Hexapeptide derived from prothymosin alpha attenuates cisplatin-induced acute kidney injury

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Abstract

Background. Prothymosin alpha (ProTα) is a nuclear protein expressed in virtually all mammalian tissues. Previous studies have shown that ProTα exhibits protective effects against ischemia-induced cell death in various cell types. Recently, the 6-residue peptide P_6Q (NEVDQE), the modified form of the active 6-residue core (51–56) in ProTα, has also been shown to protect effect against retinal ischemia. However, it remains to be elucidated whether P_6Q is effective against acute kidney injury (AKI). Therefore, we investigated the renoprotective effect of P_6Q on cisplatin-induced AKI.

Methods. Cultured HK-2 cells were treated with cisplatin for 24 hours and cell viability was evaluated using the MTT assay. In an *in vivo* study, 8-week-old male Wistar rats were divided into control, cisplatin-treated, and cisplatin-treated with P_6Q injection groups. In the last of these, P_6Q was injected intravenously before cisplatin treatment. Then, we evaluated the renoprotective effect of P_6Q.

Results. In the study on cultured cells, pretreatment with ProTα or P_6Q prevented cisplatin-induced cell death. In the *in vivo* study, pretreatment with P_6Q significantly attenuated cisplatin-induced increases in serum creatinine and blood urea nitrogen levels, renal tubular cell injury, and apoptosis. Moreover, P_6Q attenuated the mitochondrial apoptotic pathway and accelerated Akt phosphorylation after cisplatin-induced renal damage.

Conclusion. Taken together, our findings indicate that P_6Q can attenuate cisplatin-induced AKI and suppress the mitochondrial apoptotic pathway via Akt phosphorylation. These data suggest that P_6Q

has potential as a preventative drug for cisplatin-induced AKI.

Keywords: prothymosin alpha, renoprotective peptide, cisplatin nephrotoxicity, apoptosis, acute kidney injury.

Introduction

Cisplatin is one of the most common anti-cancer drugs used to treat solid cancers [1, 2]. However, it is associated with multiple adverse effects, including nephrotoxicity [3]. The incidence of cisplatin nephrotoxicity is 20–30%, limiting its clinical application [4]. Cisplatin nephrotoxicity is related to its preferential uptake by proximal tubular cells. After cisplatin administration, cisplatin is freely filtered at the glomerulus and taken up into the renal tubular cells, primarily by the copper transporter protein 1 (Ctr1) and organic cation transporter 2 (OCT2) [4, 5]. Then, cisplatin induces tubular cell injury via oxidative stress, inflammation, hypoxia, and cell death (apoptosis and necrosis) [6, 7]. These mechanisms have been targeted to prevent cisplatin nephrotoxicity, but no ideal treatment has been established.

Prothymosin (ProTα) is a small (~12.5 kDa), highly acidic, nuclear protein expressed in virtually all mammalian tissues [8, 9]. ProTα has multiple functions in cell survival, proliferation, and immunogenicity [8-13]. Several studies have reported a therapeutic effect of ProTα in multiple disease models. For example, ProTα has been shown to prevent ischemia-induced apoptosis in cardiocytes via Akt activation [8] and ischemia-induced damage in the retina [14]. Different forms of ProTα have different effects. Recently, it was reported that the active core 30-residue peptide of ProTα (P_{30}, residues 49–78) is essential for neuroprotection against brain and retinal ischemia as well as serum-starvation stress [15]. Furthermore, a 6-residue peptide (P_6, 51–56: NEVDEE) from ProTα also has

69 been reported to have a neuroprotective effect against retinal ischemia, and a modified P_6, P_6Q
70 (NEVDQE) showed a stronger protective effect than the unmodified peptide [16]. Although the
71 mechanism of action of P_6Q is not fully understood, the deteriorative factors, including oxidant stress,
72 inflammation, hypoxia, and apoptosis, in retinal ischemia are shared with acute kidney injury,
73 including cisplatin nephrotoxicity [6, 7, 17, 18]. Therefore, we hypothesized that P_6Q would prevent
74 cisplatin nephrotoxicity and have tested this hypothesis.

Materials and Methods

Cell culture and drug treatment

HK-2 cells, a proximal tubular cell line derived from healthy human kidney, were purchased from the ATCC (CRL-2190; Manassas, VA, USA). HK-2 cells were cultured in Keratinocyte Serum-Free (KCSF) medium supplemented with 5 ng/ml epidermal growth factor, 0.05 mg/ml bovine pituitary extract (17005042; Life Technologies), 100 U/ml penicillin (15140122; Life Technologies), and 100 μg/ml streptomycin (15140122; Life Technologies) in 75-cm^2 tissue culture flasks in a 5% CO_2 atmosphere at 37 ℃. For drug treatments, HK-2 cells were seeded at a density of 1×10^4 cells/well in 96-well plates and treated with 12.5 μM cisplatin (479306-1G; Sigma-Aldrich, Saint Louis, MO, USA) for 24 hours. Pretreatment with 0-80 μM of recombinant human ProTα (CSB-YP0190000HU; CUSABIO, Wuhan, China) or 0-100 μM of P_6Q peptide (275754; GL Biochem, Shanghai, China) was carried out 30 minutes before cisplatin treatment.

Cell viability assay

Cell viability was evaluated using a CytoSelect™ MTT Cell Proliferation Assay kit (CBA-252; Cell Biolabs Inc., San Diego, CA, USA), according to the manufacturer's instructions. Absorbance was measured at 540 nm with a microplate reader (MULTISKAN FC; Thermo Scientific, Kanagawa, Japan).

93

94 **Animals**

95 All studies were performed on 8-week-old male Wistar rats (Japan SLC Inc., Shizuoka, Japan),
96 housed in standard rodent cages in a light- and temperature-controlled room in the Biomedical
97 Research Center, Center for Frontier Life Sciences, Nagasaki University, with free access to laboratory
98 food and tap water.

