

# The Sphingosine Kinase 2 Inhibitor Opananib Protects Against Acute Kidney Injury in Mice

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**Introduction:** Acute kidney injury (AKI) is a common multifactorial adverse effect of surgery, circulatory obstruction, sepsis or drug/toxin exposure that often results in morbidity and mortality. Sphingolipid metabolism is a critical regulator of cell survival and pathologic inflammation processes involved in AKI. Opananib (also known as ABC294640) is a first-in-class experimental drug targeting sphingolipid metabolism that reduces the production and activity of inflammatory cytokines and, therefore, may be effective to prevent and treat AKI.

**Methods:** Murine models of AKI were used to assess the in vivo efficacy of opananib including ischemia-reperfusion (IR) injury induced by either transient bilateral occlusion of renal blood flow (a moderate model) or nephrectomy followed immediately by occlusion of the contralateral kidney (a severe model) and lipopolysaccharide (LPS)-induced sepsis. Biochemical and histologic assays were used to quantify the effects of oral opananib treatment on renal damage in these models.

**Results:** Opananib suppressed the elevations of creatinine and blood urea nitrogen (BUN), as well as granulocyte infiltration into the kidneys, of mice that experienced moderate IR from transient bilateral ligation. Opananib also markedly decreased these parameters and completely prevented mortality in the severe renal IR model. Additionally, opananib blunted the elevations of BUN, creatinine and inflammatory cytokines following exposure to LPS.

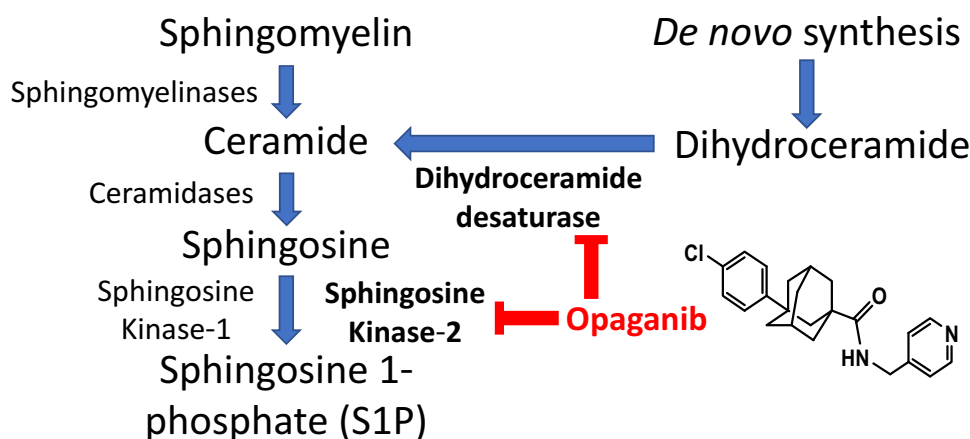
**Conclusion:** The data support the hypotheses that sphingolipid metabolism is a key mediator of renal inflammatory damage following IR injury and sepsis, and that this can be suppressed by opananib. Because opananib has already undergone clinical testing in other diseases (cancer and Covid-19), the present studies support conducting clinical trials with this drug with surgical or septic patients at risk for AKI.

**Keywords:** opananib, sphingosine kinase, sphingolipid, acute kidney injury, ischemia-reperfusion injury, lipopolysaccharide, sepsis

## Introduction

Acute kidney injury (AKI) is one of the most common and serious complications following major surgery or sepsis. For example, AKI occurs in up to 30% of patients who undergo cardiac surgery,<sup>1</sup> of which there are more than 900,000 cardiac procedures performed in the United States alone each year. Mortality rates are over 20% for AKI patients following cardiac surgery<sup>2</sup> and up to 50% in patients that require renal replacement therapy,<sup>1</sup> with survivors needing extended stays in the intensive care unit and often continuing dialysis. The pathogenesis of AKI involves multiple pathways with overlapping hemodynamic, inflammatory, and nephrotoxic processes leading to kidney injury.<sup>1,3</sup> AKI frequently follows ischemia-reperfusion (IR) of the kidney that occurs not only in cardiac surgery but also in other scenarios such as aortic aneurysm repair,<sup>4</sup> trauma, organ transplantation<sup>5</sup> and Covid-19.<sup>6</sup> Additionally, sepsis which occurs in at least 1.7 million adults in the US per year frequently results in inflammatory damage to the kidneys resulting in more than 200,000 deaths annually. Other than supportive care medications, there are currently no drugs approved for AKI prevention or therapy. Consequently, there is a large unmet clinical need for drugs that can prevent and/or treat AKI resulting from surgery, Covid-19 or sepsis.

Inflammation is a critical component of AKI, and sphingolipids are known to regulate cellular signaling pathways in inflammation (reviewed in).<sup>7-9</sup> Briefly, inflammatory cytokines, including TNF $\alpha$  and IL-6, activate sphingomyelinases that hydrolyze sphingomyelin to form ceramide which is then cleaved by ceramidases to yield sphingosine (Figure 1). Sphingosine



**Figure 1** Inhibition of sphingolipid metabolism by opaganib. Opaganib (also known as ABC294640) inhibits sphingosine kinase-2 (SK2) resulting in reduced synthesis of sphingosine 1-phosphate (S1P), thereby suppressing pathologic inflammation. In parallel, opaganib also inhibits dihydroceramide desaturase (DES1) resulting in accumulation of dihydroceramides, which promotes autophagy.

kinases (SKs) phosphorylate the primary hydroxyl of sphingosine, forming sphingosine 1-phosphate (S1P) which promotes inflammation and cell proliferation. Two SK isozymes (SK1 and SK2) have different kinetic parameters and expression patterns in normal and diseased tissue and are therefore direct regulators of the equilibrium of ceramides, sphingosine, and S1P. Increasing attention is being paid to the roles of sphingolipid metabolism in renal pathologies (reviewed in).<sup>10–12</sup> However, there are no approved products that modulate sphingolipid metabolism for the treatment and/or prevention of AKI.

