~37.5°C (9, 28). A midline laparotomy was made, and the left renal pedicle subjected to 45 min of ischemia using a microvascular clamp followed by reperfusion for up to 5 days. The contralateral kidney was excised at reperfusion. Extended-release buprenorphine (0.65 mg/kg sc, QD) was administered before the animals were returned to their cages. A sham group comprised animals that had the left kidney manipulated without subjection to ischemia. While all animals were monitored twice daily, postsurgical animals were monitored up to 4 times/day. Since mortality was anticipated in the study and one of the end points was an improvement in survival, before the start of the study it was determined that animals in extremis, adjudged by a trained laboratory animal facility observer blinded to the treatment groups, would be humanely euthanized. Drug effects were assessed on animals that succumbed to renal dysfunction or euthanasia (CO<sub>2</sub>). For the determination of kidney parameters [urine output, serum creatinine (SCr), and blood urea nitrogen (BUN)], urine was collected over successive 24-h periods and blood drawn from the retroorbital sinus. Baseline values were recorded from animals at least 3 days before surgery. Postischemic values were collected every 24 h up to 96 h. SCr and BUN were determined in serum samples by a commercial laboratory. To evaluate treatment effects, 24 h after the onset of reperfusion, animals ( $n = 36$  animals/group) were randomized to BB3 (2.0 mg/kg iv) or its vehicle. BB3 was synthesized using a three-step process by a commercial manufacturer. BB3 drug product was prepared at a concentration of 3 mg/ml in polyethylene glycol 300-Tween 80-PBS [5:1:4 (vol/vol/vol)]. An infusion volume of 0.2 ml was delivered into the penile vein via a slow push. Animals were administered vehicle or BB3 once daily until

euthanization. To identify any effects of BB3 on urine output in healthy, i.e., non-IR kidneys, three animals each were administered vehicle or BB3 and housed in metabolic cages for a period of 24 h. To determine if the effect of BB3 on urine output was associated with better preservation of the renal microarchitecture and given that the control group experienced uniform mortality by 96 h, a subset of animals was euthanized at 52 h of reperfusion after two administrations (at 24 or 48 h) of BB3 or vehicle. Given the highly consistent response to BB3 in this model and low variability, 3 animals/group were deemed sufficient. To determine the effects of BB3 on kidney cMet phosphorylation, animals were subjected to 45 min of renal ischemia and 24 h of reperfusion. Vehicle ( $n = 3$ ) or BB3 (2 mg/kg iv,  $n = 3$ ) was administered at 24 h, the animals were euthanized ~20 min later, and kidney samples stored in formalin until analysis.

## Histopathology

**Tubular regeneration.** Kidney sections were incubated with mouse anti-rat monoclonal proliferating cell nuclear antigen (PCNA) antibody (Dako, Carpinteria, CA) and quantified in a blinded fashion. For tubular regeneration, PCNA-positive nuclei were counted in several fields in each slide and the mean of each section and group was evaluated.

**Tubular injury.** Histopathological examination of formalin-fixed, paraffin-embedded, 4- to 5- $\mu$ m-thick longitudinal cross-sections of the left kidney stained with hematoxylin and eosin was performed. Specimens were examined in a blinded manner under light microscopy, as previously described (43) with minor modifications. Briefly, three high-power fields (at  $\times 40$  magnification, representing ~50 tubules) from the cortex and outer medulla of each kidney were examined and graded for predominant injury patterns including tubular necrosis and tubular dilation (43). Overall kidney injury was graded on a scale of 0–4.

**Tubular apoptosis.** TUNEL staining of renal tissue sections was performed using The DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) according to the manufacturer's instructions. The fluorescein-stained area of cells undergoing apoptosis was captured using confocal microscopy (Leica) and quantified using Bioquant image-analysis software (Bioquant Life Science, Nashville, TN).

