Usefulness of urinary biomarkers for nephrotoxicity in cynomolgus monkeys treated with gentamicin, cisplatin, and puromycin aminonucleoside

Hiroshi Uchino1,2, Junko Fujishima1, Kaori Fukuoka1, Teppei Iwakiri1, Akira Kamikuri1, Hidenori Maeda1 and Kazuhiro Nakama1

1Shin Nippon Biomedical Laboratories, Ltd., Drug Safety Research Laboratories, 2438 Miyanoura, Kagoshima 891-1394, Japan
2SNBL USA, Ltd., 6605 Merrill Creek Parkway, Everett, Washington 98203, USA

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ABSTRACT — The objective of this study was to investigate the availability of novel urinary biomarkers (BMs) such as total protein, albumin, β2-microglobulin, clusterin, cystatin C, neutrophil gelatinase-associated lipocalin (NGAL) for the detection of acute nephrotoxicity in cynomolgus monkeys. Animals (total 9 males/3 groups) were administered gentamicin (GM) subcutaneously at 40 mg/kg for 7 days, cisplatin (CDDP) intravenously at 3 mg/kg once and puromycin aminonucleoside (PAN) intravenously at 20 mg/kg for 7 days. Two-hr urine on Days 0, 3, and 6, and 16-hr urine and blood on Days 1, 4, and 7 were collected. Novel urinary BMs and conventional clinical pathology parameters were evaluated in parallel to histopathological and electron microscopic examinations on the kidneys at termination. Urinary BMs and enzymes increased earlier than serum creatinine and blood urea nitrogen, particularly in 2-hr urine after dosing on Day 0, urinary albumin was increased in all groups and urinary NGAL with the highest magnitude of change rate among urinary BMs was observed in the GM and CDDP groups. Degeneration/necrosis and hyaline droplet of renal tubule, cellular cast and dilatation of renal tubule, and hypertrophy of podocytes were observed in the GEN, CDDP, and PAN groups, respectively. These results showed that the increases of urinary BMs reflected the agent-specific renal damages and these urinary BMs could be useful for the detection of segment-specific nephrotoxicity. Urinary albumin and NGAL are the most useful BMs to estimate glomerular and distal tubular damages, respectively, as well as proximal tubular damage in cynomolgus monkeys.

Key words: Urinary biomarker, Nephrotoxicity, Cynomolgus monkeys

INTRODUCTION

The kidney is one of the main toxic targets for drug candidates but accurate detection of nephrotoxicity is often difficult in the early stage of drug development (Hoffmann et al., 2010). Urinalysis and urine chemistry tests such as urine volume, specific gravity, semiquantitative dipstick analysis (protein, glucose, ketones, urobilinogen, bilirubin, and occult blood), sediment examination, and electrolyte concentration measurements (sodium, potassium, and chloride) are conducted to monitor renal function in routine toxicity studies but they rarely contribute meaningful and useful information (Hall and Everds, 2003). Serum creatinine (sCRE) and blood urea nitrogen (BUN) have long been used as serum BMs for the detection of drug-induced nephrotoxicity. sCRE and BUN are insensitive or nonspecific to diagnose and monitor acute nephrotoxicity because these parameters can be easily influenced by many physiological functions. sCRE and BUN can show an increase in nonrenal-related causes of alteration like congestive heart failure, shock, and dehydration. It has also been reported that gastrointestinal bleeding can lead to an increase in sCRE without negative impact on the kidney. The increase in the levels of sCRE and BUN is due to the decreases of more than 50% in glomerular filtration rate with renal epithelial cell death occurring after the cell damage with increases in the levels of novel urinary BMs (Fergusston et al., 2008; Vaidya
et al., 2008; Fuchs and Hewitt, 2011). Sensitive methods for a prediction of nephrotoxicity in toxicity studies and identification of renal damage are extremely important for safety in clinical practice and in all stages of the drug-development process (Bonventre et al., 2010).