Opaganib (Figure 1) is an orally-active isozyme-selective inhibitor of SK2, and is competitive with respect to sphingosine.<sup>13,14</sup> Opaganib depletes S1P and elevates ceramide in tumor cells, suppresses signaling through pERK, pAKT and NFκB, and promotes autophagy and/or apoptosis.<sup>13–17</sup> Opaganib also down-regulates c-Myc in a variety of tumor cell lines.<sup>17–20</sup> Because it acts as a sphingosine mimetic, opaganib also inhibits dihydroceramide desaturase (DES1), increasing levels of dihydroceramides<sup>18</sup> and promoting autophagy in those cells. Opaganib has antitumor activity in a wide range of mouse models,<sup>13,17–19,21–25</sup> as well as anti-inflammatory activity in several rodent models.<sup>26–30</sup> Preclinical and certified Good Laboratory Procedure toxicology studies with opaganib in rodents and dogs demonstrated a lack of significant systemic toxicity, lack of hematologic suppression, and lack of organ-specific toxicity. This supported moving opaganib into clinical trials and the Investigational New Drug application was allowed by the FDA. A Phase I clinical trial with opaganib administered to patients with advanced solid tumors demonstrated that it is well-tolerated even with treatment of >40 weeks, and provided disease stabilization in most patients.<sup>31</sup> Opaganib is currently in Phase II clinical testing in patients with cholangiocarcinoma (NCT03377179) or prostate cancer (NCT04207255). Because of its anti-inflammatory and antiviral properties, opaganib has been evaluated in hospitalized patients with Covid-19 pneumonia.<sup>32</sup> A Phase 2a study that demonstrated that patients receiving oral opaganib required less supplemental oxygen and achieved earlier hospital discharge.<sup>33</sup> Subsequently, a Phase 2/3 multinational randomized, placebo-controlled study enrolled 475 adult subjects hospitalized with severe Covid-19. This trial confirmed the safety of opaganib for these patients and demonstrated a clinical benefit to patients requiring lower oxygen supplementation (62% reduction in rate of ventilation and death).

Because opaganib has anti-inflammatory activity in several rodent models, we hypothesized that it will provide protection against AKI in vivo. In the present studies, we examine the ability of opaganib to suppress AKI using two well-established models in mice, specifically renal IR injury and lipopolysaccharide (LPS)-induced sepsis. These models were chosen to assess potential protective effects of opaganib against AKI induced by very different challenges representing a large number of patients with a need for improved therapies.

## Materials and Methods

### Chemicals and Animals

Opaganib (also known as ABC294640) was synthesized as previously described<sup>13</sup> and dissolved in 0.375% Tween 80 in Phosphate-Buffered Saline (PBS). Lipopolysaccharide (L2030) and 3,3',5,5'-tetramethylbenzidine (860336) were

purchased from Sigma-Aldrich (St. Louis, MO, USA). C57BL/6 mice (Strain # 000664) were purchased from Jackson Labs (Bar Harbor, ME, USA), and were maintained at the Hershey Center for Applied Research (Hummelstown, PA, USA). Research was conducted under the supervision of the IACUC of the Penn State College of Medicine.

## Renal Ischemia-Reperfusion Protocols

Multiple protocols for the induction of renal IR injury have been used to study AKI (reviewed in)<sup>34,35</sup> and provide mild (unilateral renal IR), moderate (bilateral renal IR) or severe (unilateral IR with contralateral nephrectomy) pathologies. Variation among mice is highest in the mild and moderate IR models, but improves in the severe IR model.<sup>34,35</sup> Therefore, we have used both a moderate and severe IR model in the present studies.

### Moderate Model

Male C57/BL6 mice (approximately 24 g) were dosed with opaganib (50 mg/kg) or vehicle (0.1 mL in 0.375% Tween 80 in Phosphate-Buffered Saline (PBS)) by oral gavage (n = 4–6/group/experimental run), immediately followed by intraperitoneal injection of ketamine/xylazine for anesthesia. Surgery was performed on a heated surface using homeothermic pads to ensure the maintenance of animal body temperature. A midline incision was made and the two renal pedicles were located and clamped for 25 min. Total blockage of the renal pedicle and thus artery was confirmed after several minutes as the kidney was seen to be dark red to purple in color. After the scheduled time elapsed, the clamps were removed and the kidney was observed to ensure reperfusion as indicated by returning to its original color. Pre-warmed (37 °C) sterile saline (1 mL) was instilled into the peritoneum at the time of closing which used sutures for musculature and wound clips for the skin incision. Animals were maintained on the homeothermic pad until awakening from anesthesia and were carefully monitored post-operatively for morbidity according to the IACUC protocol. Control mice (“sham IR”) underwent surgery without clamping of the pedicles.

