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## Myristoylated Protein Kinase C Epsilon Inhibitor Preserves Renal Function in a Mouse Model of Acute Bilateral Kidney Ischemia-Reperfusion Injury

**Keywords:** Delayed Graft Function; Ischemia Reperfusion; Kidney; Protein Kinase C Epsilon; YT-001

#### Abstract

Delayed graft function (DGF) is a post kidney transplant complication in which kidney function and urine production is delayed for days, weeks or months. During this period, the patient must be maintained on dialysis. DGF is a consequence of ischemia reperfusion (I/R) injury as the kidney is exposed to ischemia when harvested and reperfusion once transplanted. The re-introduction of oxygenated blood underlies I/R damage. DGF occurs in up to 30% of kidney transplant recipients, which suggests that therapeutic approaches are needed to improve patient outcomes.

This study investigates the role of protein kinase C epsilon (PKCɛ) inhibition in maintaining kidney function in a murine model of bilateral kidney I/R. Measurements of glomerular filtration rate (GFR) and serum creatinine (Cr) were used to assess kidney function. Ischemia was induced in male C57BL/6J mice by clamping kidney pedicles bilaterally for 19 minutes followed by 96 hours of reperfusion. A cell permeable peptide that inhibits PKCɛ (N-myristic acid-EAVSLKPT; YT-001) interaction with downstream receptors,given at the time of reperfusion, significantly maintained GFR, blunted serum Cr elevation to a greater extent, and prevented PKCɛ translocation to renal epithelial cell membranes compared to the scrambled control peptide (N-Myr-LSETKPAV).

These findings suggest that YT-001 given upon the re-institution of blood flow after kidney transplant would potentially decrease the incidence of DGF and the subsequent downstream morbidity and mortality. Based upon these findings, YT-001 was granted Orphan Drug Status by the FDA in 2023.

#### Abbreviations

3,3'-Diaminobenzidine: DAB; Acute kidney injury: AKI; Creatinine: Cr; Delayed graft function: DGF; Dihydrobiopterin: BH2; Endothelial nitric oxide synthase: eNOS; Extracorporeal shockwave lithotripsy: ESWL;Glomerular filtration rate: GFR;Hydrogen peroxide: H2O2;Molecular weight: MW;Myristoylated: Myr;Phosphate buffered saline: PBS; Protein kinase C epsilon: PKCe;Reactive oxygen species: ROS;Receptor for activated C kinase-1: RACK-1; Transactivator of transcription: TAT; Tetrahydrobiopterin: BH4

#### Introduction

Ischemic injury occurs when tissues are subjected to a cessation of blood flow leading to cellular dysfunction and damage. Ischemic injury leads to a reduction of ATP from the mitochondria, which compromises  $Na^+/K^+$  ATPase and, in turn, leads to acute renal failure that is indicated by decreased glomerular filtration rate (GFR) and

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increased serum creatinine (Cr). Timely restoration of blood flow (i.e., reperfusion) to the kidney is essential to restore kidney function. However, reperfusion of blood flow after prolonged ischemia leads to the generation of reactive oxygen species (ROS) in blood thatwill exacerbate injury to the previously ischemic tissue, lead to kidney fibrosis and compromise renal function months later [1,2]. Hence, Ischemia-reperfusion (I/R) in the kidney is a well-recognized mechanism of injury following kidney transplant [1]. There are over twenty thousand kidney transplants per year in the United States alone, and approximately 30% of transplant recipients experience acute kidney injury (AKI) which progresses to the clinical diagnosis of delayed graft function (DGF) within one week after transplantation [2].

DGF is characterized by endothelial dysfunction, inflammation, and ROS. These processes culminate in significant kidney damage, manifesting as inadequate urine production. Current interventions are largely supportive with fluid and electrolyte management via dialysis; however, these measures do not address the mechanisms of I/R injury to prevent or mitigate DGF [3-9]. Patient outcomes following DGF include the need for dialysis, graft rejection, and high morbidity compared to those without DGF [10-12].

