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Effects of the Administration of p-{N,N-Bis(2-chloroethyl)amino}-4-phenyl acetyl-amino-2,6-piperidinedione (CK-15) on Rat Kidney

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ABSTRACT *: To evaluate the renal toxicity of the antitumor agent, p-{N,N-Bis(2-chloroethyl)amino}-4-phenyl acetyl-amino-2,6-piperidinedione (CK-15), rats were treated with CK-15 (acute: 50 mg/Kg, i.p., single and subacute: 5 mg/Kg, i.p., daily for 7 days). The changes in the body weights, water consumption, kidney weights and urine volume after and during the treatment were observed. The concentrations of urinary creatinine and protein, the activities of N-acetyl-β-D-glucosaminidase (NAG), alanine aminopeptidase (AAP), γ-glutamyl transpeptidase (γ-GT) and lactate dehydrogenase (LDH) in 24 hr urine were also determined. The body weight, water consumption, and urine volume were decreased after the acute and subacute administration. However, the weights of kidney were not changed after the treatments. The excretion of creatinine was significantly decreased 1 day after acute administration but, returned to the control value. In subacute administration, the excretion of creatinine was gradually decreased. However, the protein excretion did not changed in both treatment. Those indicate that CK-15 might decrease the metabolic rate of muscle. The urinary activities of NAG, AAP, γ-GT, and LDH were significantly affected by the drug treatment. The urinary activities of NAG, AAP and γ-GT were significantly increased 1 day after the acute administration and then returned to the control value. However, the urinary activities of LDH were not changed in acute treatment. In subacute treatment, although the urinary activities of NAG were not changed, those of AAP and γ-GT were significantly increased 2.3 times at 3 days during the subacute administration. Also the urinary activities of LDH were significantly increased at 7 day after the administration. These results indicate that the high and subacute administration might induce a damage in the kidney cells. Furthermore the present results suggest that the toxic effects of CK-15 might be due to the accumulation of the metabolites.*

Key Words *: CK-15, Creatinine, N-acetyl-β-D-glucosaminidase, Alanine aminopeptidase, γ-Glutamyl transpeptidase, Lactate dehydrogenase*

I. INTRODUCTION

One of the most threatening diseases in recent years is cancer. Although various methods are applied in cancer treatment, chemotheraphy with antitumor agents is getting more attentions. However, it is known that antitumor agent is toxic to various organs such as blood, liver, kidney and so on. To develope more valuable compound, it is important index to observe the kidney toxicity of the synthesized antitumor agent. These toxicities are usually related to the chemical structure of the agents. Also, their metabolites often have toxic effects.

It has been reported that p-{N,N-Bis(2-chloroethyl) amino}-4-phenyl acetyl-amino-2,6-piperidinedione (CK-15) is bifunctional alkylating agent which express its cytotoxic and antitumor effects by cross-linking cellular DNA (Choi *et al.*, 1998; Trual *et al.*, 1990).

It has been reported that in the damaged kidney, the glomerular filtration rates (GFR) are changed and total levels of some urinary enzymes are increased. Thus, the level of excreting creatinine and the activities of the enzymes in the urine can be used as an indicator of renal damage without injury to the examinee (Hofmeister *et al.*, 1986; Ohata *et al.*, 1987; Shin *et al.*, 1989; Wachamuth *et al.*, 1982).

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ABBREVIATION: p-{N,N-Bis(2-chloroethyl)amino}-4-phenyl acetyl-amino-2,6-piperidinedione, CK-15; N-acetyl-β-D-glucosaminidase, NAG; Alanine aminopeptidase, AAP; Gamma-glutamyl transpeptidase, γ-GT; Lactate dehydogenase, LDH

Antitumor agents are usually administered for a long period. Although the in vitro antitumor activities of CK-15 in various cell types is more potent than the other derivatives (Choi *et al.*, 1998), the toxic effects of this agent are not sufficiently identified.