### Severe Model

The severe IR model was performed in a similar manner to the moderate model with the exception that the right kidney pedicle was tied off and the kidney was removed. Immediately thereafter, the left kidney was clamped for 45 min before reperfusion was allowed. Animals were maintained on the homeothermic pad until awakening from anesthesia and were carefully monitored post-operatively for morbidity according to the IACUC protocol. Control mice (“sham IR”) underwent surgery without kidney removal or clamping of the pedicles.

## Lipopolysaccharide Model of Sepsis

Male C57/BL6 mice (approximately 20 g) were dosed with opaganib (100 mg/kg) or vehicle by oral gavage 1 hr before (–1); 2 hr after (+2); or both before and after (–1, +2) intraperitoneal injection of LPS (5.0 mg/kg) (n = 4–6/group/experimental run). Animals were carefully monitored for morbidity according to the IACUC protocol. Control mice (“sham”) did not receive LPS injections.

## Blood Chemistry and Cytokine Analyses

At the indicated times after completion of the IR surgery or LPS-treatment, mice were anesthetized with ketamine/xylazine and blood was harvested by cardiac puncture. The kidneys were then surgically removed and euthanasia was ensured by cervical dislocation. For the IR experiments serum chemistry values were obtained using a Roche Cobas Mira Plus automated chemistry analyzer on a fee-for-service basis from the Penn State Department of Comparative Medicine in Hershey, PA. Because of a change in instrumentation, plasma was analyzed using a VetScan VS2 Chemistry Analyzer (Zoetis, Boston, MA, USA) and Comprehensive Diagnostic Profile 2 rotors (10023220) for the LPS studies. Cytokine analyses of plasma were conducted on a Luminex 100 Multi-analyte System at the Cytokine Laboratory of the University of Maryland School of Medicine on a fee-for-service basis.

## Myeloperoxidase (MPO) Assay

Kidney MPO activity was determined as a measure of granulocyte and monocytes infiltration into the kidney. At the time of collection, one half of each kidney was removed and snap-frozen in a methanol/dry ice bath and stored at  $-80^{\circ}\text{C}$  until the time of the MPO assay. The kidney samples were thawed and homogenized in 10 volumes (weight/volume) of cold PBS, and the homogenate was centrifuged at  $22,000 \times g$  for 15 min. The resulting pellet was resuspended and assayed for MPO by quantifying the metabolism of tetramethylbenzidine as described by Fitzpatrick et al.<sup>36</sup>

## Kidney Histology

At the time of collection, one half of each kidney was placed in 10% formalin for at least 24 h and then dehydrated through graded alcohols. The kidney tissues were then embedded in paraffin wax and sections ( $5\text{ }\mu\text{m}$ ) were stained with hematoxylin and eosin according to standard protocols. Kidney histology was quantitatively scored for 4–6 mice/treatment group and 4 areas/slide using a scale of 1 to 3 for each of five parameters (tubule cell swelling, tubular dilatation, edema, epithelial necrosis and tubular casts) that were added to generate a total Histology Score (range = 5–15).

## Statistical Analyses

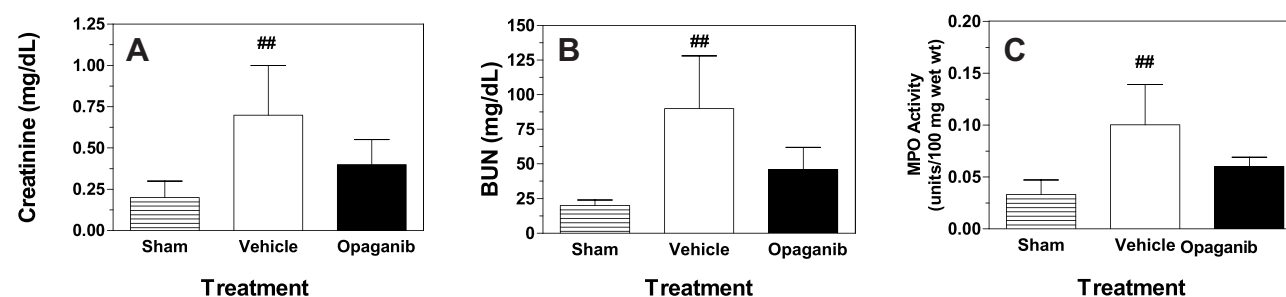
Using GraphPad (San Diego, CA, USA) Prism 5 software, survival rates were compared using the Kaplan–Meier approach with the Gehan-Breslow-Wilcoxon test. Other data shown in bar graphs were analyzed by one-way ANOVA using the Tukey post hoc test. Differences among treatment groups were considered to be statistically significant when  $p < 0.05$ .