In previous *in vivo* porcine myocardial I/R studies, YT-001, given at the beginning of reperfusion resulted in a marked reduction in infarct area and restoration of post-reperfusion cardiac function [13]. YT-001 is known to confer protection by inhibiting the generation of ROS from uncoupled endothelial nitric oxide synthase (eNOS) and mitochondrial ATP-sensitive potassium channels [14-19]. YT-001 reduced serum ROS (e.g.,  $H_2O_2$ ) levels when given at reperfusion in an *in vivo* rat hindlimb I/R model [14-15]. (Figure 1) illustrates the pathway following I/R induced ROS injury in the heart and proposed pathway for the kidney. Extracorporeal shockwave lithotripsy (ESWL) is a procedure used to break up kidney stones and causes ischemic-like injury during the 15-minute ESWL procedure. YT-001 attenuated ESWL-induced kidney I/R injury, in part, by preventing the generation of ROS in blood flow to the kidney [20].

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I/R insult activates PKCe via inflammatory cytokines and opening of mitochondrial ATP-sensitive potassium channels, resulting in mitochondrial ROS production. Oxidative stress leads to oxidation of tetrahydrobiopterin (BH4) to dihydrobiopterin (BH2) which promotes eNOS uncoupling. Increased plasma BH2 levels promote uncoupled eNOS activity to produce ROS in lieu of nitric oxide when activated byPKCe phosphorylation [21].

YT-001 prevents the interaction between activated PKCɛ and its receptor for activated C kinase-1 (RACK-1) resulting in decreased ROS production from renal epithelial cells as shown in (Figure 2) [22]. Selective inhibition of PKCɛ translocation, an upstream regulator of eNOS, may mitigate ROS-related damage involved in kidney I/R injury.

#### EAVSLKPT (V1-V2)

Activated PKC $\epsilon$  binds RACK-1 for translocation to phosphorylate ROS-producing targets, including uncoupled eNOS and mitochondrial ATP-sensitive K+ channels [21-22]. Panel B: PKC $\epsilon$  inhibitor impedes the interaction between PKC $\epsilon$  and RACK-1 and subsequent translocation.PKC $\epsilon$  binds to the variable region within the RACK-1 binding site (i.e., V1-V2 region) of PKC $\epsilon$  to regulate translocation to cellular proteins to phosphorylate its substrate (e.g. eNOS) [22].

DGF remains a major clinical challenge. In this study, we investigated the cell-permeable, PKCɛ inhibitor, YT-001, as a potential therapeutic to prevent I/R-induced AKI.Myr- conjugation to the N-terminus of the peptide cargo facilitates intracellular delivery via an anchoring mechanism and simple diffusion into the target tissue [21]. The current study used a scrambled control peptide (*N*-Myr-LSETKPAV) which would not be able to inhibit the binding of PKCɛ to RACK-1 (Figure 2). The scrambled peptide is the optimal control because it eliminates the variables of molecular weight (MW) and peptide binding.

to mitigate kidney I/R injury by comparing the effects of YT-001 to control peptide. Kidney injury was characterized by decreased GFR, elevated serum Cr and increased PKCɛ localization to kidney epithelium in Immunohistochemistry (IHC) analysis. The expected outcome was that YT-001 would improve indices of kidney function and reduce PKCɛ localization to kidney epithelial cell membranes compared to controls.

The aim of this study was to investigate the role of PKCE inhibition

#### Materials and Methods

All animal studies have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and were approved by the Institution's Animal Care and Use Committee at the University of Alabama at Birmingham.

#### **Chemicals and Reagents**

The scrambled control peptide is *N*-Myr-LSETKPAV; MW = 1054, and the active peptide is *N*-Myr-EAVSLKPT (YT-001); MW = 1054, both provided by Genemed Synthesis, Inc., San Antonio, TX. Anti-PKC $\epsilon$  antibody was used in IHC analysis of PKC $\epsilon$  localization in kidney I/R samples (EMD Millipore, Burlington, MA; catalog number 06-991) as described previously [23].