Therefore, the present study is designed to evaluate the renal cytotoxicity of CK-15.

II. METHODS

1. Animal and materials

Male Spraque-Dawley rats weighing 200~250 g, housed under 12 hour light/dark cycle, 23±1°C, 60±5% humidity, were used. All animals had free access to food and water. CK-15 was synthesized according to the method of Choi *et al.* (1998) MPS-1 (micropartition system-1) and membrane were purchased from Amicon (Denver, U.S.A.). The diagnostic kit for γ-GT was purchased from Gilford (Cleveland, U.S.A.). The other chemicals were purchased from Sigma (St. Louise, U.S.A).

2. Animal treatment

Rats were adapted in metabolic cages for 5 days before the administration of CK-15. CK-15 was dissolved in ethanol: propylene glycol: DMSO (2 : 6 : 2) and administered. Rats were injected intraperitonially in a dose of 50 mg/kg for the acute treatment. In subacute treatment, rats were treated daily for 7 days in dose of 5 mg/kg, i.p., The doses were chosen according to our preliminary study. Individual rat was kept in metabolic cages. After and during the administration of drug, 24 hour-urine was collected and volume was measured at each rats. Also, the kidneys were removed and weighed after the acute and subacute administration.

3. Pretreatment of urine for enzyme determinations

It has been reported that enzyme inhibitors are contained in urine. To remove the inhibitors, micropartition systems were employed (Leathwood *et al.*, 1969; Ohata *et al.*, 1987); 24 hour-collected urine was centrifuged at 800×g, 4°C for 5 min. The diluted supernatant was added to MPS-1 and centrifuged at 1500×g, 5°C for 50 min. The filtrate in the lower tube of MPS-1 was used for creatinine quantification. To dissolve the enzymes attached to the membrane, phosphate buffered saline (PBS, 50 mM KH_2PO_4-K_2HPO_4 in saline, pH 7.4) was applied to the upper tube of MPS-1 and vortexed. After the same procedure was repeated, the washing solution was mixed with the first fluid. Then it was used as the source for enzyme determinations. Enzyme activities were represented as creatinine ratios.

4. Creatinine measurement

Creatinine was determined by Jaffe reaction (Rock *et al.*, 1987); The diluted filtrate and 0.36 M picric acid were mixed. After 30 sec, 1.4 M NaOH was added to stop the reaction and 15 min stabilization followed. The detection wavelength for quantification was 500 nm.

5. Protein concentration

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

6. N-acetyl-β-D-glucosaminidase activity (NAG)

The activity of NAG was determined by Maruhn method (1976); 0.25 ml of substrate (5 mM p-nitrophenol-N-acetyl-β-glucosaminide in 50 mM Citric acid-K_2HPO_4-KOH buffer, pH 4.2) was added to 0.05 ml of enzyme sample. After 40 min incubation at 37°C, the reaction was stopped by adding 0.1 M borate buffer (H_3PO_4-KOH, pH 10.5) and the absorbance at 406 nm was determined.

7. Alanine aminopeptidase activity (AAP)

The activity of AAP was determined by Jung and Scholz method (1980); 0.08 ml of enzyme sample and 0.8 ml of substrate (2 mM L-alanine-4-nitroanilide) were incubated at 37°C for 20 min. The reaction was stopped by adding 20% sodium dodecyl sulfate (SDS) and the absorbance at 406

nm was determined.

8. Gamma-glutamyl transpeptidase activity (γ-GT)

The activity of γ-GT was determined by Szasz method (1974); The diagnostic kit for γ-GT was applied to the 0.02 ml of enzyme sample. After 10 min incubaton at 25°C, the reaction was stopped by adding 20% SDS and the absorbance at 406 nm was determined.