European Medicines Agency (EMA), US Food and Drug Administration (FDA), and Japanese Pharmaceuticals and Medical Devices Agency (PMDA) qualified the 7 kinds of urinary kidney BMs: total protein (TP), albumin (ALB), β₂-microglobulin (B2M), clusterin (CLU), cystatin C (CysC), kidney injury molecule-1 (KIM-1), and trefoil factor 3 (TFF3). The BMs are considered acceptable in the context of non-clinical drug development for the detection of drug-induced acute toxicity in renal tubules and glomeruli. KIM-1, ALB, CLU, and TFF3 could be detected for the drug-induced acute renal tubular alteration, while TP, B2M, and CysC could be for acute glomerular alterations/damage and/or impairment of kidney tubular absorption in rats. In addition to 7 urinary kidney BMs, EMA and FDA also qualified renal papillary antigen-1 (RPA-1) for detecting renal tubular alterations, particularly in the collecting duct in rats (EMA, 2009, 2010; FDA, 2008, 2010; PMDA, 2010). Furthermore, EMA and FDA qualified an additional 2 novel urinary BMs: NGAL (lipocalin-2) and osteopontin (OPN) which showed elevations by proximal tubular degeneration/necrosis in rats (EMA, 2014; FDA, 2014). The urinary increases in TP, ALB, B2M, and CysC are derived from the damage of glomerulus and proximal tubule, while CLU and NGAL are from that of the proximal and distal tubules in humans (Bonventre et al., 2010; Vlasakova et al., 2014).

Many investigations of novel urinary BMs for the early detection of the segment-specific nephrotoxicity in rats have been reported but only a few investigations were conducted in cynomolgus monkeys with agents such as GM and triple reuptake inhibitor (Guha et al., 2011; Gautier et al., 2016). The early detection of nephrotoxicity in rodent and non-rodent species, particularly non-human primates, is extremely useful for early strategic decision in drug development because toxicity studies generally require non-rodent and rodent species. Non-human primates are used when it is scientifically demonstrated that none of the other non-rodent species is appropriate for the purpose of the study. The cynomolgus monkey is the most widely used species in non-human primates and is the most appropriate animal to characterize safety of many biotechnology-derived pharmaceuticals. In addition, cynomolgus monkeys anatomically and physiologically homologous to humans are commonly used in toxicity studies in order to provide a better interpretation prior to initiation of human clinical studies and translation into humans (SCHEER, 2017). These have been investigated with the novel urinary BMs for drug-induced nephrotoxicity in proximal and/or distal tubules induced by GM and triple reuptake inhibitor, the human-specific commercial kits were partially employed for the analysis of novel urinary BMs. The analytical methods of novel urinary BMs which were validated using matrices of cynomolgus monkeys need to be employed in further investigation. To date, urinary BMs for the detection of drug-induced nephrotoxicity in glomeruli in cynomolgus monkeys have not been reported despite being proclaimed the novel urinary BMs for the detection of glomerular damage. There is a need to investigate the availability of novel urinary BMs for the early detection of the xenobiotic-induced segment-specific nephrotoxicity in comparison to the conventional clinical pathology parameters such as sCRN, BUN, and urinary enzymes in cynomolgus monkeys. Therefore, we selected GM, CDDP, and PAN to investigate the early detection of the segment-specific nephrotoxicity because it has been well known that GM damages the proximal tubules, CPPD damages both the proximal and distal tubules, and PAN damages the glomeruli in cynomolgus monkeys (Robertson, 1998; Working et al., 1998; Gautier et al., 2016).

The objective of the present study was to mainly investigate the availability of the urinary BMs in cynomolgus monkeys receiving GM, CDDP, and PAN which have different modes of action for induction of acute nephrotoxicity. The urinary BMs (TP, ALB, B2M, CLU, CysC, and NGAL) for early detection of renal damage were compared with serum BMs (sCRN and BUN) and urinary enzymes such as N-acetyl-β-D-glucosaminidase (NAG), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), and lactate dehydrogenase (LDH). The increases in urinary enzyme (NAG, ALP, and GGT) are due to the lesion of proximal tubules and that in LDH is the damage of entire nephron (Clemo, 1998). We selected the serum and urinary BMs and enzymes which have been validated using each matrix of cynomolgus monkeys at Shin Nippon Biomedical Laboratories, Ltd., Drug Safety Research Laboratories (SNBL DSR).