## Immunohistochemistry for cMet, phosphorylated Met, NGAL and KIM-1, phosphorylated Akt, and Bcl-2.

Kidney tissue sections (4  $\mu$ m thick) from formalin-fixed, paraffin-embedded tissue samples were deparaffinized and treated with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. For heat-induced antigen retrieval, tissue sections were immersed in 0.01 mol/l citrate buffer (pH 6.0) and treated in a microwave oven for 20 min at 620 W. After being cooled for 30 min at room temperature, samples were immersed in blocking buffer and then incubated with primary antibodies including those against rat cMet (Santa Cruz Biotechnology, Paso Robles, CA), phosphorylated (p)Met (Cell Signaling, Danvers, MA), pAkt (R&D Systems, Minneapolis, MN), Bcl-2 (Santa Cruz Biotechnology, Dallas, TX), neutrophil gelatinase-associated lipocalin (NGAL; BOSTER, Pleasanton, CA), and kidney injury molecule-1 (KIM-1) overnight at 4°C. After being washed, sections were incubated with appropriate alkaline-phosphatase-conjugated secondary antibodies for 1 h followed by a substrate mixture containing  $\beta$ -naphthyl phosphate and fast blue B and then mounted on slides. Photomicrographs of the cortex and outer medulla were taken by an observer blinded to the treatment groups, and the positive staining of each biomarker was quantified using Bioquant image-analysis software by another observer blinded to the treatment groups. All histological quantified data were expressed as arbitrary units in the figures.

## Data Analysis

Survival differences were analyzed for significance using the log-rank (Mantel-Cox) test. Other data are presented as means  $\pm$  SE. Between-group effects were analyzed by one-way ANOVA followed by a Tukey's post hoc test.  $P$  values of  $<0.05$  were considered significant.

## RESULTS

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### Renal IR

Based on a previous report demonstrating the therapeutic efficacy of BB3 in ischemic stroke, we determined whether BB3 administration, starting 24 h after insult, mitigates renal postischemic renal dysfunction. Rats subjected to a 45-min period of normothermic renal ischemia followed by contralateral nephrectomy exhibited reduced urine output (Fig. 1A) and increasing SCr (Fig. 1B) and BUN (Fig. 1C) over a 96-h reperfusion period as well as significant mortality (Fig. 1D). In contrast, animals randomized to BB3 at 24 h of reperfusion experienced a profoundly different trajectory for urine output (Fig. 1A). Whereas control animals, on average, experienced oliguria or anuria over the 24- to 96-h reperfusion period, BB3-treated animals experienced increased urine output over this period. Thus, renal dysfunction, manifested as oliguria or anuria, was more than likely the cause of death in this model. Accompanying the increase in urine output in the BB3-treated cohort were decreased SCr (Fig. 1B) and BUN (Fig. 1C) and improved survival (Fig. 1D). Importantly, in contrast to its effects on urine output after renal IR, BB3 did not influence urine output in animals ( $n = 3$  animals/group) with healthy kidneys ( $51 \pm 1.5 \text{ ml}\cdot\text{kg}^{-1}\cdot 24\text{-h vehicle}^{-1}$  vs.  $56 \pm 3 \text{ ml}\cdot\text{kg}^{-1}\cdot 24\text{-h BB3}^{-1}$ ,  $P = 0.3$ ). Taken together, these results suggest that the drug effects on urine output are limited to the postischemic kidney, that the drug has an immediate and profound impact on postischemic renal function, and that the drug mitigates renal IR dysfunction, at least in part, by augmenting urine output.