99

100 **Animal experimental protocol**

101 Wistar rats were divided into three groups: control (CTL), which received 16 ml/kg saline
102 intraperitoneally and 3 ml/kg phosphate-buffered saline (PBS) intravenously (n = 3); P_6Q, which
103 received 16 ml/kg saline intraperitoneally and 30 mg/kg P_6Q in PBS intravenously (n = 3); cisplatin
104 (Cis), which received 8 mg/kg cisplatin intraperitoneally and 3 ml/kg PBS intravenously (n = 5). In
105 addition to these three groups, we evaluated renal function in two additional groups: cisplatin with
106 P_6Q (Cis + P_6Q), which received cisplatin 8 mg/kg intraperitoneally and 30 mg/kg P_6Q
107 intravenously (n = 5); cisplatin with control peptide (Cis+CTL peptide), which received 8 mg/kg
108 cisplatin intraperitoneally and 30 mg/kg control peptide intravenously (n = 4). The Cis + CTL peptide
109 group was added as a control for nonspecific effects of peptide injection. Control peptide was a
110 mixture of N (70-47-3; GL Biochem, Shanghai, China) and EVDQE (713038; GL Biochem,

111 Shanghai, China). P_6Q and control peptide were injected 30 minutes before cisplatin treatments.

112 The doses and timing for administration of P_6Q were selected on the basis of a pilot study.
113 Pretreatment with P_6Q at 30 mg/kg, but not 10 mg/kg, attenuated cisplatin nephrotoxicity. We found
114 that administration of P_6Q alone at a dose of 30 mg/kg did not affect renal function. Furthermore,
115 posttreatment with P_6Q at a dose of 30 mg/kg did not attenuate cisplatin nephrotoxicity. Therefore,
116 we selected pretreatment with P_6Q at a dose of 30 mg/kg. Five days after cisplatin treatment, rats
117 were sacrificed and the blood and kidneys collected. Serum creatinine (Cr) and blood urea nitrogen
118 (BUN) levels were measured using enzymatic methods (SRL, Tokyo, Japan). Dissected kidneys were
119 fixed with 4% paraformaldehyde in PBS (pH 7.4) immediately after sampling and were embedded
120 in paraffin. For histology, 3-μm-thick paraffin-embedded tissue sections were stained with Periodic
121 Acid-Schiff (PAS) stain.

122

123 **Immunohistochemistry**

124 Paraffin-embedded tissue sections were examined immunohistochemically using an indirect method.
125 The following antibodies were used: rabbit anti-active caspase-3 (1:50) (AB3623; Millipore, Temecula,
126 CA, USA), rabbit anti-caspase 9 (1:30) (10380-1-AP; Proteintech Group, Chicago, IL, USA), rabbit
127 anti-phosphorylated Akt (pAkt) diluted at 15 μg/ml (AF887; R&D systems, Minneapolis, MN, USA),
128 mouse anti-hypoxia-inducible factor 1α (HIF-1α) (1:40) (NB100-105; Novus Biologicals, Littleton,

CO, USA), mouse anti-ED-1 (1:400) (MCA341R; Serotec, Kidlington, UK), mouse anti-4-hydroxy-2-nonenal (4-HNE) (1:50) (MHN-100P; Jaica, Shizuoka, Japan), and goat anti-caspase 1 (1:50) (sc-1597; Santa Cruz Biotechnology, TX, USA).

After deparaffinization, for active caspase-3, pAkt, and ED-1 staining, sections were treated with proteinase K (P2308; Sigma-Aldrich, Saint Louis, MO, USA) for 15 minutes at 37 °C for antigen retrieval. For caspase 9 and HIF-1α staining, the sections were heated to 95 °C for 15 minutes in 10 mM citrate buffer (pH 6.0) for antigen retrieval. Sections were treated with 0.3% H_2O_2 in methanol for 20 minutes to inactivate endogenous peroxidase activity, then incubated with a blocking solution [500 μg/ml normal goat IgG in 1% bovine serum albumin (BSA) in PBS, 500 μg/ml normal rabbit IgG dissolved in 1% BSA in PBS, or 10% normal goat serum] for 30 minutes at room temperature (RT). For caspase-9 and pAkt staining, sections were stained with the avidin-biotin complex using a Vectastain Elite ABC-HRP kit (PK-6101; Vector Laboratories, Burlingame, CA, USA) after reacting with the primary antibody overnight at 4 °C. For active caspase-3 and 4-HNE staining, sections were stained with the mouse Envision kit (K4000; Dako) or rabbit Envision kit (K4002; Dako) after reacting with the primary antibody overnight at 4 °C. For HIF-1α, ED-1, and caspase-1 staining, after reacting with the primary antibodies for 1 hour or overnight at RT, sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin antibody (P0448; Dako) or goat anti-mouse immunoglobulin antibody (P0447; Dako) diluted at 1:100 or 1:200 for 1 hour at RT. Antibody

147 binding was visualized by treating the sections with H_2O_2 and 3,3'-diaminobenzidine
148 tetrahydrochloride (DAB). Finally, after counterstaining with methyl green, sections were dehydrated
149 and mounted. For all specimens, negative controls were prepared using normal IgG instead of primary
150 antibody.
151
152 **Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining**
153 To identify renal tubular cell apoptosis, TUNEL staining was performed as described previously [19].
154 In each sample, the numbers of TUNEL-positive nuclei were counted in 10 fields at ×400
155 magnification.
156
157 **Histological analysis**
158 We carried out a semiquantitative analysis of the morphological changes in the outer medulla and
159 corticomedullary junction at day 5 after cisplatin treatment. Renal tubular injury was scored and
160 analyzed as described previously [20].
161 For the semiquantitative evaluation of the positive area for immunohistochemistry, images were
162 digitized using ImageJ software (National Institute of Health, MD, USA). Positive areas in the outer
163 medulla and the corticomedullary junction were examined using a light microscope (Nikon ECLIPSE
164 E600; Nikon, Tokyo, Japan). For each sample, five areas were selected and the positive area was

identified at ×200 magnification. For each sample, the numbers of ED-1-positive cells were counted in 10 fields at ×400 magnification.

Western blotting

Whole kidney protein was extracted by using CelLytic™ MT Cell Lysis Reagent (C3228; Sigma-Aldrich, St. Louis, MO, USA). Extracts were mixed with sample buffer solution (AE-1430; ATTO, Tokyo, Japan) and heated at 95 ℃ for 5 minutes. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA in Tris (hydroxymethyl) aminomethane-buffered saline (pH 7.2–7.4) containing 0.05% Tween-20 (TBS-T) for 2 hours and incubated with primary antibodies against pAkt (0.4 μg/ml) or total Akt (2 μg/ml) (MAB2055; R&D systems, Minneapolis, MN, USA) overnight at RT. Membranes were washed with TBS-T five times and incubated with HRP-conjugated goat anti-rabbit (1:2500) or goat anti-mouse (1:1000) immunoglobulin antibody for 1 hour at RT. The membranes were washed five times again, and the signals were visualized using Pierce ECL Plus western blotting substrate (170-5061; Bio-Rad Laboratories, Hercules, CA, USA) and quantified using the ChemiDoc™ MP System (170-8280J1; Bio-Rad Laboratories, Hercules, CA, USA).

182 **Statistical analyses**

183 Data are expressed as means ± standard errors. Differences among groups were examined for
184 statistical significance using repeated measures analysis of variance (Tukey's Honestly Significant
185 Difference tests). All statistical analyses were performed using JMP version 13 software (SAS Institute
186 Inc., Cary, NC, USA). Differences were considered statistically significant at a *P*-value less than 0.05.