**MATERIALS AND METHODS**

**Chemicals**

GM (GENTACIN Injection 60, injectable product of gentamicin sulfate, CAS No. 1405-41-0), CDDP (IA-call, injectable product of cisplatin, CAS No. 15663-27-1), and PAN (reagent grade, CAS No. 58-58-2, assay: minimum 96.0%) were purchased from MSD K. K. (Tokyo, Japan),
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Nippon Kayaku Co., Ltd. (Tokyo, Japan), and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. GM, CDDP, and PAN were dissolved in physiological saline (Japanese pharmacopoeia, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) at concentrations of 20, 0.6, and 4 mg/mL, respectively.

Animals and husbandry

This study was approved by the Institutional Animal Care and Use Committee and was performed in accordance with the animal welfare bylaws of SNBL DSR, which is accredited by AAALAC International. Nine male cynomolgus monkeys weighing 2 to 4 kg and aged 3 to 4 years, purpose-bred in Cambodia and maintained at SNBL, were used. Animals were accepted for use in this study based on the physical examinations such as tuberculosis test, clinical observation, body weight, and blood examination performed during the quarantine period. The animals were housed individually in stainless steel cages [680 mm (D) × 620 mm (W) × 770 mm (H)] at a conventional facility at a temperature of 23°C to 29°C, humidity of 30% to 70%, and 12-light/dark cycle (lights on from 07:00 to 19:00). Approximately 108 g (approximately 12 g × 9 pieces) of pellet food (HF Primate J 12G 5K9J, Purina Mills, LLC, Gray Summit, MO, USA) were provided to each animal daily, and water was available ad libitum.

Experimental design

Three male cynomolgus monkeys per group were used (total 9 animals in 3 groups). These animals were given a daily subcutaneous injection of GM at a dose level of 40 mg/kg for 7 days (Nos. 1 to 3), single intravenous injection of CDDP at 3 mg/kg (Nos. 4 to 6), and daily intravenous injection of PAN at 20 mg/kg for 7 days (Nos. 7 to 9). These dosages and administration routes to animals were set based on the results of in-house pilot studies in which increased urinary BMs and slight histopathological renal lesions were observed but severe findings were not in clinical observation. The day before dosing was designated as Day -1 and the first day of dosing as Day 0. All animals were observed once or more daily throughout the experimental period, and body weight was measured on Days -7 and -1 in the non-dosing period, and Days 0, 4, and 7 (before sacrifice) in the dosing/observation period. Two-hr urine were collected on Days -6, -3, 0, 3, and 6 from a urine collection pan placed beneath the animal cage under room temperature from immediately after dosing. Sixteen-hr urine were collected on Days -5, -2, 1, 4, and 7 on ice-chilled containers from a urine collection pan placed beneath the animal cage from approximately 17:00 to 09:00 on the following day. Food was removed during the urine collection, and a mesh net was attached onto the urine collection pan to avoid contamination of vomitus and diarrheal stool. Blood for serum chemistry was collected on Days -5, -2, 1, 4, and 7, and its sampling volume was approximately 3 mL per sampling point. The overview of experiment design (Fig. 1) illustrates the timeline of dosing, sampling of urine and blood, and sacrifice during the study.