#### Animal Care

Mice were permitted continuous access to food and water. They were kept on a 12-hour light and 12-hour dark cycle in a temperaturecontrolled room. Thirteen male C57BL/6J mice (25–30g) were used in this study to limit protective effects of estrogen. To minimize pain and distress, mice received treatment with Buprenex (0.05-0.1mg/ kg; SC) pre-incision for preoperative care. It was also administered immediately pre-op,12 hours and 24 hours post-op. After surgery, the mice were hydrated with 0.5 mL intraperitoneal sterile saline

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and placed under a warm light for recovery from anesthesia for 1-2 hours until complete recovery. The mice were observed closely for surgical complications. Any mice with a weight loss of more than 20% were humanely euthanized. At the end of the experiment, mice were put under deep isoflurane anesthesia prior to harvesting tissues and euthanized via exsanguination while still under anesthesia.

#### In Vivo kidney I/R

Mice were anesthetized with ketamine/xylazine prior to the procedure. Animals were divided into two groups (YT-001 and scrambled peptide [control]) and under aseptic precautions, an incision was made in the left and right loin, and the renal pedicles were exposed, secured and clamped with a micro- serrefine vascular clamp (Fine Science Tools) for 19 minutes to induce bilateral ischemia (i.e. both kidneys). Complete ischemia is indicated by color change of the kidney from red to dark purple (not pale or blanching) in minutes. The kidneys are internalized during the 19 minutes ischemia and the skin incision is kept moist with saline soaked gauze. One minute before unclamping, six mice were given YT-001 (1.6 mg/ kg; approximately 20 µM serum concentration), and seven were given scrambled control peptide via tail vein injection one minute prior to reperfusion. At the end of ischemia, clamps were removed to allow reperfusion, which was confirmed visually. Kidneys were returned to the abdominal cavity in their original position. The incisions were closed with 4-0 prolene sutures, and the animals were allowed to recover.

#### Creatinine (Cr) Measurement

Blood draws were collected retro-orbitally at baseline, 24, 72, and 96 hours, and serum Cr was measured using LCMS-MS as previously described [24-25]. Reperfusion continued until sacrifice at 96 hours.

#### Glomerular Filtration (GFR) Measurement

GFR was measured using methods as previously described [26].

FITC-sinistrin (40 mg/mL) in phosphate buffered saline (PBS) was administered to mice using 0.15 mg FITC-sinistrin per gram body weight. Mice were given 2.5% isoflurane to induce anesthesia and maintained with 1.0-1.5% isoflurane. The mice were then placed prone on a heating pad, and fur was removed dorsally using an electric razor. A thin layer of depilation cream was applied to the shaved area using a cotton swab and then removed after 1-3 minutes with warm water. A 2.5 x 3 cm<sup>2</sup> transdermal GFR monitor was applied to the GFR device. The battery was then connected to the GFR device and placed on the shaved skin over the ribs using the adhesive. The device was secured with white tape and left untouched for 3 minutes before FITCsinistrin injection to allow for steady background reading. The tails of the mice were then warmed in preparation for tail vein injection. FITC-sinistrin injections were administered with an insulin syringe in one smooth but rapid bolus. Mice were then placed in their own cage to recover from anesthesia. GFR measurements were recorded for 1.5 hours before the GFR device removal. The battery was then removed from the GFR device. The GFR device was connected to the computer using a USB, and data were read via software as previously described [25, 27].

#### Immunohistochemistry (IHC)

HC localization of PKCε was performed as previously described [23]. At the end of the 96-hour reperfusion period, animals were euthanized. Kidneys were then removed, sectioned, fixed with 4% neutral- buffered formalin, and embedded with paraffin. Tissues were placed on a slide warmer overnight at 55°C. Sections were de-paraffinized, and the tissues were washed in PBS three times. 10 mM citrate buffer (0.2% sodium citrate and 0.2% tween in dH2O) was microwaved until boiling and then poured into the slide holder. Slides were added to the solution, covered in foil and then left to sit for 20 minutes. Slides were then rinsed in PBS three times before protein block was added. After 10 minutes, slides were rinsed in PBS