Results

ProTα and hexapeptide derived from ProTα attenuated cisplatin-induced tubular cell cytotoxicity

First, we examined the protective effects of ProTα and P_6Q on cisplatin-induced tubular cell cytotoxicity by MTT assays. When HK-2 cells were treated with 12.5 μM cisplatin for 24 hours, the viability of cisplatin-treated cells decreased to 82.1–82.6% (Figure 1A and 1B). However, this cytotoxicity was significantly attenuated by co-treatment with ProTα. For example, 20 μM ProTα increased the viability of cisplatin-treated cells to 94.5% (Figure 1A). Similarly, co-treatment with P_6Q significantly attenuated cisplatin-induced cytotoxicity; treatment with 100 μM P_6Q increased the viability of cisplatin-treated cells to 91.2% (Figure 1B). These results suggested that both ProTα and modified hexapeptide P_6Q had a protective effect on cisplatin-induced cytotoxicity in renal tubular cells.

P_6Q attenuated cisplatin-induced acute kidney injury in rats

We next examined the protective effects of P_6Q on cisplatin nephrotoxicity *in vivo*. In contrast with the CTL and P_6Q groups, serum Cr and BUN levels peaked on day 5 after cisplatin treatment in the Cis group. However, P_6Q injection significantly suppressed serum Cr and BUN levels on day 5. Control peptide did not suppress serum Cr and BUN levels (Figure 2 A, B). Furthermore, in contrast

205 with the CTL and P_6Q groups, histologic examination of renal tissue on day 5 revealed extensive
206 tubular damage and intratubular cast formation in the outer medulla and corticomedullary junction in
207 the Cis group. The tubular damage score increased in the Cis group. However, P_6Q treatment
208 significantly suppressed these histological changes and decreased the tubular damage score. Control
209 peptide did not suppress histological change (Figure 2 C-F, K). Because renal tubular cell apoptosis is
210 one of the most crucial factors in cisplatin nephrotoxicity [6, 7], we analyzed apoptotic renal tubular
211 cells by TUNEL staining. As shown in Figure 2 G-J and L, TUNEL-positive apoptotic cells were more
212 numerous around the corticomedullary junction in the Cis group than in the Cis+P_6Q group. These
213 results indicated that P_6Q prevented cisplatin-induced AKI, as well as tubular apoptosis *in vivo*.
214
215 **P_6Q suppressed the mitochondrial apoptotic pathway via Akt activation**
216 Previous reports have shown that ProTα suppresses the mitochondrial apoptotic pathway [8].
217 Therefore, we performed immunohistochemical detection of active caspase-3, a standard marker for
218 apoptosis, and of caspase-9, a marker for mitochondrial apoptosis. The expression of active caspase-
219 3 and caspase-9 increased in the Cis group on day 5 after cisplatin treatment. As hypothesized, P_6Q
220 suppressed the expression of both caspase-3 (Figure 3 A-D) and caspase-9 (Figure 3 E-H). These
221 results suggested that P_6Q suppressed the cisplatin-induced mitochondrial apoptotic pathway.
222 Next, we performed immunohistochemistry and western blot analyses for pAkt, a critical regulator

223 of the anti-apoptotic effect of ProTα [8]. Immunohistochemical analysis of pAkt demonstrated that
224 the sole administration of P_6Q enhanced pAkt expression significantly compared with that in the CTL
225 group. Moreover, both analyses indicated that pAkt expression was higher in the Cis group than in the
226 CTL group but that the difference was not significant. On the contrary, pAkt expression was highest
227 in the Cis + P_6Q group (Figure 4 A-G). Taken together, these results indicated that P_6Q enhanced Akt
228 phosphorylation and suppressed the cisplatin-induced mitochondrial apoptotic pathway.
229
230 **P_6Q did not affect cisplatin-induced renal oxidative stress, inflammation, and hypoxia**
231 Cisplatin induces renal tubular damage via hypoxia, inflammation, and oxidative stress, all of
232 which can be an upstream causes in the induction of apoptosis [6, 7]. Therefore, we investigated the
233 effect of P_6Q on these factors. Immunohistochemical analyses of HIF-1α, ED-1, caspase-1, and 4-
234 HNE were performed on day 5 after cisplatin treatment. As shown in Figure 5 A-P, cisplatin
235 increased HIF-1α, caspase-1, and 4-HNE expression, as well as the frequency of ED-1-positive
236 cells, but P_6Q did not suppress any of these factors. These results suggested that the renoprotective
237 effect of P_6Q is independent of these pathways.
238

Discussion

In this study, we found that ProTα and its derivative, P_6Q, significantly reduced cisplatin-induced cytotoxicity. We also found that P_6Q also inhibited cisplatin-induced AKI successfully by suppressing renal tubular cell apoptosis through the acceleration of Akt activation.

As for the mechanism of the anti-apoptotic effect of P_6Q, previous studies have reported that ProTα prevented apoptosis via inhibition of apoptosome formation or Akt activation [23]. Apoptosome formation is a critical event in mitochondrial apoptotic pathways [24], and Akt activation also suppresses mitochondrial apoptotic pathways via inhibition of proapoptotic Bcl-2 family members and caspase-9 [25, 26]. In our study, pretreatment with P_6Q suppressed cisplatin-induced caspase-9 expression; moreover, P_6Q accelerated Akt phosphorylation. Therefore, these results indicate that Akt activation may be one of the critical mechanisms in the anti-apoptotic action of P_6Q, similar to that of ProTα. Furthermore, in our pilot study P_6Q posttreatment did not attenuate cisplatin nephrotoxicity. This result suggested that preactivation of anti-apoptotic factor by P_6Q might be important for renoprotective action.

On the other hand, a previous study showed that preconditioning with ProTα inhibited the expression of proinflammatory cytokines in a retinal ischemia model [14]; this is in accordance with the finding that the inflammatory pathway is one of the major inducers of apoptosis [22]. However, in our study, P_6Q did not inhibit the infiltration of inflammatory cells and expression of caspase-1 that might promote the secretion of proinflammatory cytokines. Hypoxia and oxidative stress are also major

258 inducers of apoptosis, and the central domain of ProTα (residues 32–52) is known to be related to the
259 anti-oxidative stress effect via Nrf2/Keap1 pathway [13]. However, P_6Q did not affect cisplatin-
260 induced hypoxia and oxidative stress in this study. Therefore, the mechanism of P_6Q cytoprotective
261 effect may be different depending upon cell or tissue type.