Clinical Pathology

Urine volume for 2- and 16-hr urine was measured using a graduated cylinder, and then the partial urine was aliquoted to the container for analysis. Initially, the occult blood (OB) in 2-hr urine was analyzed using test paper and Clinitek Atlas XL (Sparton Medical Systems, Schaumburg, IL, USA), the results were graded as follows: 0, negative; 1, hemolyzed trace; 2, small; 3, moderate; 4, large, and then centrifuged at 550 × g for 5 min at 20°C (Inverter Refrigerated Centrifuge, Kubota Manufacturing Corporation, Tokyo, Japan). The urine sediments were observed microscopically, and findings were classified and graded based on the number of cells per high-power field as follows: 0, <1; 1, 1 to 4; 2, 5 to 9; 3, 10+. The supernatant for 2- and 16-hr urine was centrifuged at 1,700 × g for 5 min at 4°C (Inverter Refrigerated Centrifuge), and then TP, B2M, ALB, ALP, GGT, LDH (Wako Pure Chemical Industries, Ltd.) and NAG (Shionogi & Co., Ltd., Osaka, Japan) were analyzed using an autoanalyzer (JCA-BM6070, JEOL, Ltd., Tokyo, Japan). The urine samples for CLU, CysC and NGAL were stored at -70°C or below until analysis and were analyzed using commercial kits (Human Clusterin ELISA and Human Cystatin C ELISA, BioVendor - Laboratorní medicína, Brno, Czech Republic; Monkey NGAL ELISA Kit, Bio Porto Diagnostics A/S, Hellerup, Denmark) within the duration of the frozen stability. sCRN and BUN (Wako Pure Chemical Industries, Ltd.) in serum were also analyzed using an autoanalyzer (JCA-BM6070). These methods of analysis were validated including the frozen stability using serum and urine samples from cynomolgus monkeys in SNBL DSR. Measured values below the lower limit of quantification were handled as a lower limit of quantification. The urinary excretions (normalized by urine volume) for each parameter were calculated from each concentration and urine volume for 2- and 16-hr urine, and then these excretions were divided by the duration of collection time (2 for 2-hr urine and 16 for 16-hr urine) to calculate the urinary excretion per hr. The change rates of excretion per hr in urinary BMs and enzymes after dosing/observation period were calculated.
based on the average in the non-dosing period individually and evaluated. sCRN and BUN were also calculated in the same manner. We evaluated the results of the urinary excretion of BMs per hr without urinary creatinine correction because it has been reported that the correction possibly overestimates the degree of acute kidney damage in rats (Tonomura et al., 2011) and the most accurate method to qualify BMs requires the collection of timed urine specimens to estimate the urinary excretion (Waikar et al., 2010).

Pathology

After blood sampling on Day 7, all 9 animals were weighed, then anesthetized by an intravenous injection of sodium pentobarbital (64.8 mg/mL, 0.4 mL/kg), and euthanized by exanguination. External appearance and internal organs and tissues were examined macroscopically. The kidneys were fixed in 10% neutral buffered formalin. The fixed kidneys were trimmed to axial transverse section, embedded in paraffin, thin sectioned, and stained with hematoxylin-eosin (HE) and Periodic acid-Schiff reaction (PAS) for histopathological examination. Cortical and medullary kidney sections of approximately 1 mm thickness were pre-fixed in 3% glutaraldehyde and fixed in 1% osmium tetroxide. They were embedded in epoxy resin, ultra-thin sectioned, double stained with uranyl acetate and lead citrate, and photographed using a transmission electron microscope (JEM-1200EX, JEOL Ltd.). Histopathological and electron microscopic findings were classified and graded as follows: -, no abnormal changes (0); ±, very slight (1); +, slight (2); 2+, moderate (3); 3+, marked (4), respectively.

RESULTS

Clinical observation, body weight, and food consumption

No changes were observed in body weight or food consumption in any group. One animal in the GM group showed vomiting before dosing on Day 6 and before sacrifice on Day 7, and 2 animals in the CDDP group showed vomiting at approximately 4 hr after dosing on Day 0.