### BB3 Mitigates Renal Damage

Since IR-related AKI is associated with disruption of the renal microarchitecture and tubular cell death (apoptosis and necrosis), we determined whether the functional effects of BB3 are associated with a reduction in cell death and/or tubular regeneration. Given the significant mortality in the vehicle-treated cohort by 96 h after reperfusion, these histopathological experiments were conducted at ~52 h of reperfusion after animals had been dosed with the drug at 24 and 48 h. As shown in Fig. 2, A and B, and consistent with the published literature, this model of AKI is associated with increased tubular KIM-1 expression. Treatment with BB3 reduced the increase observed with postischemic KIM-1 in the kidney (Fig. 2, A and B). In addition, BB3 reduced the increased renal NGAL expression in this model of AKI (Fig. 2, C and D). Associated with renal IR injury, there was an increase in tubular apoptosis in the outer medulla and cortical regions of the kidney (Fig. 3A). Consistent with its effects on tubular injury markers, BB3 treatment reduced tubular epithelial apoptosis (Fig. 3B). The effects of BB3 on the renal microarchitecture were examined in hematoxylin and eosin-stained sections. Compared with the sham cohort, at 52 h after reperfusion, outer medulla and cortical kidney sections in the IR-vehicle cohort exhibited loss of nephrons, hemorrhage, tubular dilation, and severe acute tubular necrosis (Fig. 4, A–D, respectively). In contrast, kidneys in the IR-BB3 cohort exhibited better preservation of nephron mass and reduced hemorrhage, tubular dilation, and acute tubular necrosis (Fig. 4, A–D, respectively). We sought to determine whether BB3, in addition to its cytoprotective effects, impacts tubular regeneration, which can be visualized using anti-PCNA antibody (Fig. 5A). As shown in Fig. 5B, drug treatment enhanced postischemic tubular regeneration.

### Mechanism Underlying BB3 Renoprotection

It has been previously reported that BB3 cytoprotection is mediated via activation of the HGF signaling path. A series of experiments was therefore undertaken to determine if activation of the HGF signaling pathway is the basis for BB3 renoprotection in the setting of IR injury. The literature indicates that tubular epithelial cMet phosphorylation underlies the renoprotective and reparative actions of HGF (28, 31, 32). Immunohistochemical analyses of kidneys were therefore undertaken to determine the effects of BB3 on tubular epithelial cMet. As shown in Fig. 6, A and B, in kidneys from animals subjected to 45 min of renal

ischemia and 24 h of reperfusion, treatment with BB3 was associated with a greater than threefold increase in the phosphorylation of tubular cMet in the outer medulla and cortical regions of the kidneys. Phosphorylation of Akt and upregulation of cell survival proteins including Bcl-2 are pivotal events mediating the tissue-protective actions of the HGF-cMet axis (28, 32). Immunohistochemical analysis of 52-h reperfused kidneys showed an approximately fourfold increase in tubular pAkt staining in the BB3-treated cohort (Fig. 7, A and B), which correlated with the increased tubular pMet status and effects of this drug on maintaining tubular integrity and attendant preservation of renal function. As shown in Fig. 7, C and D, treatment with BB3 was associated with a four- to fivefold increase in renal Bcl-2 expression, a phenomenon consistent with activation of the cMet-Akt axis.

## DISCUSSION

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This study demonstrates that the small molecule BB3, with HGF-like activity, administered starting 24 h after renal IR, improves survival, augments urine output, reduces SCr and BUN, attenuates tubular injury and tubular cell death, and promotes tubular regeneration. In fact, to the best of our knowledge, this is the first demonstration of a successful intervention 24 h after renal ischemia by a HGF-like small molecule.

Tubular cellular injury and death are hallmark features of AKI, with emerging evidence suggesting that in addition to acute tubular necrosis, apoptotic death also contributes to tubular loss (28, 32). The cells of the renal proximal tubule are under a large metabolic demand due to their role in bulk reabsorption of glomerular filtrate, rendering the epithelial cells lining this segment highly susceptible to injury after IR. Tubular epithelial cells respond to such an insult with shedding of the brush border or cell death due to either necrosis or apoptosis (28, 32, 43). Identifying an effective strategy to promote tubular survival and/or regeneration is essential for minimizing kidney damage and accelerating repair and recovery after AKI. This is important because IR-related AKI is associated with increased risk of renal replacement therapy, chronic kidney disease, and mortality (18, 29). Despite decades of research, there is no effective therapy for patients with AKI. Hydration and/or supportive dialysis remain the mainstay for management of this population.