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and the primary antibody diluted in PBS (10  $\mu$ L antibody in 500  $\mu$ L PBS) was added. After 190 minutes, the slides were rinsed in PBS 3 times and HRP-conjugated goat anti-rabbit antibody was added [28]. After 15 minutes, the slides were rinsed four times in PBS. 10 µL of 3,3'-Diaminobenzidine (DAB) chromagen was added to 200 µL of DAB substrate and swirled to mix. The solution was added to the tissue and allowed to sit for 1-10 minutes until tissues turned light brown. The tissues were rinsed four times in PBS, then Mayer's hematoxylin was added. After 4 seconds tissues were rinsed in dH<sub>2</sub>O. Tissues were then placed in bluing solution for 30 seconds and rinsed with dH<sub>2</sub>O again. Finally, a coverslip was placed over tissues with aqueous mounting medium. Samples with more PKCe expression retained more brown staining and therefore produced more positive signals. Signal positivity was measured using Aperio Image Scope via an algorithm designed by Core Facility at the University of Pennsylvania. The pen tool was used to outline the area of interest. The area was then analyzed via Positive Pixel Count v9.

#### **Statistical Analysis**

Results are reported as mean  $\pm$  standard error of mean (SEM). Student's t-tests were used for statistical analysis. Significance was established at the 95% confidence level (P < 0.05). Sample size is indicated by (n=).

#### Results

#### Serum Creatinine (Cr)

At baseline, serum Cr levels were the same in both treated and control groups. Following I/R, serum Cr increased statistically in both groups, but recovery in the YT-001 treated animals was significantly faster and occurred to a greater extent compared to scrambled peptides over the 96-hour period. YT-001 reduced serum Cr (from  $0.07\pm0.01$  mg/dL at 0hr, to  $0.40\pm0.14$  mg/dL at 24 hr,  $0.15\pm0.02$  mg/dL at 72 hr, and  $0.13\pm0.02$  mg/dL at 96 hr; 24-96hr, p<0.05) relative to control (from  $0.08\pm0.01$  mg/dL at 0 hr, to  $0.94\pm0.20$  mg/dL at 24 hr,  $0.56\pm0.11$  mg/dL at 72 hr, and  $0.44\pm0.09$  mg/dL at 96 hr). (Figure 3) depicts the serum Cr time course of YT-001 vs scrambled peptide.



levels in murine renal ischemia-reperfusion.

#### **Glomerular Filtration Rate (GFR)**

Prior to ischemia, GFR was the same in both groups of animals. Following reperfusion, GFR was maintained to a significantly greater extent in YT-001 treated animals compared to those given scrambled peptide. GFR declined compared to baseline throughout the 96-hour reperfusion period with greatest reduction at 24 hours in both the YT-001 and scrambled groups. However, YT-001 maintained a higher GFR throughout reperfusion (from 212.20±17.51 µl/min at 0 hr, to 94.18±21.62 µl/minat 24 hr, 100.24±3.87 µl/min at 72 hr, and 110.08±10.78 µl/min at 96 hr, 24-96 hr, p<0.05) compared to control (from 230.26±23.75 µl/min at 0 hr, to 45.89±11.58 µl/min at 24 hr, 51.20±6.77 µl/min at 72 hr, and 67.59±11.27 µl/min at 96 hr). (Figure 4) displays the GFR time course of YT-001 vs scrambled peptide (control).

Serum Cr rose to 0.68 mg/dL at 24h in scrambled peptide control mice (n=7) and to 0.40 mg/dL in YT-001 mice (n=6) from baseline values ( $0.07\pm0.007$  [YT-001] vs  $0.07\pm0.003$  [scrambled peptide]) following 19-min renal ischemic injury. YT-001 significantly attenuated serum Cr levels from 24h to 96h compared to scrambled peptide control.

\* Indicates significance compared to scrambled peptide (p < 0.05).

Glomerular Filtration Rate (GFR) in Murine Renal I/R Model. GFR levels were calculated using FITC-Sinistrin renal clearance. 1.6 mg/kg ( $\sim 20\mu$ M serum conc.) YT-001 (n=6) or scrambled peptide (n=7) were given intravenously one-min prior to reperfusion following 19-min renal ischemic injury. GFR was calculated at baseline, 24h, 72h, and 96h post-

ischemic injury. Scrambled peptide mice experienced a 71% decrease in GFR (230 $\pm$ 24 $\mu$ l/mL to 68 $\pm$ 11 $\mu$ L/mL) following the 96 hr reperfusion period. Conversely, YT-001 treated mice improved GFR to 50% of baseline pre-ischemic levels (212 $\pm$ 18 $\mu$ L/mL to 110 $\pm$ 11 $\mu$ L/mL) during the same period.