262 Taken together, we propose the following mechanism: P_6Q preconditioning activates anti-apoptotic
263 factor in tubular cells. After cisplatin administration, cisplatin is taken up by tubular cells and induces
264 inflammation, oxidative stress, and hypoxia. However, downstream apoptosis and tubular cell damage
265 are attenuated by preactivated anti-apoptotic factors. Our data point to Akt activation as a critical factor.
266 An earlier study showed that Akt activation by ProTα administration worked via binding to the plasma
267 membrane [8]; therefore, P_6Q might also activate Akt by binding to a membrane receptor. However,
268 the molecular mechanisms and pharmacokinetics of P_6Q remain unclear. Moreover, regarding the
269 relationship between P_6Q and pAkt, this study was unable to determine a cause-consequence
270 relationship. Thus, our study could not fully clarify the renoprotective mechanism of P_6Q. On the
271 contrary, co-administration of P_6Q and cisplatin or sole administration of P6Q enhanced Akt
272 phosphorylation. This result supports the hypothesis that P_6Q itself may affect Akt phosphorylation.
273 Therefore, we consider Akt to be a critical factor affecting the P6Q renoprotective effect. However
274 further studies are needed to determine the precise mechanism involved.

275 Our study showed beneficial effects from P_6Q treatment, but there are limitations for its clinical

276 application. First, the long-term beneficial effect of P_6Q is not clear. Indeed, renal function peaked on
277 day 5 after cisplatin treatment and then decreased to the same level in the Cis and Cis + P_6Q groups
278 (data not shown). However, clinical studies have shown that the severity of AKI is a major factor in
279 all-cause mortality and chronic kidney disease progression [27]. Therefore, we anticipate that P_6Q
280 preconditioning for cisplatin nephrotoxicity would have long-term beneficial effects. Second, P_6Q
281 might contribute to cancer proliferation. In fact, ProTα has been reported to be involved in cancer
282 progression and resistance to cisplatin [28, 29]. Akt activation is also known to be related to cancer
283 proliferation [30]. Therefore, it is prudent to have concerns about the potential for P_6Q to attenuate
284 anti-cancer effects. However, at least in our study, P_6Q did not induce renal or other malignant
285 alterations (data not shown). Furthermore, even if P_6Q affect cancer cells, it is controversial whether
286 the doses and frequency of P_6Q administration in this study would be high enough to cause concerns.
287 In conclusion, from our data, P_6Q shows significant promise as a preventative therapeutic for
288 cisplatin nephrotoxicity. However, before clinical use further studies are needed to investigate whether
289 P_6Q influences cancer cell proliferation, as well as determination of appropriate dosages.
290

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Conflict of interest

The authors have declared that no conflict of interest exists.

Human and Animal rights:

This article does not contain any studies with human participants.

Ethical approval: All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (IRB approval number 1506261242-5).

Informed Consent

This section is not applicable to this study.

References

1. Dasari S, Bernard Tchounwou P. Cisplatin in cancer therapy: Molecular mechanisms of action. Eur J Pharmacol. 2014; 740:364–78.

2. Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. Nat Rev Drug Discov. 2005; 4:307–20.

3. Rybak LP, Whitworth CA, Mukherjea D, Ramkumar V. Mechanisms of cisplatin-induced ototoxicity and prevention. Hear Res. 2007; 226:157–67.

4. Miller RP, Tadagavadi RK, Ramesh G, Reeves WB. Mechanisms of cisplatin nephrotoxicity. Toxins. 2010; 2:2490–518.

5. Manohar S, Leung N. Cisplatin nephrotoxicity: a review of the literature. J Nephrol. 2018; 31:15–25.

6. Yao X, Panichpisal K, Kurtzman N, Nugent K. Cisplatin nephrotoxicity: a review. Am J Med Sci. 2007; 334:115–24.

7. Pabla N, Dong Z. Cisplatin nephrotoxicity: Mechanisms and renoprotective strategies. Kidney Int. 2008; 7:994–1007.

8. Cannavo A, Rengo G, Liccardo D, Pironti G, Scimia MC, Scudiero L, De Lucia C, Ferrone M, Leosco D, Zambrano N, Koch WJ, Trimarco B, Esposito G. Prothymosin alpha protects cardiomyocytes against ischemia-induced apoptosis via preservation of Akt activation. Apoptosis. 2013; 18:1252–61.

9. Mosoian A. Intracellular and extracellular cytokine-like functions of prothymosin α: implications for the development of immunotherapies. Future Med Chem. 2011; 3:1199–208.

10. Emmanouilidou A, Karetsou Z, Tzima E, Kobayashi T, Papamarcaki T. Knockdown of prothymosin α leads to apoptosis and developmental defects in zebrafish embryos. Biochem Cell Biol. 2013; 91:325–32.

11. Ioannou K, Samara P, Livaniou E, Derhovanessian E, Tsitsilonis OE. Prothymosin alpha: a ubiquitous polypeptide with potential use in cancer diagnosis and therapy. Cancer Immunol Immunother. 2012; 61:599–614.

12. Jiang X, Kim H-E, Shu H, Zhao Y, Zhang H, Kofron J, Donnelly J, Burns D, Ng SC, Rosenberg S, Wang X. Distinctive roles of PHAP proteins and prothymosin-alpha in a death regulatory pathway. Science. 2003; 299:223–6.

13. Ueda H, Matsunaga H, Halder SK. Prothymosin α plays multifunctional cell robustness roles in genomic, epigenetic, and nongenomic mechanisms. Ann N Y Acad Sci. 2012; 1269:34–43.

14. Halder SK, Matsunaga H, Ishii KJ, Ueda H. Prothymosin-alpha preconditioning activates TLR4-TRIF signaling to induce protection of ischemic retina. J Neurochem. 2015; 135:1161–77.

15. Halder SK, Matsunaga H, Yamaguchi H, Ueda H. Novel neuroprotective action of prothymosin alpha-derived peptide against retinal and brain ischemic damages. J Neurochem. 2013; 125:713–23.

16. Ueda H, Halder SK, Matsunaga H, Sasaki K, Maeda S. Neuroprotective impact of prothymosin alpha-derived hexapeptide against retinal ischemia-reperfusion. Neuroscience. 2016; 318:206–18.

17. Minhas G, Sharma J, Khan N. Cellular stress response and immune signaling in retinal ischemia-reperfusion injury. Front Immunol. 2016; 7:444.

18. Katai N, Yoshimura N. Apoptotic retinal neuronal death by ischemia-reperfusion is executed by two distinct caspase family proteases. Invest Ophthalmol Vis Sci. 1999; 40:2697–705.

19. Song N, Endo D, Song B, Shibata Y, Koji T. 5-aza-2′-deoxycytidine impairs mouse spermatogenesis at multiple stages through different usage of DNA methyltransferases. Toxicology. 2016; 361–362:62–72.

20. Miyaji T, Kato A, Yasuda H, Fujigaki Y, Hishida A. Role of the increase in p21 in cisplatin-induced acute renal failure in rats. J Am Soc Nephrol. 2001; 12:900–8.