A hallmark feature of the HGF/cMet pathway is the rapid, sustained, and localized upregulation of the receptor after tissue injury. Immunohistochemical studies have indicated that cMet is upregulated in renal tubules after AKI. Site-specific association between cMet induction and structural injury in AKI suggests that upregulation of this receptor is a defensive response of the kidneys to protect tubular epithelial cells (28, 31, 32, 45, 48). Consistent with this view, tubule-specific depletion of cMet aggravates kidney injury induced by toxic or ischemic insult and is evidenced by renal dysfunction, structural injury, and apoptosis (28, 31, 32). The ligand for cMet, HGF, is released from stores into the circulation and is rapidly upregulated in multiple organs after AKI, suggesting that the HGF/cMet signaling system targets its action to injured tissue by site-specific upregulation of its receptor while maximally mobilizing its ligand. However, HGF upregulation is transient and HGF is rapidly cleared by the liver, thus having a very short systemic half-life (<4 min) (1, 2). Supplementing HGF in the form of exogenously administered protein or gene therapy protects renal epithelial cells from both apoptotic and necrotic death and preserves the structural and functional integrity of renal tubules in AKI secondary to a variety of insults (9, 14, 27, 31, 33, 37).

The short half-life of HGF necessitates continuous exogenous administration to achieve a therapeutic effect. Furthermore, administration of HGF, as gene or protein therapy, is impractical in a clinical setting. As previously reported (4), BB3, a small molecule identified via a search engine combining phage display and molecular modeling, emulates the tissue-protective activity of HGF. In the setting of ischemic stroke, BB3 has a therapeutic window of at least 6 h in the rat. In that study (4), the neurobehavioral benefit conferred by BB3 was accompanied by enhanced cMet phosphorylation in the ischemic cortex. Given the challenges associated with successful interventional therapy after ischemic stroke, these data support the

notion that the HGF/cMet pathway represents an expanded window for pharmacologic intervention in multiple tissues and that BB3 is an effective therapeutic modality for mitigating tissue dysfunction after injury.

The renoprotective effects of BB3 observed in this study are consistent the aforementioned effects of activating the HGF/cMet pathway. When administered starting 24 h after a 45-min period of normothermic ischemia, BB3 attenuated mortality, augmented urine output, and decreased the elevations in BUN and SCr. While this drug does not affect urine output in healthy animals, even a single administration of BB3 24 h after IR had an immediate and profound impact on renal physiology evidenced by the trajectories of urine output, SCr, and BUN.

Consistent with a previous report, BB3 shares the HGF/cMet signaling pathway. In the reperfused kidney, treatment with BB3 resulted in increased phosphorylation of kidney cMet which immunohistochemical analysis showed localized to renal tubules. Activation of this pathway was associated with reduced expression of KIM-1 and NGAL, two of the most highly induced proteins in the kidney after ischemic or nephrotoxic AKI. KIM-1, a type 1 transmembrane glycoprotein, is expressed only minimally in the normal adult kidney but is dramatically upregulated in the S3 segment of the proximal tubule in postischemic kidneys and in proximal tubular epithelial cells in biopsies from patients with acute tubular necrosis (19, 38). The reduction in KIM-1 staining in the BB3-treated cohort suggests that the drug abrogates renal proximal tubule injury after IR. Preclinical transcriptome profiling studies have identified *Ngal* to be one of the most upregulated genes in the kidney very early after AKI in animal models (11, 12).