\*Indicates significance compared to scrambled peptide (p < 0.05).

Figure 4: Time course of YT-001 vs scrambled peptide.

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#### Immunohistochemistry (IHC)

IHC showed increased brown pigmentation in scrambled peptide compared to YT-001treated tissue as seen in (Figure 5)These signals were quantified as mean positive signals as shown in (Table 1). The data indicate that there was a significant decrease in localization of PKC $\epsilon$  in kidney exposed to YT- 001. DAB chromogen reaction resulted in a brown precipitate indicating detection of PKC $\epsilon$ . YT-001 (Panel A) treatment attenuated PKC $\epsilon$  localization in tubular epithelium compared to scrambled peptide (Panel B). YT-001 resulted in a significant decrease in the number of positive signals (N positive) and positivity (ratio of positive signals to all signals) in whole-kidney samples

\* Indicates significance compared to scrambled peptide (p < 0.05) in (Table 1).

#### Discussion

#### **Summary of Major Findings**

This study demonstrated that i.v. administration, just prior to reperfusion, of YT-001 (1.6 mg/kg) successfully reduced evidence of kidney injury in an *in vivo* mouse model of I/R injury. In YT-001 treated animals, Cr levels increased to a significantly lower value compared to scrambled peptide controls, and YT-001 treated animals maintained a higher GFR compared to scrambled controls at 24 hours, 72 hours, and 96 hours. Furthermore, IHC results demonstrated a marked reduction in the detection of PKCɛ in the YT-001treated mice, suggesting a resultant decrease in PKCɛ uptake in the vascular and epithelial tissues of the treated kidneys. These findings help delineate the way for further targeted pre-clinical experiments to better characterize the profile and pharmacodynamics of this peptide.

#### Mechanism of Action Related to Various Organ Systems

Previous literature has established the role of PKCɛ in activating pro-fibrotic cytokines leading to neutrophil recruitment and promoting the release of ROS via uncoupled eNOS activity in I/R injuries [29-32]. PKCɛ expression is known to increase in acute I/R changes to the myocardium, in stroke, and in transplanted kidneys



Figure 5: PKCc IHC staining of YT-001 and scrambled peptide treated kidney sections following I/R (19 min/96 hrs).

Group	N Positive	N Negative	N Total	Positivity
	(mean positive signal)	(mean negative signal)	(N Positive + N Negative)	(N Positive/ N Total)
Myr-PKCe-	$9.85 \times 10^7 \pm 1.65 \times 10^7 *$	3.06x10 <sup>8</sup> ±3.55x10 <sup>7</sup>	4.04x10 <sup>8</sup> ±4.37*10 <sup>7</sup>	2.44x10 <sup>-1</sup> ±2.92x10 <sup>-2</sup> *
Myr-PKCe-scram	$1.77 \times 10^8 \pm 3.14 \times 10^7$	3.06x10 <sup>8</sup> ±2.67x10 <sup>7</sup>	4.83x10 <sup>8</sup> ±4.01*10 <sup>7</sup>	3.58x10 <sup>-1</sup> ±5.03x10 <sup>-2</sup>

**Table 1:** Comparison of PKCɛ IHC staining signal for YT-001 and scrambled peptide control treated kidney sections following I/R.

through mitochondrial and tubular damage [33-35]. PKC $\epsilon$  inhibitors have been shown tohave protective effects from I/R injury on various organs. Previous data have shown that the use of PKC $\epsilon$  inhibitors or PKC $\epsilon$  deficiency reduce the myocardial infarct size and improve overall kidney transplant function [13, 35]

#### PKCE as a Target to Decrease I/R Injury

As mentioned above, in previous studies, PKCɛ inhibition exhibits prevention of I/R injury in an *in vivo* porcine heart I/R model, as well as 90% restoration of cardiac function, and reduction of infarct size by 70%[13]. PKCɛ inhibition also demonstrates reduction in H2O2 levels in the blood in a rat hindlimb I/R model [14-15] and in ESWL [20]. Additionally, PKCɛ inhibition via a trans activator of transcription (TAT) conjugated peptide (*N*-YGRKKRRQRRR-EAVSLKPT) effectively reduces cardiac fibrosis and inflammation in a murine cardiac transplant model [36]. Together, these studies demonstrate a strong basis for targeting PKCɛ to improve kidney I/R injury.