21. Sancho-Martínez SM, Piedrafita FJ, Cannata-Andía JB, López-Novoa JM, López-Hernández FJ. Necrotic concentrations of cisplatin activate the apoptotic machinery but inhibit effector caspases and interfere with the execution of apoptosis. Toxicol Sci. 2011; 122:73–85.

22. dos Santos NAG, Carvalho Rodrigues MA, Martins NM, dos Santos AC. Cisplatin-induced nephrotoxicity and targets of nephroprotection: an update. Arch Toxicol. 2012; 86:1233–50.

23. Qi X, Wang L, Du F. Novel small molecules relieve prothymosin alpha-mediated inhibition of

363 apoptosome formation by blocking its interaction with Apaf-1. Biochemistry. 2010; 49:1923–30.

364 24. Schafer ZT, Kornbluth S. The apoptosome: physiological, developmental, and pathological

365 modes of regulation. Dev Cell. 2006; 10:549–61.

366 25. Pugazhenthi S, Nesterova A, Sable C, Heidenreich KA, Boxer LM, Heasley LE, Reusch JE.

367 Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding

368 protein. J Biol Chem. 2000; 275:10761–6.

369 26. Zhou H, Li XM, Meinkoth J, Pittman RN. Akt regulates cell survival and apoptosis at a

370 postmitochondrial level. J Cell Biol. 2000; 151: 483–94.

371 27. Chawla LS, Kimmel PL. Acute kidney injury and chronic kidney disease: An integrated clinical

372 syndrome. Kidney Int. 2012; 82(5):516-524.

373 28. Suzuki S, Takahashi S, Takahashi S, Takeshita K, Hikosaka A, Wakita T, Nishiyama N, Fujita T,

374 Okamura T, Shirai T. Expression of prothymosin alpha is correlated with development and

375 progression in human prostate cancers. Prostate. 2006; 66:463–9.

376 29. Lin Y Te, Liu YC, Chao CCK. Inhibition of JNK and prothymosin-alpha sensitizes hepatocellular

377 carcinoma cells to cisplatin. Biochem Pharmacol. 2016; 122:80-89.

378 30. Bellacosa A, Kumar CC, Di Cristofano A, Testa JR. Activation of AKT kinases in cancer:

379 implications for therapeutic targeting. Adv Cancer Res. 2005; 94: 29–86.

380

381 **Figure legends**

382 **Figure 1: Viability of cisplatin-treated HK-2 cells in the presence of ProTα or P_6Q**

383 Cell viability was measured using the MTT assay. (A) ProTα prevented cisplatin-induced cytotoxicity.
384 HK-2 cells were pretreated with or without ProTα (0–80 μM) for 30 minutes and then cultured in the
385 presence or absence of 12.5 μM cisplatin for 24 hours. (B) P_6Q also prevented cisplatin-induced
386 cytotoxicity. HK-2 cells were pretreated with or without P_6Q (0–100 μM) for 30 minutes and then
387 cultured in the presence or absence of 12.5 μM cisplatin for 24 hours. *$P < 0.05$; Tukey's honestly
388 significant difference test; error bars indicate means ± standard error. n = 8 for each group.

389

390 **Figure 2: Evaluation of renal function, morphological changes, and tubular cell apoptosis in the**
391 **kidneys**

392 (A and B) Serum levels of blood urea nitrogen (BUN) and creatinine (Cr) 5 days after cisplatin (Cis)
393 treatment. In the Cis group, both serum BUN and Cr increased compared with the levels in the control
394 (CTL) and P_6Q groups. In the Cis＋P_6Q group, pretreatment with P_6Q prevented the increases in serum
395 BUN and Cr, but the CTL peptide failed to prevent them. (C-G and M) Periodic acid-Schiff (PAS)
396 stain of the kidneys (magnification ×200). (C and D) In the CTL and P_6Q groups, most tubular cells
397 appeared to be normal. (E) In the Cis group, tubular injury, including protein casts, vacuolation, and
398 desquamation of the epithelial cells in the renal tubules, was observed. (F) In the Cis＋P_6Q group,

399 tubular injury was decreased. (G) In the Cis＋CTL peptide group, the tubular injury was not decreased
400 compared with Cis group. (M) Bar graph showing the tubular injury score assessed by PAS staining.
401 (H-L and N) TUNEL stain of the kidneys (magnification ×400). (H and I) In the CTL group, few
402 positive nuclei were observed. (J) In the Cis group, the number of TUNEL-positive nuclei significantly
403 increased. (K) In the Cis＋P_6Q group, the number of TUNEL-positive nuclei significantly decreased
404 compared. (L) In the Cis＋CTL peptide group, the number of TUNEL-positive nuclei remained high.
405 (N) Bar graph of counts of TUNEL-positive nuclei. (A and B) n = 4–9 for each group. (M and N) n =
406 3, CTL and P_6Q groups; n = 5, Cis and Cis＋P_6Q groups; n = 4 Cis + CTL peptide group. *$P < 0.05$;
407 Tukey's honestly significant difference test; error bars indicate means ± standard error.
408
409 **Figure 3: Immunohistochemistry for active caspase-3 and caspase-9 in the kidneys**
410 (A-D) Immunohistochemical analyses of active caspase-3 in kidney (magnification ×200). (A) In the
411 control (CTL) group, little active caspase-3 expression was observed. (B) In the cisplatin (Cis) group,
412 active caspase-3 expression was increased. (C) In the Cis＋P_6Q group, active caspase-3 expression
413 was significantly decreased relative to Cis alone. (D) Bar graph showing active caspase-3-positive
414 areas. (E-H) Immunohistochemical analysis of caspase-9 in the kidneys (magnification ×200). (E) In
415 CTL rats, little caspase-9 expression was observed. (B) In the Cis group, caspase-9 expression was
416 significantly increased. (C) In the Cis＋P_6Q group, caspase-9 expression was significantly decreased

417 relative to that in the Cis group. (D) Bar graph showing the caspase-9-positive area; n = 3, CTL group;

418 n = 5, Cis and Cis＋P_6Q groups. *P < 0.05; Tukey's honestly significant difference test; error bars

419 indicate means ± standard error.

420

421 **Figure 4: Immunohistochemistry and western blotting for pAkt in the kidney**

422 (A-E) Immunohistochemical analysis of pAkt in the kidney (magnification ×200). (A) In the control

423 (CTL) group, little pAkt expression was observed. (B) In the P_6Q group, pAkt expression was

424 significantly increased over that in the CTL group. (C) In the cisplatin (Cis) group, pAkt expression

425 was increased, but not significantly over that in the CTL group. (D) In the Cis＋P_6Q group, pAkt

426 expression was significantly increased over that in the other groups. (E) Bar graph showing pAkt-

427 positive areas. (F and G) Western blotting for pAkt. (F) Trends of the western blotting results for pAkt

428 were consistent with those of the immunohistochemistry results. (G) Densitometric analysis of

429 pAkt/Akt ratios by western blotting. Bar graph showing pAkt/Akt signal intensities. (E) n = 3, CTL

430 and P_6Q group; n = 5, Cis and Cis＋P_6Q group. (G) n = 3, CTL group; n = 5, Cis and Cis＋P_6Q group.