Clinical pathology

No changes were observed in 2- or 16-hr urine volume when compared with the average in the non-dosing period in any groups. Positive OB was observed in 2-hr urine of each 1 animal on Days 6 (grade 1) and 0 (grade 4) in the GM and CDDP groups, respectively, and in 2 and/or 3 animals on Days 3 (grade 1 and 2) and 6 (grade 2 to 4) in the PAN group. The 2-hr urine values with positive OB were excluded from the evaluation of the changes of BMs to avoid any effect of falsely increased BM levels due to OB. In urine sediment, epithelial and granular casts were observed in 1 animal in each the GM and CDDP groups on Days 3 and 6, respectively, and renal tubular cells were...
observed in all animals on Day 6 and 3 in the GM and CDDP groups, respectively, but not in the PAN group.

With regard to the change rates of urinary excretion per hr and serum BMs in the GM group, TP, ALB, B2M, and NGAL in 2-hr urine were slightly to markedly increased (grade for urinary BMs and enzymes: slightly, >3-fold; moderately, >6-fold; markedly, >12-fold, when compared with the average values in non-dosing period in each animal) on Day 0 (5.0 to 8.9-, 6.3 to 6.9-, 8.0 to 10-, and 3.4 to 139-fold, respectively) as the increased level of urinary BMs. Cys C and CLU in 2-hr urine were moderately to markedly increased on Day 6 (7.7 to 12- and 18 to 26-fold, respectively). Urinary enzymes ALP, GGT, and LDH in 2-hr urine were slightly increased on Day 0 (3.6-, 3.2-, and 3.3-fold, respectively) and NAG in 16-hr urine was slightly increased on Day 1 (3.0 to 4.0-fold). The mean fold changes of TP, ALB, B2M, and NGAL on Day 0 were higher than those of urinary enzymes (ALP, GGT, LDH, and NAG) (Fig. 2 and 4). sCRN and BUN were slightly to markedly increased (grade for serum BMs: slightly, >1.5-fold; moderately, >3-fold; markedly, >6-fold, when compared with the average values in non-dosing period in each animal) on Day 7 in 1 animal or more (2.3 to 13- and 1.6 to 7.0-fold, respectively) (Figs. 3 and 4).

In the CDDP group, TP, ALB, Cys C, and NGAL in 2-hr urine were slightly to markedly increased on Day 0 (7.8-, 20-, 4.4-, and 178 to 238-fold, respectively), while CLU in 16-hr urine was slightly to markedly increased on Day 1 (4.4 to 37-fold) and B2M in 2-hr urine were markedly increased on Day 6 (38-fold). All urinary enzymes (NAG, ALP, GGT, and LDH) in 2-hr urine were slightly to markedly increased on Day 0 (4.3-, 37-, 6.0-, and 8.1-fold, respectively). The change rates for NGAL in 2-hr urine on Day 0 were higher than ALP which showed the highest increase among the urinary enzymes and CLU in 16-hr urine was increased earliest on Day 1 among all 3 compounds (Fig. 2 and 4). sCRN and BUN were slightly increased on Day 1 (both 1.5-fold) (Figs. 3 and 4).

In the PAN group, ALB in 2-hr urine was slightly to moderately increased on Day 0 (3.5 to 7.4-fold), while NGAL in 16-hr urine was slightly increased on Day 7 (3.0-fold) but TP, B2M, Cys C, and CLU were not increased. LDH in 2-hr urine was slightly increased on Day 0 (3.0-fold) but NAG, ALP, and GGT were not increased. The mean fold changes of ALB on Day 0 were higher than LDH. ALB in 2-hr urine on Day 0 was higher than that in 16-hr urine on Day 1 (Figs. 2 and 4). sCRN and BUN were slightly increased on Day 4 and 7 (1.5- and 1.6 to 2.0-fold), respectively (Figs. 3 and 4).

### DISCUSSION

The objective of this study was to investigate the availability of the novel urinary BMs such as TP, ALB, B2M, CLU, Cys C, and NGAL for an early detection of acute nephrotoxicity and a comparison with serum BMs and urinary enzymes in cynomolgus monkeys receiving GM at 40 mg/kg subcutaneously for 7 days, CDDP at 3 mg/kg intravenously once and PAN at 20 mg/kg intravenously for 7 days.