#### **Peptide Conjugation**

Previous clinical studies of TAT-conjugated PKCe demonstrate no safety or immunogenicity concerns during phase II trials investigating the treatment of orthopedic pain and post herpetic neuralgia [37-38]. Based on these findings, it is likely that a myrconjugated YT-001 would have a similar safety profile. TAT is a sequence (YGRKKRRQRRR) of primarily positive charged amino acids (i.e. arginine [R] and lysine [K]) that facilitates intracellular delivery of the cargo sequence via an endocytic mechanism. Whereas, myr is a naturally occurring fatty acid that facilitates cargo delivery via simple diffusion [21, 39-40].

#### Prevention of Polymorphonuclear (PMN) Infiltration

It is well studied that PKCE inhibition attenuates PMN infiltration of the heart and the resultant cytokine mediated tissue damage [14-15, 41-43]. The mechanism by which PKCE infiltrates the heart and leads to damage suggests that the beneficial effects of PKCE inhibition on I/R injury is likely to be translational across organs and therefore decrease kidney damage following I/R [25, 44]. I/R injury induces superoxide release from renal mitochondria [16-19]. Superoxide, in turn, activates cytokine release, which then activates PKCE. Once activated, PKCE stimulates both the renal mitochondrial ATP dependent K+ channels and uncoupled eNOS, exponentially increasing superoxide release and overall oxidative stress [15, 19, 45]. Superoxide release from mitochondrial ATP dependent K+ channels via PKCe activation is known to be a type of preconditioning cardioprotective response prior to ischemia, however, during reperfusion superoxide release from this source is thought to exacerbate reperfusion injury and increase infarct size [15-16]. Thus, a PKCe inhibitor would prevent PKCe activation of both mitochondria and uncoupled eNOS, preventing the downstream injurious effects of ROS in I/R injury [13-15, 29-30, 46-47]

### Comparison of Results to Prior Studies in Regards to Kidney Function

A prior *in vivo* experiment by Fuller et al. 2012 evaluated the effect of sotrastaurin (inhibitor of independent PKC isoforms) on I/R injuries of a transplanted kidney, which shows a significant

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improvement in kidney function through reduced apoptosis and extended renal cold preservation [48]. Both experiments were different in several ways. First, although this experiment inhibits a different isoform of PKC, outcomes are similar which further emphasizes the importance and versatility of PKC inhibitors as therapeutic targets. Second, the study by Fuller et al. used different outcome measurements excluding Cr levels, such as urine analyses and serial histological tissue sampling. Therefore, results are not directly comparable due to different endpoints.

#### Limitations

The pre-clinical and translational nature of this study limits the ability to extrapolate the results in a clinical setting. Further preclinical and clinical studies (in particular, over a longer time-period) are still needed to achieve this goal. Since only one dose was tested, a dose-response with multiple doses will provide additional, valuable information.

#### Conclusion

In summary, PKCɛ inhibition by YT-001 had a nephroprotective effect in *in vivo* I/R kidney injury. Based upon previous studies with YT-001 in other I/R models and data from the literature, it is likely that YT-001 inhibits ROS generation and therefore the underlying pathology of DGF. In addition, by preserving/improving the health of these donor kidneys, it is possible that fewer kidneys will be rejected by transplant surgeons, thereby increasing the size of the donor pool. PKCɛ inhibitors should be investigated further as a possible therapeutic target for kidney transplant in the future. Future studies will evaluate the long-term (9-month) protection of kidney function in mice treated with YT-001 vs. control peptide.

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