431 *P < 0.05; Tukey's honestly significant difference test; error bars indicate means ± standard error.

432

433 **Figure 5: Immunohistochemical analyses of HIF-1α, ED-1, caspase-1, and 4-HNE in the kidney**

434 (A-D) Immunohistochemical analysis of HIF-1α in the kidneys (magnification ×200). (A) In the

435 control (CTL) group, little HIF-1α expression was observed. (B-C) In the cisplatin (Cis) group and
436 Cis＋P_6Q group, HIF-1α expression was significantly increased over that of the CTL group. However,
437 there was no difference between the Cis and Cis＋P_6Q groups. (D) Bar graph showing HIF-1α-
438 positive area. (E-H) Immunohistochemical analysis of ED-1 in the kidneys (magnification ×400). (E)
439 In the CTL group, few ED-1-positive cells were observed. (F-G) In the Cis group and Cis＋P_6Q group,
440 the numbers of ED-1-positive cells were significantly increased over that of the CTL group. However,
441 there was no difference between the Cis and Cis＋P_6Q groups. (H) Bar graph showing numbers of
442 ED-1-positive cells. (I-L) Immunohistochemical analysis of caspase-1 in the kidneys (magnification
443 ×200). (I) In the CTL group, little caspase-1 expression was observed. (J and K) In the Cis group and
444 Cis＋P_6Q group, caspase-1 expression was significantly increased. However, there was no difference
445 between the Cis and Cis＋P_6Q groups. (L) Bar graph showing caspase-1-positive areas. (M-P)
446 Immunohistochemical analysis of 4-HNE in the kidneys (magnification ×200). (M) In the CTL group,
447 little 4-HNE expression was observed. (N-O) In the Cis group and Cis＋P_6Q group, 4-HNE expression
448 was significantly increased. However, there was no difference between the Cis and Cis＋P_6Q groups.
449 (P) Bar graph showing 4-HNE-positive areas. (D, H, L, P) n = 3, CTL group. N = 5, Cis, and Cis＋
450 P_6Q group. *$P < 0.05$; Tukey's honestly significant difference test; error bars indicate means ± standard
451 error.

Figure 1

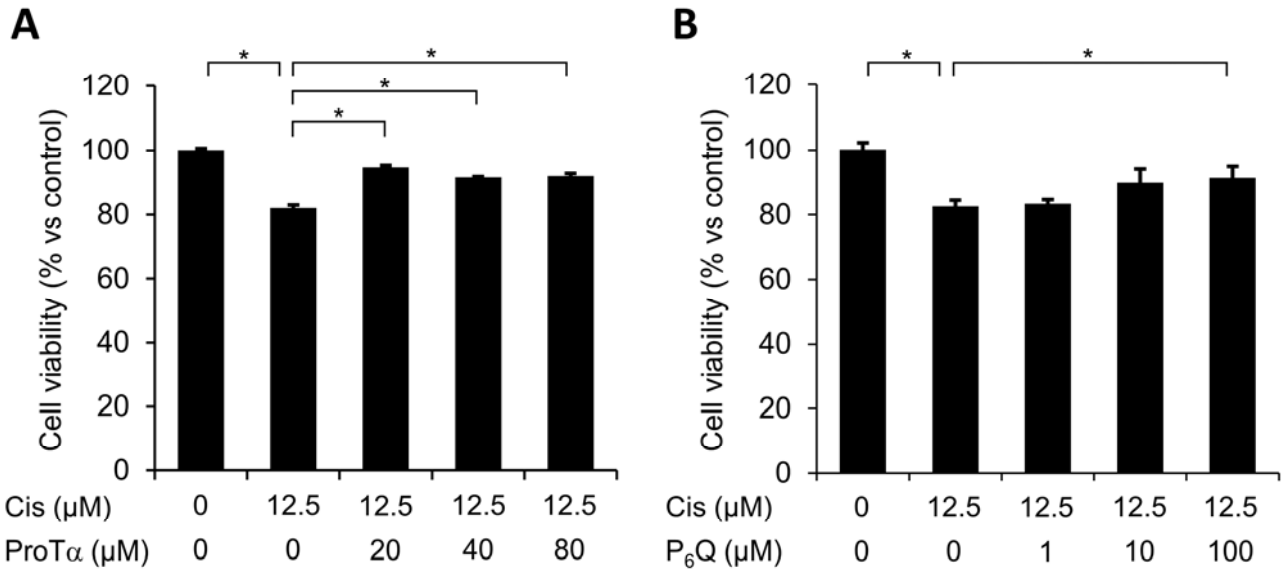

A
B
Cell viability (% vs control)
120
100
80
60
40
20
0
*
Cis (μM) 0 12.5 12.5 12.5 12.5
ProTα (μM) 0 0 20 40 80
P6Q (μM) 0 0 1 10 100