In the GM group, all 10 urinary BMs and enzymes increased earlier than sCRN and BUN. The increased TP, ALB, B2M, NGAL, ALP, GGT, and LDH on Day 0 were considered to be mainly related to the pathological lesions in the renal proximal tubules observed in histopathological and electronmicroscopic examinations. The levels of TP in 2-hr urine on Day 0 were similar to Day 3; however, ALB, B2M, and NGAL decreased on Day 3 compared with Day 0. CLU specifically protects against GM-induced renal tubular cell damage in vitro model (Girton et al., 2002), suggesting that these observations may be
Fig. 2. Mean fold changes of (a) urine volume, (b) TP, (c) ALB, (d) B2M, (e) Cys C, (f) CLU, and (g) NGAL in excretion per hr of urinary BMs, and (h) NAG, (i) ALP, (j) GGT, and (k) LDH in excretion per hr of urinary enzymes. Each represents the group mean ± S.D. (n = 3) for GM, CDDP, and PAN groups. Two-hr urine was collected on Days -6, -3, 0, 3, and 6, and 16-hr urine was collected on Days -5, -2, 1, 4, and 7 (Days -5, -2, 1, 4, and 7 indicate the day for the end of the collection of 16-hr urine, respectively). The values for 2-hr urine with positive occult blood were excluded from the evaluation to avoid false increase of these BMs (GM, Day 6 in 1 animal; CDDP, Day 0 in 1 animal; PAN, Days 3 and 6 in 2 and 3 animals, respectively).
related to the action of cytoprotection to renal tubular cell. The maximum increases in urinary BMs and enzymes on Days 6 and/or 7 reflected the severe renal damages, suggesting that the daily repeated dosing of GM continuously damaged the proximal tubules up to Day 7. It has been reported that elimination of GM in plasma is saturated due to nephrotoxicity due to the repeated GM dosing in cynomolgus monkeys (Gautier et al., 2016). Among 10 urinary BMs and enzymes, the highest increase of NAGL was noted in 2-hr urine on Day 0, suggesting that NAGL is the most useful BM to estimate the proximal tubular damages in the early stage after administration. The increased levels of TP, ALB, and B2M in 2-hr urine on Day 0 were higher than that of ALP, GGT, and LDH.
Urinary BMs such as TP, ALB, B2M, and NGAL are superior to the urinary enzymes from the point of view of the early detection of the damage of the proximal tubules.

In the CDDP group, TP, ALB, Cys C, NGAL, NAG, ALP, GGT, and LDH, except for B2M and CLU, increased earlier than sCRN and BUN. Those BMs in 2-hr urine on Day 0 were related to the damages of both the proximal and distal tubules, and the increases of urinary enzyme were considered to be due to the cytotoxicity by CDDP. It has been reported that CDDP damages the proximal tubules, particularly the S3 segment in animal models, while the site of damage involves either the distal tubules and collecting ducts or the proximal and distal tubules in humans (Yao et al., 2006). These changes in urinary BMs were considered to be due to the damage of both the distal and proximal tubules based on the histopathological tubular lesions. NGAL, in 2-hr urine, showed the highest increase on Day 0 and CLU, in 16-hr urine, increased earliest on Day 1 among all 3 compounds. The increases in CLU have been reported in cynomolgus monkeys treat-
Table 2. Electronmicroscopic findings.