## Results

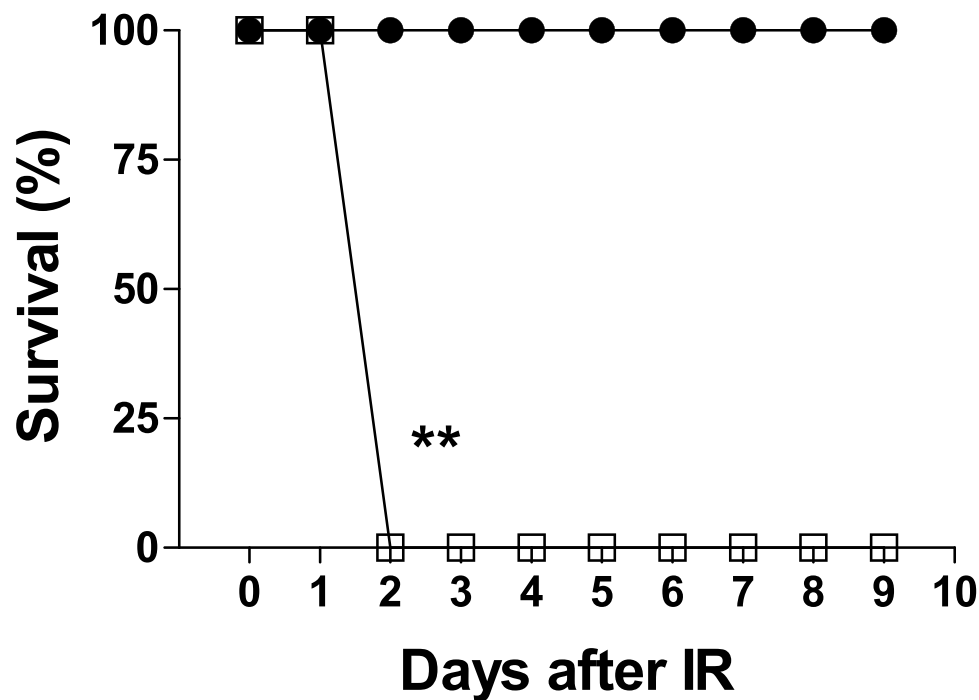
### Opaganib Improves Kidney Function and Prevents Mortality Following Renal IR

Opaganib has efficacy in other inflammation models at oral doses of 50–100 mg/kg.<sup>26–30</sup> To evaluate the effects of opaganib in the moderate IR model, mice were treated with 0 or 50 mg/kg opaganib and bilateral occlusion of the kidneys was maintained for 25 min. Ischemia-reperfusion resulted in significant increases in creatinine (Figure 2A) and BUN (Figure 2B) levels in vehicle-treated mice compared to non-IR sham animals ( $p < 0.01$  for each). Mice treated with opaganib had substantially lower creatinine and BUN levels at 24 hr after reperfusion than did IR mice that received the vehicle. Similarly, IR promoted significant elevation of renal MPO activity ( $p < 0.01$ ) reflecting granulocyte infiltration into the kidneys, and this was attenuated in IR mice that received opaganib (Figure 2C). However, because of the inherent variability of the moderate IR model,<sup>34,35</sup> these differences between the vehicle- and opaganib-treatment groups did not reach the target level for designation as significant for creatinine ( $p = 0.12$ ), BUN ( $p = 0.07$ ) and MPO ( $p = 0.09$ ).

To reduce inter-animal variability, the effects of opaganib were evaluated using the severe IR model involving nephrectomy and immediate occlusion of the contralateral kidney for 45 min. Mice were treated with opaganib (50 mg/kg) or vehicle by oral gavage prior to severe renal IR and were carefully monitored. In this model, vehicle-treated IR mice consistently required sacrifice because of morbidity on post-surgery Day 2 (Figure 3). In marked contrast, mice that

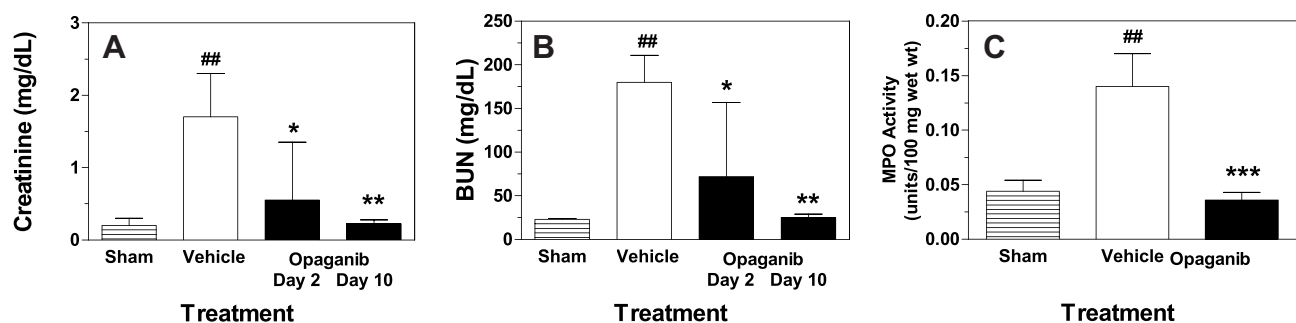


**Figure 2** Opaganib improves renal function after moderate ischemia-reperfusion. Mice were treated with vehicle or opaganib (50 mg/kg) by oral gavage immediately prior to bilateral clamping of the kidney pedicles for 25 min. No surgery was performed in the Sham treatment group. Blood was drawn 24 h after reperfusion and assayed for serum creatinine (A) and BUN (B) levels. Additionally, kidney lysates were assayed for myeloperoxidase activity (C). Bars show the mean  $\pm$  SD values for Sham-IR (n=5), vehicle-treated (n=5) and opaganib-treated (n=4) groups. ##Indicates  $p < 0.01$  compared with the Sham treatment group.