Figure 2

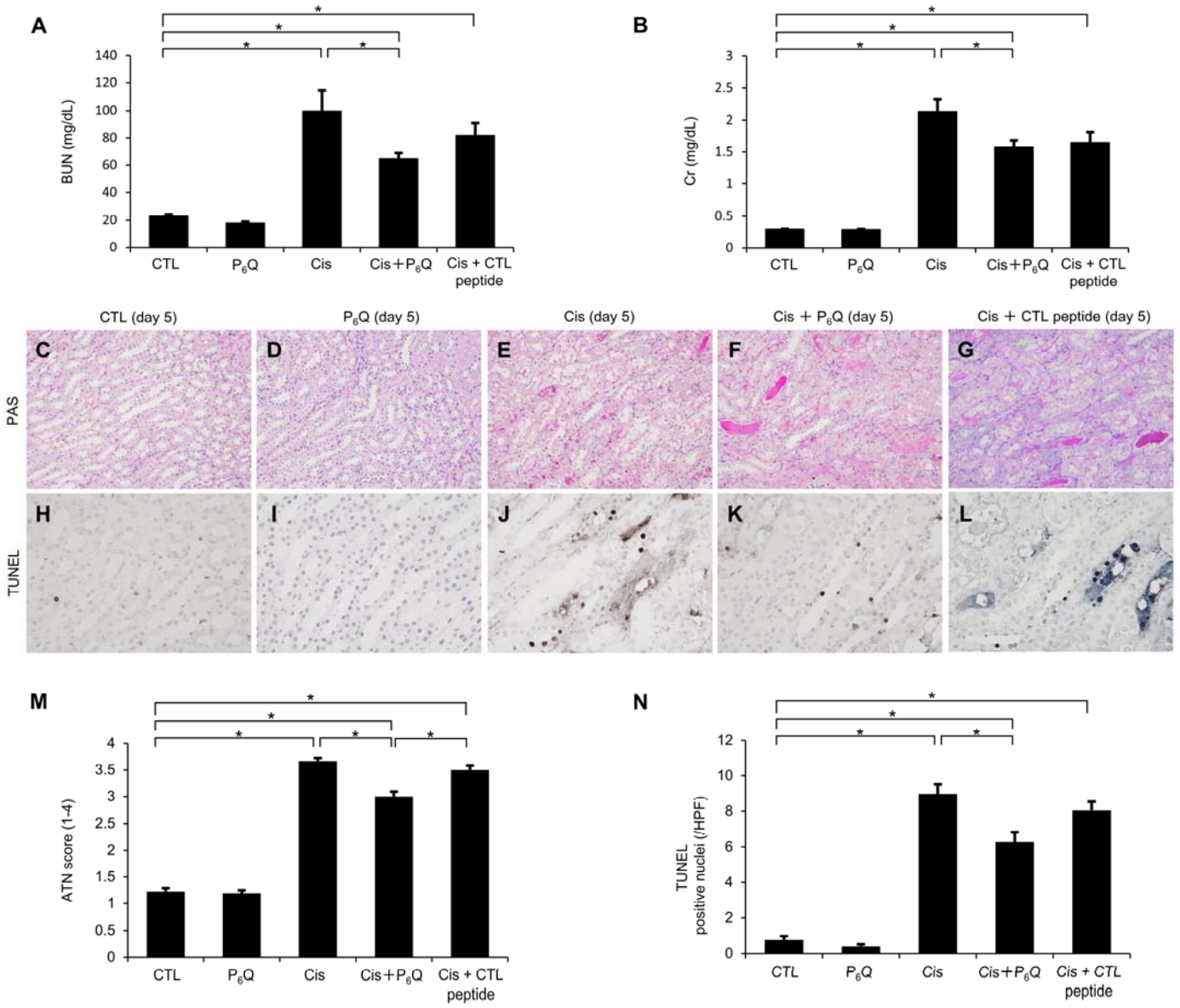

A
BUN (mg/dL)
140
120
100
80
60
40
20
0
CTL
P6Q
Cis
Cis+P6Q
Cis + CTL peptide
B
Cr (mg/dL)
3
2.5
2
1.5
1
0.5
0
CTL
P6Q
Cis
Cis+P6Q
Cis + CTL peptide
CTL (day 5)
P6Q (day 5)
Cis (day 5)
Cis + P6Q (day 5)
Cis + CTL peptide (day 5)
PAS
TUNEL
C
D
E
F
G
H
I
J
K
L
M
ATN score (1-4)
4
3.5
3
2.5
2
1.5
1
0.5
0
CTL
P6Q
Cis
Cis+P6Q
Cis + CTL peptide
N
TUNEL positive nuclei (/HPF)
12
10
8
6
4
2
0
CTL
P6Q
Cis
Cis+P6Q
Cis + CTL peptide

Figure 3

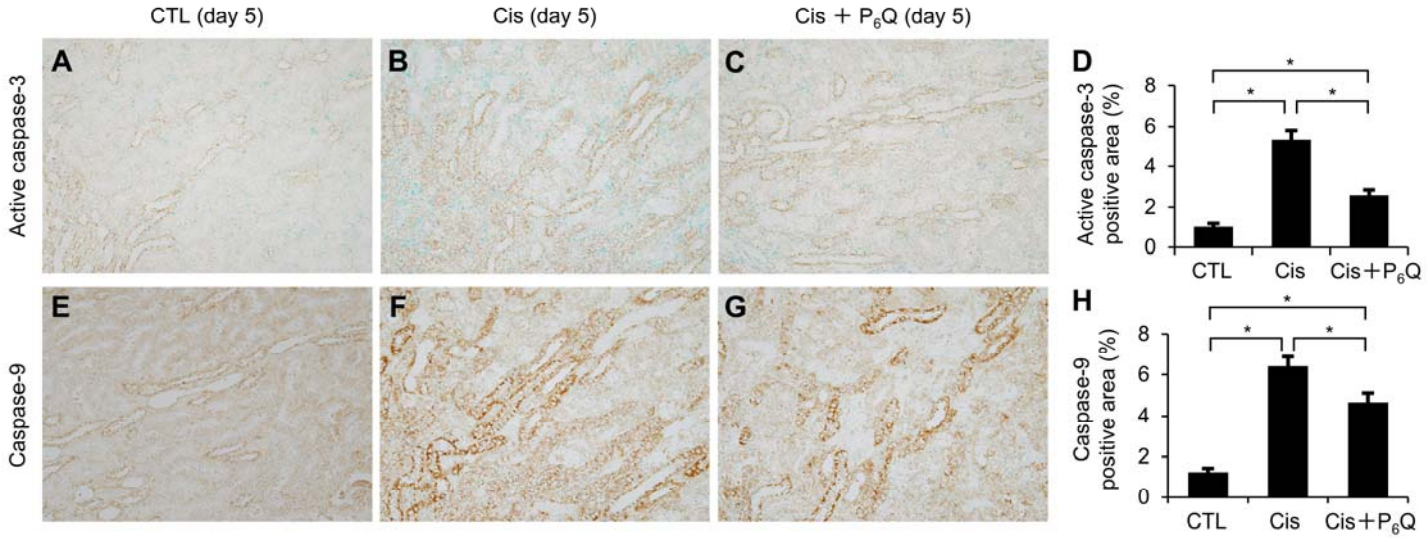

CTL (day 5)
Cis (day 5)
Cis + P6Q (day 5)
A
B
C
D
E
F
G
H
Active caspase-3
Caspase-9
Active caspase-3 positive area (%)
Caspase-9 positive area (%)
0
2
4
6
8
CTL
Cis
Cis+P6Q
*