<table>
<thead>
<tr>
<th>Findings / grade</th>
<th>GM</th>
<th>CDDP</th>
<th>PAN</th>
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<tbody>
<tr>
<td>Proximal tubule (pars convoluta)</td>
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<td></td>
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<tr>
<td>Enlargement of lysosome</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Increase of lysosome</td>
<td>-</td>
<td>2</td>
<td>1</td>
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<tr>
<td>Myelinoid body in tubular lumen</td>
<td>2</td>
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<td>-</td>
</tr>
<tr>
<td>Myelinoid body in lysosome</td>
<td>1</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Proximal tubule (pars recta)</td>
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<tr>
<td>Decreased microvilli</td>
<td>NE</td>
<td>-</td>
<td>2</td>
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<tr>
<td>Protrusion of cytoplasm</td>
<td>NE</td>
<td>1</td>
<td>-</td>
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<tr>
<td>Distal tubule</td>
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<tr>
<td>Dilatation of endoplasmic reticulum</td>
<td>3</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Dilatation of intercellular gap</td>
<td>3</td>
<td>-</td>
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<tr>
<td>Glomerulus</td>
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<tr>
<td>Fusion of foot process in podocytes</td>
<td>3</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Dilatation of rough endoplasmic reticulum</td>
<td>3</td>
<td>-</td>
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Numbers in table represent “number of animals with findings”.
NE: not examined.
Grade: 0, No abnormal changes; 1, Very slight; 2, Slight; 3, Moderate.

Fig. 5. Histopathological lesions induced by GM, CDDP, and PAN. H&E staining. (A) GM at 40 mg/kg for 7 days. Slight hyaline droplet (left arrow), moderate degeneration/necrosis (right arrow) in renal tubular epithelium (No. 2). (B) CDDP at 3 mg/kg. Very slight cellular cast in tubular lumen (No. 4). (C) PAN at 20 mg/kg for 7 days. Very slight hypertrophy of podocytes (No. 8). (D) Normal.
ed with triple reuptake inhibitor, a selective distal tubules and/or collecting duct toxicant, has been reported (Guha et al., 2011), suggesting that CLU may be a useful BM for the detection of damage of the distal tubules than that of proximal tubules. The increased levels of NGAL in 2-hr urine on Day 0 were higher than that of ALP which showed the highest increase among urinary enzymes. NGAL is superior to the urinary enzymes from the point of view of the early detection of the damage of the proximal and/or distal tubules.

In the PAN group, ALB and LDH increased earlier than sCRN and BUN but TP, B2M, Cys C, CLU, NGAL, NAG, ALP, and GGT did not. The increases in ALB and LDH in 2-hr urine on Day 0 were related to the glomerular damages based on the histopathological glomerular lesion. ALB outperformed LDH in detecting the glomerular damages due to its higher magnitude of change rate.

It has been reported that acute and reversible proteinuria (albuminuria) can be induced by intravenous administration of PAN within 24 hr in rats (Robertson, 1998). Positive OB observed in 2-hr urine in 2 and 3 animals on Days 3 and 6, respectively, were probably due to glomerular damages because it has been reported PAN produces glomerular pathological changes that resembles human minimal change disease with hematuria (Robertson, 1998). The levels of ALB in 2-hr urine on Day 0 were higher than those of 16-hr urine on Day 1, and similar high ALB levels were observed in 2- and 16-hr urine of animals with OB on Days 3 to 7 (data not shown). ALB in 2-hr urine could be a useful BM to detect the glomerular damages but urinary enzymes are not. Furthermore, the increases in NGAL in 2-hr urine with positive OB on Days 3 and 6 (data not shown) were considered to be due to the damage of the glomerulus because the usefulness

![Fig. 6. Electronmicroscopic lesions induced by GM, CDDP, and PAN. (A) GM at 40 mg/kg for 7 days. Moderate enlargement and increase of lysosome (left arrow), myelinoid body (right arrow) in lysosome in pars convoluta in proximal tubule (No. 2). (B) CDDP at 3 mg/kg. Slight decrease in microvilli in pars recta in proximal tubule (No. 4). (C) PAN at 20 mg/kg for 7 days. Very slight fusion of foot process in podocyte in glomerulus (No. 8). (D) Normal. Normal foot process of podocyte.](image-url)
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of NGAL for the detection of glomerular damage as well as renal tubular damage has been reported (Kuwabara et al., 2009) and the increases in NGAL have been reported in rats treated with PAN (Tonamura et al., 2010).