Immunohistochemical studies have demonstrated that NGAL protein, barely detectable in control mouse kidney, is upregulated predominantly in proximal tubules within 1–3 h of ischemia (11, 12). Kidney biopsies in subjects with AKI have demonstrated an intense accumulation of immunoreactive NGAL in cortical tubules, confirming NGAL as a sensitive index of established AKI in humans (13). The reduction in NGAL immunostaining with BB3 is consistent with protection of BB3 against tubular injury. Supporting this notion are our findings that treatment with BB3 mitigated tubular dilation, tubular apoptosis, and acute tubular necrosis. In addition to cytoprotection, tubular regeneration represents an important mechanism by which cMet signaling improves outcome in AKI (13, 28, 32). While cytoprotection prevents subsequent or additional injury, tubular regeneration is key to renal repair. Consistent with this reparative aspect of HGF/cMet activation, treatment with BB3 enhanced tubular regeneration in the postischemic kidney.

Experimental evidence indicates that activation of the HGF/cMet pathway effects tissue protection via activation of AKT and upregulation of cell survival proteins including Bcl-2 (9, 10, 28, 32, 35). In proximal tubular epithelial cell cultures, HGF has been shown to promote tubular cell survival by inhibiting apoptosis via activating Akt phosphorylation and upregulation of cytoprotective proteins. HGF rescued human proximal tubular cells from cyclosporin-induced apoptosis by enhancing Akt phosphorylation and upregulating Bcl-2 (10). Bcl-2 expression was enhanced and tubular atrophy reduced in HGF-transfected rats subjected to unilateral ureteral obstruction (35). In vivo, deletion of tubular cMet correlates with a marked reduction in Akt signaling and loss of downstream antiapoptotic and proliferative signaling (28, 39). In fact, the cMet-pAkt signaling cascade is a nodal pathway for cytoprotection and proximal tubular repair as activation is critical for promoting survival and repair of sublethally injured proximal tubule cells after IR, providing a sufficient reservoir of cells for subsequent repair. In the present study, treatment with BB3 was associated with increased pAkt immunostaining in the tubules and increased renal Bcl-2 expression. Taken together, these data suggest that activation of the cMet-pAKT signaling pathway and upregulation of Bcl-2 mediates, at least in part, the effects of BB3 on the renal microarchitecture and renal function.

The literature indicates that the renoprotective effects of HGF are mediated, at least in part, via its vasoactive effects on the renal arterioles, preservation of endothelial integrity, and promotion of neoangiogenesis. Measuring the effect of BB3 on these variables was beyond the scope of this study, and it

is possible that BB3 renoprotection may also be mediated via such activities. In good laboratory practice safety studies with BB3, no effect of this drug has been observed in mean arterial pressures. Nevertheless, HGF-like effects of this drug on renal blood flow cannot be excluded.

In summary, BB3 administration, 24 h after renal IR, mitigates tubular injury and death, augments renal output, reduces the elevations in BUN and SCr, and improves survival. Consistent with a previous report, these actions of BB3 appear to be mediated via activation of the HGF signaling pathway. Based on these data and the safety profile of this drug, BB3 is now in clinical trials in AKI postcardiopulmonary bypass and in patients presenting with delayed graft function after kidney transplantation ([21](#), [22](#), [24](#)).

## GRANTS

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## DISCLOSURES

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One or more authors own stock in Angion Biomedica Corporation.

## AUTHOR CONTRIBUTIONS

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P.N. and I.D.G. conception and design of research; P.N. and L.P. analyzed data; P.N. and L.P. interpreted results of experiments; P.N. and L.P. prepared figures; P.N. drafted manuscript; P.N., M.Y., S.L.F., and M.R.W. edited and revised manuscript; P.N. approved final version of manuscript; B.D., K.J., J.L., and L.P. performed experiments.

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## Figures and Tables

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**Fig. 1.**

BB3 attenuates mortality and improves postischemic renal function. Starting at 24 h after 45 min of renal ischemia and contralateral nephrectomy, animals ( $n = 36$  animals/group) were randomized (arrow) to BB3 (2 mg/kg iv) or vehicle treatment administered daily. BB3 augmented postischemic renal output (*A*), decreased the elevation in serum creatinine (SCr; *B*) and blood urea nitrogen (BUN; *C*), and improved survival (*D*). \* $P < 0.05$ , vehicle vs. BB3.