**Figure 3** Opaganib promotes survival after severe ischemia-reperfusion. Mice were treated with vehicle (□, n=6) or 50 mg/kg opaganib (●, n=4) by oral gavage immediately prior to induction of severe kidney ischemia-reperfusion by ligating and removing the right kidney and then clamping the left kidney for 45 min. Mice were monitored for morbidity daily, and the percentages of surviving animals are shown. \*\*Indicates  $p < 0.01$  compared with the Vehicle treatment group.

received 50 mg/kg opaganib preoperatively all appeared robust and maintained body weight until they were sacrificed on post-surgery Day 10 ( $p < 0.001$ ). Blood was drawn from the mice at sacrifice (Day 2 or Day 10 for opaganib-treated mice) for BUN and creatinine analyses. Both creatinine (Figure 4A) and BUN (Figure 4B) levels were highly elevated in the vehicle-treated IR animals compared with sham IR animals ( $p < 0.01$ ), indicating post-surgical renal failure. Creatinine and BUN concentrations were substantially higher, but less variable, than in the moderate IR model. In the severe IR model, animals treated with opaganib had significantly reduced serum creatinine and BUN levels at 48 hr post-IR ( $p < 0.05$  compared with vehicle) with values returning to normal by Day 10 ( $p < 0.01$  compared with Vehicle on Day 2). Similarly, MPO activity was elevated in vehicle-treated IR animals at 48 hr compared to sham IR controls (Figure 4C), and the increase in MPO activity was completely blocked in mice treated with opaganib ( $p < 0.001$  compared with Vehicle).



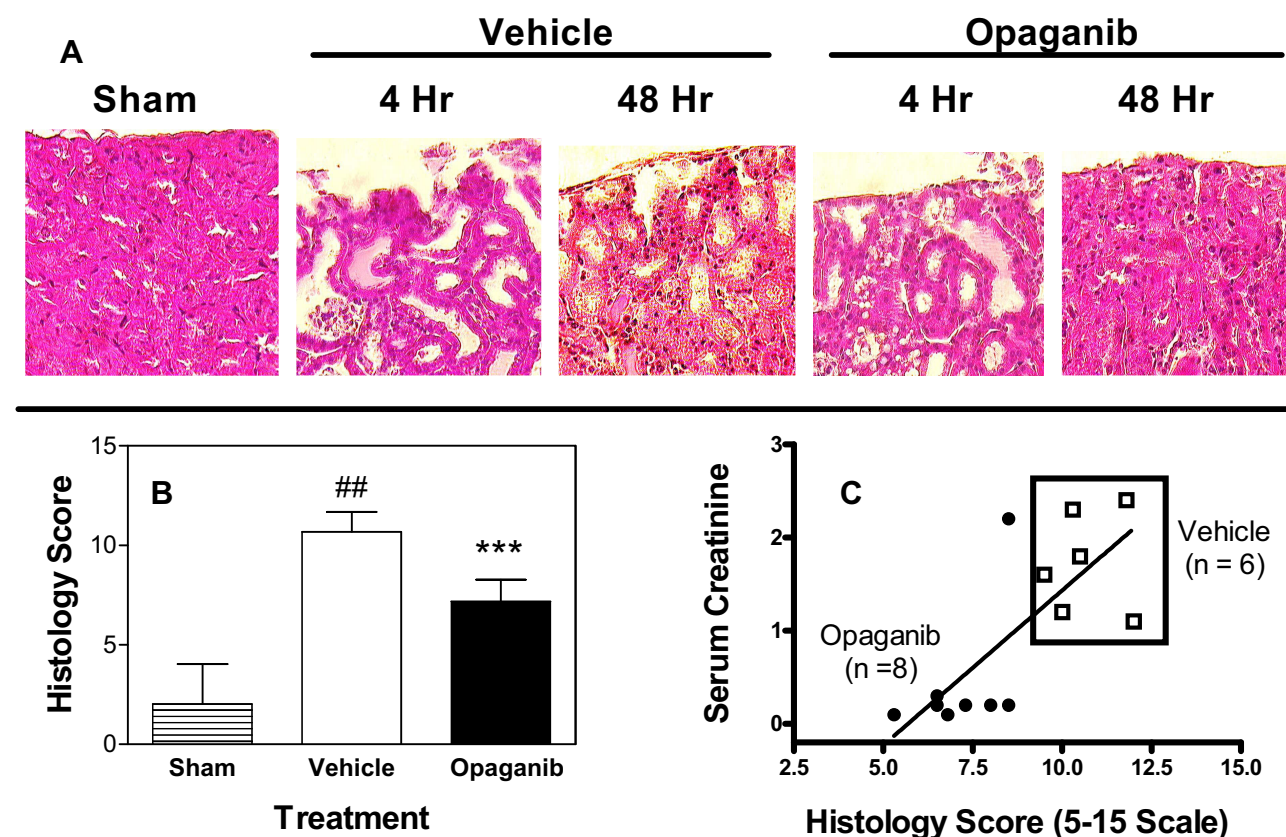
**Figure 4** Opaganib improves renal function after severe ischemia-reperfusion. Mice were treated with vehicle or 50 mg/kg opaganib by oral gavage immediately prior to induction of severe kidney ischemia-reperfusion. No surgery was performed in the Sham treatment group. Serum levels of creatinine (A) and BUN (B) were measured on Day 2 or Day 10 (opaganib treatment group only). Kidneys were harvested on Day 2 for myeloperoxidase (MPO) assays (C). ##Indicates  $p < 0.01$  compared with the Sham treatment group. \*Indicates  $p < 0.05$ , \*\*Indicates  $p < 0.01$ , and \*\*\*Indicates  $p < 0.001$  compared with Vehicle treatment group.