9. Lactate dehydogenase activity (LDH)

The activity of LDH was determined by Bergmeyer and Bernt method (1974); Enzyme sample was incubated with NADH (1 mg/ml) and phosphate buffer (0.1 M K_2HPO_4-KH_2PO_4 containing 0.2% Triton X-100, pH 7.4) at 30°C for 5 min. The decreased optical density at 340 nm during the incubation was used to calculate the activity.

10. Statistics

Statistical significance was determined by Student's t-test.

III. RESULT AND DISCUSSION

Changes in the body weight during and after the administration of CK-15 are shown at Fig. 1. In acute administration of CK-15, the body weight was significantly decreased. Also some rats were dead from 4 days and the mortality is 33.3% at 5 days after the acute administration. In subacute administration of CK-15, the changes of body weight and the death were not observed, but the rate of growth was significantly decreased. Changes in water consumption are shown in Table 1. Water consumption was significantly decreased by both treatments. The decreases in water consumption were profound in the acute treatment, which showed maximum at 3 days after the treatment. During the subacute administration, the decreased water consumption was similar ratios compared to the control. However, after the acute and the subacute administrations, the kidney weight per

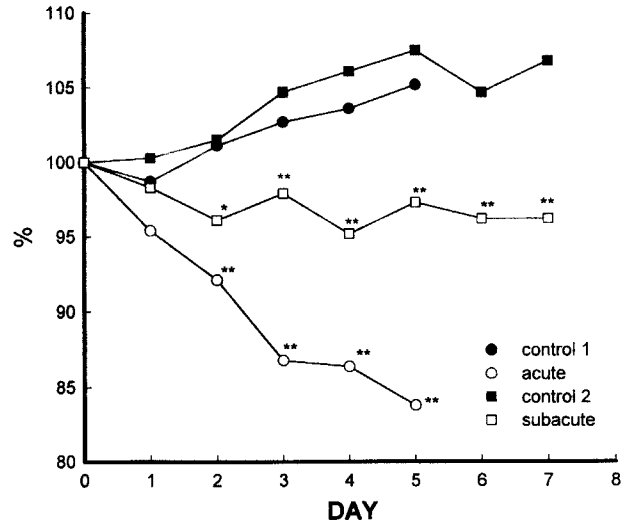

Fig. 1. Changes in the body weight during and after the administration of CK-15. Rats were treated either 50 mg/kg for acute or daily 5 mg/kg for 7 days, i.p., of CK-15. The values represent mean±S.E. for 6 animals. The 100% value represents the body weight before the administration of CK-15. *, ** mean significantly different from the respective control values at $p<0.05$ and $p<0.01$, respectively.

Table 1. Changes in water consumption after and during the administration of CK-15

		1 day	3 day	5 day	7 day
Acute	Control	14.1±1.4	15.6±1.0	16.5±0.9	
	Treated	6.3±0.5**	3.6±1.0**	9.3±0.6**	
Subacute	Control	19.8±1.3	17.4±0.9	20.9±1.2	25.3±1.9
	Treated	12.5±2.4*	13.4±2.1	15.0±1.0**	15.8±2.5*

Rats were treated either 50 mg/kg for acute or daily 5 mg/kg for 7 days, i.p., of CK-15. The consumed water for 24 hr were measured. The values represent mean± S.E. for 6 animals. *, **mean significantly different from the respective control values at $p<0.05$ and $p<0.01$, respectively.

body weight and the appearance of kidneys were not changed. These results indicate that CK-15 induced the reduction in water consumption and the decrease in growth rate. Also the occurrence in both the maximum decrease in water consumption and mortality after the acute treatment was superimposed. It is known that the water consumption is depend on the various body conditions, such as physical, psychological and neuronal states. Although the exact mechanisms was not clear, these results suggest that the compound might affect the consumatory behaviors and reduce the metabolic rate of the animals.

Table 2 shows the changes in the urine volume.