**Fig. 2.**

BB3 attenuates markers of tubular injury. *A*: in animals ( $n = 3$  animals/group) subjected to 45 min of renal ischemia, contralateral nephrectomy, and 52 h of reperfusion, tubular kidney injury molecule (KIM)-1 expression was evident in outer medulla and cortex sections of the kidneys. *B*: treatment with BB3, at 24 and 48 h after reperfusion, mitigated the increase in tubular KIM-1. *C* and *D*: the ischemia-reperfusion (IR)-mediated increase in renal neutrophil gelatinase-associated lipocalin (NGAL), which was reduced with BB3 treatment. a.u., arbitrary units.  $*P < 0.05$ , vehicle vs. BB3.

**Fig. 3.**

BB3 attenuates tubular epithelial apoptosis. *A*: in animals ( $n = 3$  animals/group) subjected to 45 min of renal ischemia, contralateral nephrectomy, and 52 h of reperfusion, examination of the outer medulla and cortical kidney sections showed that several tubular epithelial cells from the vehicle-treated cohort were FITC-TUNEL positive. *B*: treatment with BB3, at 24 and 48 h of reperfusion, mitigated tubular apoptosis.  $*P < 0.05$ , vehicle vs. BB3.

**Fig. 4.**

BB3 preserves renal structure and attenuates acute tubular necrosis. The outer medulla and renal cortex (hematoxylin and eosin stained) in sham animals ( $n = 3$ ) or animals ( $n = 3$  animals/group) subjected to 45 min of renal ischemia, contralateral nephrectomy, and 52 h of reperfusion were evaluated and graded (scale: 0–4) for hemorrhage, tubular dilation, and tubular necrosis. *A*: representative micrographs showing widespread damage in the renal microarchitecture of an IR-vehicle kidney compared with an IR-BB3 kidney or sham kidney. Treatment with BB3 at 24 and 48 h of reperfusion mitigated the disruption in renal architecture, reducing hemorrhage (*B*), tubular flattening/dilation (*C*), and acute tubular necrosis (*D*). \* $P < 0.05$ , vehicle vs. BB3.

**Fig. 5.**

BB3 promotes tubular regeneration. *A*: punctate proliferating cell nuclear antigen (PCNA) staining ( $\times 20$ , arrows) of nuclei within regenerating tubules in BB3-treated animals ( $n = 3$ ). *B*: postischemic tubular regeneration was augmented in BB3- versus vehicle-treated animals.  $*P < 0.05$ , vehicle vs. BB3.

**Fig. 6.**

Phosphorylation of tubular cMet in postischemic kidneys. *A*: after 45 min of normothermic ischemia and 24 h of reperfusion, immunohistochemical analysis of kidneys from animals ( $n = 3$ ) treated with BB3 showed phosphorylation of tubular cMet (arrows) in the cortex. *B*: compared with the IR-vehicle ( $n = 3$ ) cohort, BB3 treatment was associated with an ~4-fold increase in cortical tubular cMet phosphorylation [phospho-Met (pMet)]. \* $P < 0.05$  vs. vehicle.

**Fig. 7.**

BB3 induces phosphorylation of tubular Akt and upregulation of Bcl-2 in postischemic kidneys. *A*: phosphorylation of tubular Akt was evident in kidneys from animals ( $n = 3$ ) treated with BB3. *B*: treatment with drug resulted in an ~4-fold increase in tubular phosphorylated (p)Akt levels compared with the IR-vehicle ( $n = 3$ ) cohort. *C*: increased tubular Bcl-2 was evident in kidneys from animals treated with BB3. *D*: compared with the vehicle cohort, treatment with BB3 resulted in a 4- to 5-fold increase in postischemic renal Bcl-2 expression. \* $P < 0.05$  vs. vehicle.

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