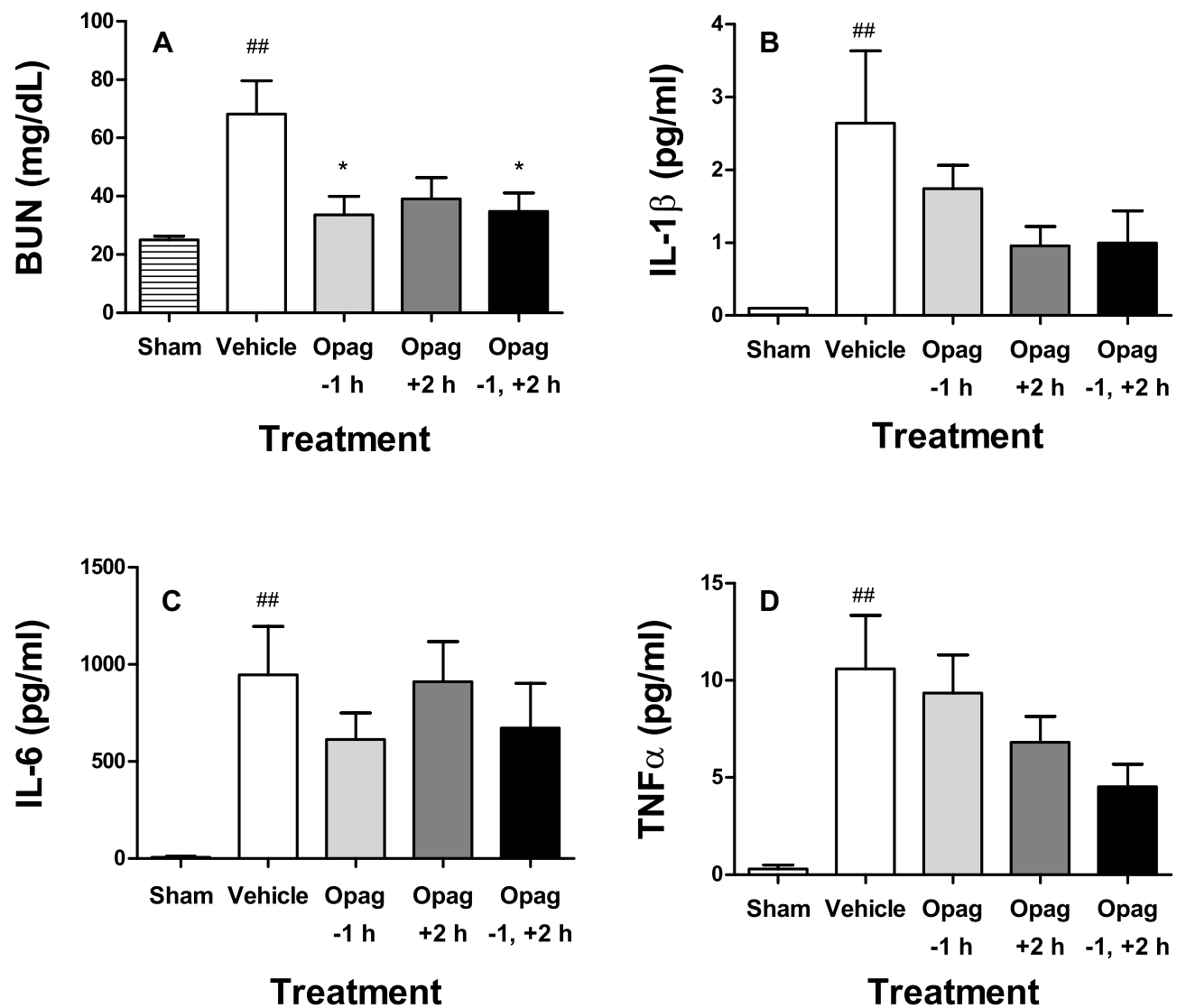
Renal thin sections were stained with hematoxylin/eosin and quantitatively scored on a scale of 1 to 3 for five parameters (tubule cell swelling, tubular dilatation, edema, epithelial necrosis and tubular casts) that were added to generate a total Histology Score. Kidneys from animals treated with opaganib had less morphologic damage (Figure 5A) and lower Histology Scores (Figure 5B,  $p < 0.001$ ) than kidneys from vehicle-treated IR mice at 48 hr after reperfusion. An excellent correlation was observed between individual mouse serum creatinine level and Histology Score ( $r = 0.7556$ ) for this relationship, suggesting a significant association between the two parameters ( $p < 0.01$ ) (Figure 5C). Overall, opaganib significantly improved kidney function and animal survival after IR surgery in this severe model of AKI.

## Opaganib Improves Kidney Function and Suppresses Inflammatory Cytokines in the LPS-Induced Sepsis Model

Lipopolysaccharide injection is widely used as a preclinical model for sepsis, resulting in transient AKI that is mediated by TLR-4-induced systemic expression of inflammatory cytokines including TNF $\alpha$  and IL-6. To evaluate the effects of opaganib in a sepsis model, mice were treated orally with 0 or 100 mg/kg opaganib before and/or after the administration of LPS (5 mg/kg). As shown in Figure 6A, LPS-treatment resulted in a significant increase in BUN levels at 24 hr in vehicle-treated mice compared with sham (non-LPS) animals of this model ( $p < 0.01$ ). Pretreatment of mice with opaganib before LPS administration significantly suppressed the increase in BUN levels ( $p < 0.05$ ). Treatment with opaganib after LPS-treatment substantially lowered BUN levels but the decrease did not reach the threshold of significance ( $p = 0.11$ ). Creatinine levels were not significantly elevated after LPS-treatment; however, IL-1 $\beta$  (Figure 6B), IL-6 (Figure 6C) and TNF $\alpha$  (Figure 6D) were all significantly induced by LPS ( $p < 0.01$ ). Increased