Figure 4

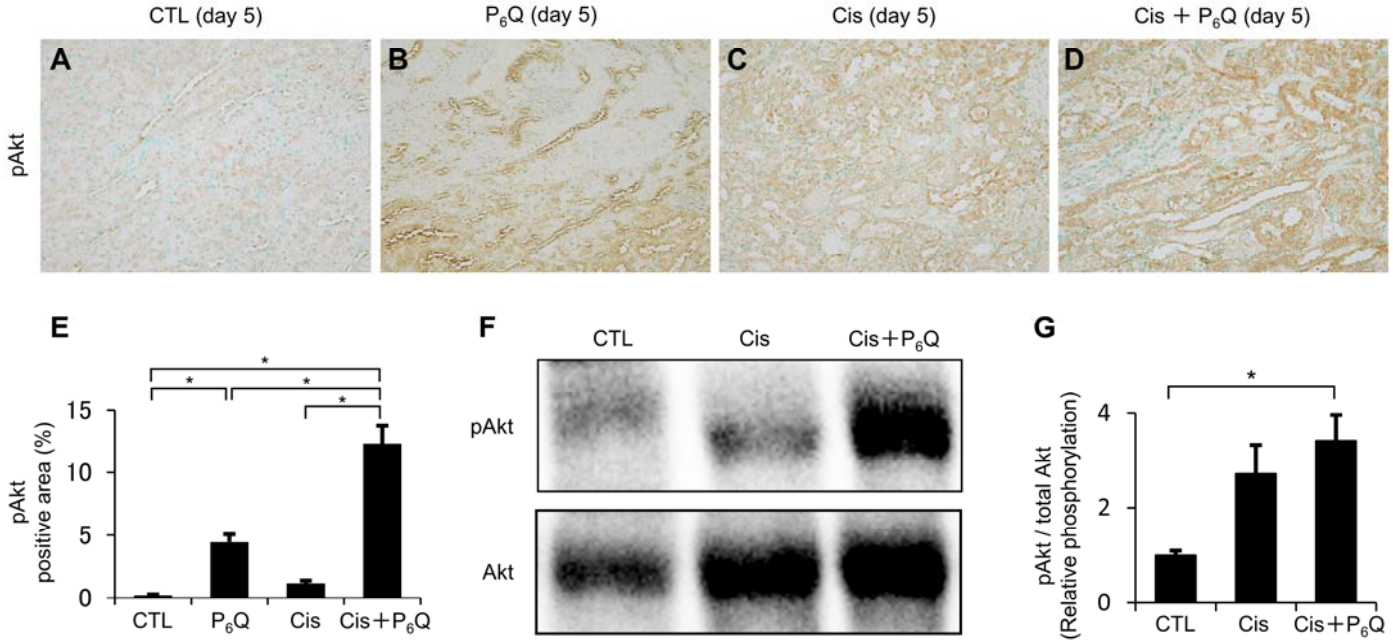
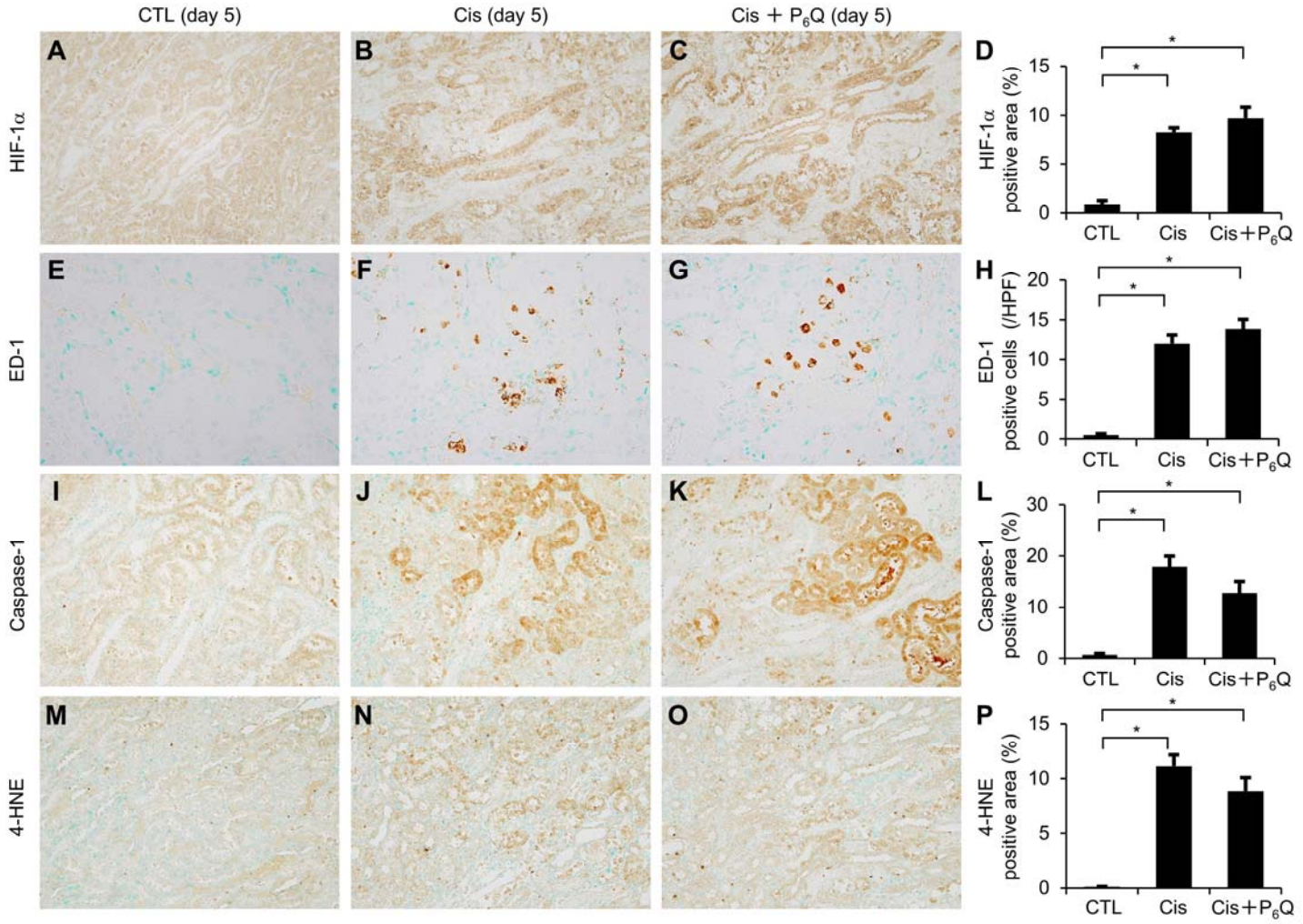

CTL (day 5)
P6Q (day 5)
Cis (day 5)
Cis + P6Q (day 5)
A
B
C
D
pAkt
E
pAkt positive area (%)
15
10
5
0
CTL
P6Q
Cis
Cis+P6Q
F
CTL
Cis
Cis+P6Q
pAkt
Akt
G
pAkt / total Akt (Relative phosphorylation)
4
2
0
CTL
Cis
Cis+P6Q

Figure 5


CTL (day 5)
Cis (day 5)
Cis + P6Q (day 5)
HIF-1α
ED-1
Caspase-1
4-HNE
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
HIF-1α positive area (%)
ED-1 positive cells (/HPF)
Caspase-1 positive area (%)
4-HNE positive area (%)
CTL
Cis
Cis+P6Q
*