The excreted urine volume for 24 hr was significantly decreased from 3 days after the acute administration. However that was not changed during the subacute treatment. Since the water consumption was decreased after the acute treatment, the decrease in the excreted urine volume might be due to the reduced water consumption. And compared to the water consumption, the delayed occurrence in the decreased urine excretion after the acute treatment suggest that the some metabolites of CK-15 might induce the toxicities. In addition, no changes in the urine excretion during the subacute administration indicate that the toxic metabolites of the compound is not accumulated.

Table 3 shows the changes in the creatinine after and during the acute and the subacute administration of CK-15. The excreted creatinine was decreased 50% at 1 day after the acute administration, but returned to the control value. However, considering the urine volume, the total amount of the excreted creatinine was reduced to 50% compared to the control. In the subacute treatment, the excreted creatinine was decreased 24.5% on 5th day and 31.4% on 7th day. Also considering the urine volume, the total amount of the excreted creatinine was decreased as same percentage. However, the level of excreted protein was not changed. It has been reported that the excreted creatinine is not changed in the damage of renal tubule and the level of creatinine was affected by the size of muscle and the food consumption (Hoffman *et al.*, 1981; Pfeifer *et al.*, 1975; Shin *et al.*, 1989). Although further study about the blood urea nitrogen and creatinine in blood is needed, the reduced amount of urinary creatinine was partly due to their reduced body weights. It has been reported that the glomerular filtration of creatinine can be used for estimating GFR (Kee, 1991) and the glomerular filtration activities are altered by hormonal or neuronal signals (Lee, 1986). The present results reveal that GFR might be decreased after both the acute and the late-stage of subacute treatment. Although the cytotoxic studies of the compound are needed, either high dose or long-term administration of CK-15 might affect the activities of kidney.

Table 2. Changes in the excretion of urine after and during the administration of CK-15

		1 day	3 day	5 day	7 day
Acute	Control	14.67±0.33	14.00±1.84	16.00±1.77	
	Treated	16.00±1.21	4.68±1.13**	7.75±1.32**	
Subacute	Control	13.83±0.48	13.17±0.48	13.17±0.79	15.00±1.65
	Treated	13.00±1.59	11.00±0.68	13.17±0.87*	13.00±1.86

Rats were treated either 50 mg/kg for acute or daily 5 mg/kg for 7 days, i.p., of CK-15. The excreted urine for 24 hr were collected and measured. The values represent mean±S.E. for 6 animals. *, **mean significantly different from the respective control values at $p<0.05$ and $p<0.01$, respectively.

Table 3. Changes in the excretion of creatinine after and during the administration of CK-15

		1 day	3 day	5 day	7 day
Acute	Control	0.54±0.07	0.55±0.06	0.53±0.07	
	Treated	0.24±0.03**	0.55±0.06	0.62±0.10	
Subacute	Control	0.93±0.05	0.85±0.05	0.94±0.04	0.83±0.08
	Treated	0.85±0.05	0.75±0.07	0.71±0.06**	0.57±0.06*

Rats were treated either 50 mg/kg for acute or daily 5 mg/kg for 7 days, i.p., of CK-15. The excreted urine for 24 hr were collected and the creatinine were measured. The values represent mean±S.E. for 6 animals. *, **mean significantly different from the respective control values at $p<0.05$ and $p<0.01$, respectively.

It has been reported that most renal toxic substances induce necrosis in proximal tubular cells and leak the lysosomal and cytoplasmic enzymes, such as NAG, AAP, γ-GT and LDH, into the urine (Ohata *et al.*, 1987, Shin *et al.*, 1990). Also Harauchi and Yoshizaki (1990) reported that the increase in the urinary enzyme activities represented the damage in the kidney and the activities ratio per creatinine were more uniform than the total activities. Table 4 shows the changes in the activities of various urinary enzymes after and during the acute and the subacute administration of CK-15. A lysosomal enzyme, NAG, was increased at 1 day and then returned to the control value after the acute treatment. However in the subacute administration, there were no changes. This results indicate that the high concentration of CK-15 induces the damage in the kidney cell. The urinary activities of AAP and γ-GT, rich in brush border, show the similar effects. The activity of urinary AAP was increased at 1 day after the acute administration and then returned to the control value. However in the subacute treatment, the urinary AAP activities were significantly increased after the third administration of CK-15 and the