**Figure 5** Opaganib attenuates histologic renal damage from severe ischemia-reperfusion. Mice were treated with vehicle or 50 mg/kg opaganib by oral gavage immediately prior to induction of severe kidney ischemia-reperfusion. No surgery was performed in the Sham treatment group. At 4 or 48 hr after reperfusion, mice were sacrificed and kidneys were harvested, fixed, sectioned and stained with hematoxylin and eosin. (A) Representative sections are shown. (B) Histology Scores were calculated as described in the Materials and Methods section. Bars show the mean  $\pm$  SD values for Sham (n=5), vehicle (n=6) and opaganib (n=8) treatment groups. ##Indicates  $p < 0.01$  compared with the Sham treatment group. \*\*\*Indicates  $p < 0.001$  compared with the Vehicle treatment group. (C) To assess the consistency of the data, serum creatinine and Histology Score values are shown for individual mice from the vehicle ( $\square$ ) and opaganib ( $\bullet$ ) treatment groups.



**Figure 6** Opaganib preserves renal function and suppresses inflammation in lipopolysaccharide-induced sepsis. Mice were treated with opaganib (100 mg/kg) or vehicle by oral gavage at 1 hr before and/or 2 hr after lipopolysaccharide (LPS) injection. No LPS was administered in the Sham treatment group. Blood was drawn 24 hr after LPS administration and assayed for BUN (A), IL-1 $\beta$  (B), IL-6 (C) and TNF $\alpha$  (D). Bars show the mean  $\pm$  SD values for n=4–6 mice per treatment group. ##Indicates p < 0.01 compared with the Sham treatment group. \*Indicates p < 0.05 compared with the Vehicle treatment group.

expression of these inflammatory proteins was generally reduced in mice treated with opaganib, particularly when opaganib was administered both before and after LPS-treatment.

## Discussion

It is well established that sphingolipid metabolism regulates pathologic inflammation underlying several disease states, and in particular, many studies have shown that SK activity regulates inflammation.<sup>7,12,37</sup> Sphingosine 1-phosphate induces NF $\kappa$ B,<sup>38</sup> which in turn can increase the proinflammatory enzymes nitric oxide synthase and cyclooxygenase-2<sup>39–41</sup> generating prostaglandins and oxidative and nitrative stress mediated that exacerbate inflammation.<sup>42</sup> Inflammatory cytokines induce adhesion molecule expression via activation of SK and NF $\kappa$ B, and these proteins facilitate infiltration of neutrophils and macrophages into inflamed tissue. Sphingosine 1-phosphate promotes granulocyte activation leading to production of reactive oxygen species<sup>43,44</sup> and activates S1P receptors further promoting inflammatory cascades at the site of tissue damage.<sup>45–47</sup> Overall, a strong body of evidence demonstrates that activation of SKs alters sphingolipid metabolism in favor of S1P formation, resulting in pro-inflammatory responses.

More specific to the current studies, the roles of sphingolipids in IR injury to the kidneys have received attention,<sup>10–12,48</sup> largely because of their direct regulation of inflammation and ROS generation.<sup>11,49–51</sup> Altered sphingolipid metabolism in response to renal ischemia was first demonstrated in 1997.<sup>52</sup> Plasma creatinine levels<sup>53</sup> and pulmonary permeability and injury<sup>54</sup> following renal IR were significantly lower in S1P receptor 3<sup>−/−</sup> mice compared with control animals, suggesting the importance of excessive S1P in IR injury. Supporting this, the S1P receptor superagonist FTY720 prevents lymphocyte egress from lymph nodes and thereby suppresses T cell influx into the kidney following IR.<sup>55,56</sup> In parallel, roles for sphingolipid metabolism in sepsis have been studied since 1997<sup>9,57–62</sup> and generally focus on regulation of NFκB-induced cytokine production.

The anti-inflammatory activity of opaganib has been shown in several in vivo models<sup>26–28,30,63</sup> and extensive preclinical toxicology studies demonstrated it does not have significant systemic, organ-specific or hematologic toxicity. In models of inflammatory bowel disease, opaganib decreased colonic levels of TNFα, IL-1β, IFN-γ and IL-6, and reduced S1P levels in colon tissue.<sup>28</sup> In liver IR injury, opaganib reduced TLR4 expression, NFκB activation, TNFα, IL-1β and CXCL-10 production, adhesion molecule expression, and liver infiltration of granulocytes.<sup>64</sup> Opaganib nearly completely ameliorated *Pseudomonas aeruginosa* (PA)-induced lung injury in mice, associated with decreased inflammatory cell and protein accumulation in the bronchoalveolar lavage fluid, and suppression of PA-induced elevation of IL-6, TNFα and H<sub>2</sub>O<sub>2</sub>.<sup>65</sup>