The results from all 3 compounds indicate that when they induced histopathological lesions in the kidney, the urinary BMs and enzymes increased earlier than serum BMs. In particular, urinary ALB and LDH increased within 2 hr after an administration of each compound. It has been reported that urinary LDH is useful for the detection of damaged nephrons in rats and dogs due to its broad distribution in the nephron (Clemo, 1998; Tonomura et al., 2010), suggesting that LDH may be a useful BM for the detection of damage of the entire nephron in cynomolgus monkeys. It has been well known that the level of ALB in urine is negligible in normal status because ALB in blood is filtered through the glomerular epithelia and is completely reabsorbed in the proximal tubular epithelia. When damages are induced in the proximal tubular epithelia by compounds, ALB is not completely reabsorbed in the proximal tubular epithelia, while when damages are induced in the glomerular epithelia, an excessive amount of ALB after filtration is not completely reabsorbed in the proximal tubular epithelia (De Loor et al., 2013). These suggested that ALB could be increased in urine in the early stage of damage of the proximal tubular or glomerular epithelia. Among all 10 urinary BMs, urinary ALB is the most useful BM to estimate glomerular and/or proximal tubular damage in cynomolgus monkeys.

The highest increases of NGAL in 2-hr urine were observed in the GM and CDDP groups on Day 0. The usefulness of NGAL to detect the damage of the renal tubules has been reported in rats (Han et al., 2012; Phillips et al., 2016) and dogs (Kai et al., 2013; Zhou et al., 2014). After renal ischemia or nephrotoxicity, intrarenal NAGL synthesis is dramatically upregulated at protein levels. The increase in urinary NGAL is detectable as early as 3 hr after damage and peaks approximately 6 hr after damage. Sustained increase in urinary NGAL was reported to be as long as 5 days post damage in humans. The increase in urinary NGAL is considered to be due to an increase in NGAL synthesis and failure of complete reabsorption of NGAL in the proximal tubular epithelia (Kai et al., 2013; Alge and Arthur, 2015). The results in the present study suggested that NGAL could be the most useful BM for the detection of earlier damages of the proximal and/or distal tubules among all urinary BMs and enzymes in cynomolgus monkey.

The urinalysis after short and long time collections (eg. 2- and 16-hr urine in the present study) are frequently conducted in routine toxicity studies. In the present study, the change patterns of urinary BMs in 2-hr urine were comparable or superior to that in 16-hr urine. Although minor differences were reported for the concentration of urinary BMs between 6-hr urine and 18-hr urine, the shorter urine collection protocols do not influence the interpretation of normalized urinary BMs data in rats (Pinches et al., 2012), suggesting that acute nephrotoxicity in cynomolgus monkeys could be easily estimated with 2-hr urine rather than 16-hr urine from the points of view of the convenience of short time collection and the change patterns of the urinary BMs.

We did not analyze KIM-1, TFF3, RPA-1, or OPN and further investigations may be needed to optimize these parameters in cynomolgus monkeys. As we investigated the usefulness of the urinary TP, ALB, B2M, CLU, CysC, and NGAL to detect drug-induced acute nephrotoxicity in the time course experiment; however, histopathology was conducted only at the end of dosing/observation (after the 7th dosing or 7 days after a single dosing). It may be necessary to examine the time course of the histopathological renal lesions on the same points as the urinary BM examination and the recovery process from the toxic damages. Investigation for sex difference in cynomolgus monkeys may also be needed because sex differences have been reported in several urinary BMs in rats given GM (Gautier et al., 2014). Because we used only 1 toxic dose of GM, CDDP, or PAN to induce the drug-specific acute nephrotoxicity, multiple lower doses than the present study are needed to be set to observe any dose-response between very slight histopathological renal lesions and sufficiently increased urinary BMs in cynomolgus monkeys.

In conclusion, our present study results indicated that urinary TP, B2M, ALB, CLU, CysC, and NGAL could be useful BMs to detect damages of the kidney earlier than BUN and sCRN. Among them, urinary ALB is the best BM for the detection of glomerular and/or proximal tubular damage and NGAL is the best BM for proximal and/or distal tubular damages.

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Conflict of interest—— The authors declare that there is no conflict of interest.
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