Table 4. Changes in NAG and AAP activities after and during the administration of CK-15

			1 day	3 day	5 day	7 day
NAG	Acute	Control	43.8±6.8	43.8±6.8	30.1±5.6	
		Treated	85.9±16.4*	92.9±35.4	52.8±13.7	
	Subacute	Control	27.2±4.6	16.5±3.3	34.8±3.7	32.1±8.1
		Treated	44.5±6.1*	38.4±8.0*	43.5±4.1	33.9±3.3
AAP	Acute	Control	72.6±3.7	88.3±20.9	49.0±8.1	
		Treated	117.4±4.9**	32.5±7.6*	59.8±9.7	
	Subacute	Control	71.8±22.3	78.6±12.9	59.8±9.7	93.0±19.0
		Treated	119.0±8.5	170.6±18.7**	154.0±3.6	202.9±37.3*
γ-GT	Acute	Control	621.0±114.3	800.1±251.1	502.6±109.9	
		Treated	1223.5±193.3*	853.1±197.9	588.4±173.4	
	Subacute	Control	326.2±69.0	347.2±54.9	323.9±74.5	379.5±82.6
		Treated	474.8±53.3	810.6±88.1**	547.4±145.6	565.2±131.9
LDH	Acute	Control	25.43±1.98	39.10±13.0	39.54±7.54	
		Treated	20.58±7.15	48.79±7.8	22.99±5.66	
	Subacute	Control	13.40±3.08	17.86±3.9	15.65±3.36	15.87±3.20
		Treated	4.16±0.30*	12.10±3.3	12.38±1.37	45.81±9.12**

Rats were treated either 50 mg/kg for acute or daily 5 mg/kg for 7 days, i.p., of CK-15. The excreted urine for 24 hr were collected and each enzyme activities were measured. The unit is nmol/min/mg creatinine. The values represent mean±S.E. for 6 animals. *, **mean significantly different from the respective control values at $p<0.05$ and $p<0.01$, respectively.

levels were maintained. Also the activity of urinary γ-GT, exist in the brush border, was increased at 1 day after acute administration and then returned to the control value. After three-days subacute treatments, the urinary γ-GT activities were increased. The results indicate that either high dose or long-term administration of CK-15 with low dose induce the increases in the urinary AAP and γ-GT activities. The increases in these urinary enzymes suggest that the kidney brush border might be damaged by the compound, CK-15. The activity of LDH, a cytoplasmic enzyme, was not changed in the acute treatment. However in the subacute treatment, the urinary activities of LDH were significantly increased after the 7th day treatment with CK-15. Although the analysis in the metabolites of CK-15 is needed, the results indicate that the cytotoxicity of the compound is appeared in the kidney cell membrane after long-term administrations with low dose. Thus it suggest that the cytotoxic metabolites of CK-15 might accumulate after the repetitive administration and induce the whole cell cytotoxicity. In summary, after the acute administration, the kidney cells in the renal tubule and brush border might be damaged by the compound and then recovered to the control condition. In the subacute administration with low dose, the kidney cells in the renal tubule were affected and then those in brush border were gradually damaged. Also a repetitive subacute administration induces the increase in membrane permeability. However the toxic effects of chronic administration are needed to be further investigated.

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REFERENCE

Bergmeyer, H.U. and Bernt, E. (1974): Lactate dehydrogenase. UV-assay with pyruvate and NADH in Methods of Enzymatic analysis (Bergmeyer, H.U. ed.). Academic Press, New York, pp 574-579.