In studies described herein, we tested the ability of opaganib to suppress AKI in two well-established mouse models of renal IR injury and in LPS-induced sepsis. In the moderate IR model (bilateral renal IR), opaganib attenuated increases in creatinine, BUN and renal granulocyte infiltration; however, the greater variability in this AKI model prevented the effects from reaching a significance level of >95% (p values were 0.07–0.12). Therefore, we switched to a severe model consisting of contralateral nephrectomy immediately prior to renal IR for 45 min. As a severe model of disease, this protocol is expected to require a high level of efficacy for a drug to mitigate the renal damage, and so provides a rigorous evaluation of its potential clinical usefulness. Orally-administered opaganib suppressed the elevation of clinical markers of AKI following severe renal IR in association with decreased neutrophil infiltration into the kidney and reduced histologic damage. In this model of severe AKI, untreated mice all died within 2 days of renal IR; whereas, all opaganib-treated mice survived the IR insult long-term. Similarly, elevations of BUN, IL-1β, IL-6 and TNFα were suppressed by opaganib in the LPS-induced sepsis model. These effects are consistent with previous studies demonstrating that activation of NFκB by TNFα in vitro is dose-dependently suppressed by opaganib,<sup>30</sup> and TNFα-induction of adhesion proteins (VCAM-1 and ICAM) involved in leukocyte recruitment and production of PGE<sub>2</sub> are strongly suppressed by opaganib.<sup>28</sup> Opaganib also suppresses titanium particle-induced TNFα and IL-6 production in RAW264.7 macrophages,<sup>66</sup> and inhibits extracellular matrix deposition in human kidney fibroblasts.<sup>67</sup>

Our focus in these initial studies was to determine if opaganib has an effect on clinically relevant endpoints indicative of AKI. With that confirmation in hand, we are pursuing additional biochemical studies that should reveal the mechanistic basis for the therapeutic effects of opaganib. Multi-organ studies characterizing the pharmacokinetics and pharmacodynamics of opaganib include pharmacokinetic and sphingolipid analyses of kidneys from opaganib-treated normal C57BL/6 mice. These analyses demonstrated that opaganib reaches pharmacologically active concentrations (24 μg/g tissue or ~63 μM) in the kidney at 2 hr after oral dosing with a half-time of elimination of approximately 16 hr. This exposure to opaganib was associated with transient decreases in renal levels of S1P and increases in sphingosine and dihydroceramide, indicating in situ inhibition of SK2 and DES1.

Mechanistically, several key biochemical pathways involved in AKI are modulated by sphingolipids, and in some cases demonstrated to be suppressed by opaganib. First, signaling through pAkt is required for the pathology of AKI,<sup>68–71</sup> and Akt inhibitors attenuate the excessive inflammation, cytokine storm, fibrosis, and thrombosis in AKI patients.<sup>72</sup> We and others have demonstrated that opaganib efficiently inhibits Akt phosphorylation in multiple cell types, and so inhibition of Akt may underlie the ability of opaganib to suppress AKI. Second, c-Myc expression and activity is pathologic following IR.<sup>73–75</sup> Similarly, LPS induces c-Myc,<sup>76–78</sup> which may underlie its pro-apoptotic actions.<sup>79</sup> Opaganib decreases the expression of c-Myc,<sup>17–20,80,81</sup> likely due to depletion of nuclear S1P destabilizing the c-Myc protein.<sup>82</sup> Therefore, inhibition of c-Myc may underlie the ability of opaganib to suppress AKI. Finally, a large body of evidence demonstrates that activation of autophagy suppresses AKI, leading to the suggestion that autophagy-inducing agents may effectively prevent and/or treat AKI (reviewed in).<sup>83–86</sup> It is well established that: dihydroceramides induce autophagy,<sup>87–93</sup> opaganib elevates dihydroceramides by inhibition of DES1,<sup>18,94,95</sup> and opaganib promotes autophagy.<sup>15,96,97</sup> Therefore, inhibition of DES1 may be involved in the ability of opaganib to suppress AKI.



Overall, sphingolipid metabolism is an emerging target for the prevention and/or treatment of AKI, and the present studies provide the first demonstration that the investigational drug opaganib protects against renal damage in two complementary models of AKI. The completed and ongoing clinical studies of opaganib demonstrate that oral opaganib is well-tolerated even by severely compromised cancer and Covid-19 patients, with treatment periods as long as 8 months for some patients. We envision that opaganib could be administered to patients at risk for developing AKI, eg in patients undergoing cardiovascular or other major surgery where periods of renal IR are expected. Similarly, trauma patients or military personnel experiencing hypovolemia or at risk for sepsis from gastrointestinal injury may benefit from opaganib treatment. Of additional interest is the potential for opaganib to mitigate sepsis from bacterial or viral infection, including SARS-CoV-2 and/or influenza viruses.

## Abbreviations

AKI, Acute Kidney Injury; DES1, dihydroceramide desaturase 1; IR, ischemia-reperfusion; LPS, lipopolysaccharide; MPO, myeloperoxidase; PBS, phosphate-buffered saline; SK, sphingosine kinase; S1P, sphingosine 1-phosphate.

## Ethical Statements

All protocols in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Penn State University College of Medicine, in compliance with US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training, which complies with the PHS Policy on Humane Care and Use of Laboratory Animals, and the United States Department of Agriculture Animal Welfare Act guidelines.

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## Disclosure

Lynn W. Maines, Staci N. Keller and Charles D. Smith are current employees and own stock in Apogee Biotechnology Corporation. Apogee Biotechnology Corporation owns patent rights to opaganib (United States Patent numbers 7,338,961, 8,063,248, 8,324,237 and 8,557,800, and multiple parallel patents in other countries) that have been licensed to RedHill Biopharma LTD. The value of these rights may be affected by the research reported in this paper. The authors report no other conflicts of interest in this work.

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