Choi, B.G., Kim, O.Y., Chung, B.H., Cho, W.J., Cheon, S.H., Choi, S.U. and Lee, C.O. (1998): Synthesis of antineoplaston A10 analogs as potential antitumor agents, *Arch. Pharm.. Res.*, **21**, 157-163.

Harauchi, T. and Yoshizaki, T. (1990): Nephrotoxic indicators in rats, *Jap. J. Pharmacol.*, **54**, 205-215.

Hoffman, J. and Hardland, R. (1981): Diurnal rhythmicity in rat kidney γ-glutamyl transpeptidase activity, *Arch. Int. Physiol. Biochem.*, **89**, 245-247.

Hofmeister, R., Bhagava, A.S. and Gunzel, P. (1986): Value of enzyme determination in urine for the

diagnosis of nephrotoxicity in rat, *Clin. Chem. Acta*, **160**, 163-167.

Jung, K. and Scholz, D. (1980): An optimized assay of alanine aminopeptidase activity in urine, *Clin. Chem.*, **2**, 1251-1254.

Kee, J.L. (1991): Laboratory and diagnostic tests with nursing implications, Appleton & Lange, Norwalk, pp 107-108.

Leathwood, P.D. and Plummer, D.T. (1969): Enzymes of rat urine: I.A. metabolism cage for the complete separation of urine and faeces, *Enzymogia*, **37**, 240-250.

Lee, K.Y. (1986): Creatine and Creatinine, Clinical chemistry test, Rhyeo-moon-gak, Seoul, pp 64-70.

Lowery, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951): Protein measurement with folin phenol reagent, *J. Biol. Chem.*, **193**, 265-275.

Maruhn, D. (1976): Rapid colorimetric assay of β-galactosidase and N-acetyl-β-D-glucosaminidase in human urine, *Clin. Chim. Acta*, **73**, 453-461.

Ohata, H., Momose, K., Takahashi, A. and Omori, Y. (1987): Urinalysis for detection of chemically induced renal damage (1): Changes in urinary excretion of enzymes and various component caused by mercuric chloride and gentamicin. *J. Toxicol. Sci.*, **12**, 341-355.

Pfeifer, U. and Scheller, H. (1975): A morphological study of cellular autophagy including diurnal variations in kidney tubules of normal rat, *J. Cell Biol.*, **64**, 608-621.

Rock, R.C., Walker, W.C. and Jennings, C.D. (1987): Nitrogen metabolites and renal function in Fundamentals of Clinical Chemistry (Tietz, N.M. ed.). Saunders, Philadelphia, pp 669-704.

Shin, D.H., Jang, D.D., Lee, S.G., Choi, J.Y. Cho, J.C. and Hyeon, S.H. (1989): Nephrotoxicity assessment by determination of urinary alanine aminopeptidase in the rat, *Report of NISR Korea*, **2**, 423-431.

Shin, D.H., Jang, D.D., Lee, S.G., Choi, J.Y., Jung, J.Y., Cho, J.C., Lee, C.W. and Park, E.B. (1990): Nephrotoxicity assessment by determination of urinary enzyme Activities in the rat, *Report of NISR Korea*, **3**, 243-261.

Szasz, G. (1974): -Glutamyl transpeptidase in Methods of Enzymtic Analysis (Bergmeyer, H.U. ed). Academic Press, New York, pp 715-720.

Trual, A.G., Kisione, K.V., Theodore, J.B., Baguley, B. C., Laurence, P.G., Wilson, W.R., Paul, D.W. and Denny, W.A. (1990): DNA-directed alkylating agents: I. Structure-activity relationships for acridine-linked aniline mustards, *J. Med. Chem.*, **33**, 1177-1186.

Wachsmuth, E.D. (1982): Quantification of acute cephaloridine nephrotoxicity in rats: Correlation of serum and 24 hour-urine analysis with proximal tubule injuries. *Toxicol. Appl. Pharmacol.*, **63**, 429-445.