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Short Communication

Histopathological and functional changes in a single-dose model of combretastatin A4 disodium phosphate-induced myocardial damage in rats

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Abstract: Cardiotoxicity is a concern in the development of microtubule-disassembling agents (MDAs) as vascular-disrupting agents of tumors. This study investigated cardiotoxicity in rats induced by a single-dose of combretastatin A4 disodium phosphate (CA4DP), an MDA and discussed the use of this rat model in nonclinical studies of MDAs. First, CA4DP (120 mg/kg) was administered to rats intravenously, and cardiac histopathology and blood biomarkers were examined after 0.5, 24, and 72 h. Next, CA4DP (120 mg/kg) was administered to rats intravenously, and the electrocardiography and echocardiography results were analyzed. The results showed that at 0.5 h after dosing, plasma creatine kinase (CK), CK-muscle/brain (CK-MB), and fatty acid binding protein 3 levels increased. At 24 h, lactate dehydrogenase (LDH)-1, CK, and CK-MB levels increased, and multifocal vacuolar degeneration of myocardial cells was observed in the apical inner layer. At 72 h, LDH-1 levels were increased, and multifocal myocardial necrosis was observed in the interventricular septum and inner layer of the apex of left ventricular wall. Furthermore, at 0.5 h, heart rate (HR), ejection fraction (EF), and cardiac output (CO) decreased. At 24 h, CO decreased. Finally, at 72 h, HR, EF, and CO decreased, and depression of the T-wave amplitude was observed. In conclusion, myocardial injury, bradycardia, and depressed cardiac function were induced in rats by a single-dose of CA4DP. The lesion distribution and electrocardiographic features suggested that myocardial injury was induced by ischemia. These findings are similar to MDA-induced cardiotoxicity in humans, and this rat model will prove useful in studies of the cardiotoxicity in humans. (DOI: 10.1293/tox.2018-0023; J Toxicol Pathol 2018; 31: 307–313)

Key words: combretastatin A4, fosbretabulin, cardiotoxicity, echocardiography, cardiac necrosis, microtubule

There has been a recent interest in microtubule-disassembling agents (MDAs) as a new class of antitumor agents that target the vasculature of solid tumors. To date, many clinical trials of MDAs as antitumor agents, particularly combretastatin A4 disodium phosphate (CA4DP; fosbretabulin) [*cis*-1-(3,4,5,-trimethoxy-phenyl)-2-(4'-methoxyphenyl) ethane-3′-0-phosphate, disodium salt], have been performed. CA4DP is the prodrug of the tubulin-disassembling agent combretastatin A4 derived from *Combretum caffrum*[1]. Preclinical studies have demonstrated that CA4DP inhibits tumor vascularization and induces central tumor necrosis[2, 3]. Although therapeutic benefits of MDAs were found in clinical trials, cardiovasculartoxicities such as hypertension, tachycardia, bradycardia, QT prolongation, and myocardial infarction were observed within 24 h after administration[4–7].

Considering this background, we studied the utility of a rat model for analyzing MDA-induced cardiotoxicity[8–10]. In a previous short-term repeated-dose study, we demonstrated that CA4DP (four doses of 60 mg/kg at intervals of 24 h and two doses of 120 mg/kg at an interval of 72 h) induced myocardial necrosis in the hearts of rats[10]. In addition, changes of electrocardiography (ECG), i.e., prolongation of the RR, PR, and QT interval and emergence of the ST junction, were detected after the second or third administration of CA4DP 50 mg/kg at intervals of 24 h. These results revealed that repeated-doses of CA4DP induced myocardial changes in rats. However, the extrapolatability of rat studies to humans remains controversial because rapid cardiac changes were not sufficiently clarified despite the fact that cardiotoxicity in humans emerged within 24 h after administration[4, 5]. Moreover, little is known about the effects of CA4DP on blood biomarkers and cardiac functions in rats, although it is reported that CA4DP induced a decrease in cardiac systolic

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Table 1. Design of *Experiment 1*

Aim	Analysis of the histopathology of the heart and blood biomarkers					
Test article	Saline	Saline	Saline	CA4DP	CA4DP	CA4DP
Dose (mg/10 mL/kg)	0	0	0	120	120	120
Time of necropsy after dosing (h)	0.5	24	72	0.5	24	72
Number of animals	3	3	3	3	3	3

function and increase in blood cardiac troponin I level[5, 7]. Therefore, here we analyzed not only histopathology and ECG but also blood biomarkers and echocardiography in order to investigate single-dose CA4DP-induced acute myocardial damage in rats and discuss the use of this rat model in nonclinical studies of MDAs.

It was thought that a single-dose of CA4DP 120 mg/kg may induce cardiac damage rapidly, because the diastolic blood pressure of CA4DP 120 mg/kg-treated rats had increased within 30 minutes and two doses of 120 mg/kg at an interval of 72 h induced myocardial necrosis with remarked infiltration of mononuclear cellsc So, we selected 120 mg/kg as the dose level of CA4DP in this study.

This study was performed in two experimental steps (*Experiments 1* and *2*). All experiments using rats were conducted in accordance with the guidance of the Institutional Animal Care and Use Committee of Yakult Central Institute or the Animal Experimentation Guidelines of the University of Tokyo and approved by the Institutional Animal Care and Use Committee of Yakult Central Institute or the Institutional Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences at the University of Tokyo. The CA4DP was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

In *Experiment 1* (Table 1), CA4DP (120 mg/10 mL/kg) or saline (10 mL/kg) was administered to rats (Crl:CD(SD), male, 6 weeks old; n = 3/group) via the caudal vein by bolus infusion. At 0.5, 24, or 72 h after administration, isoflurane anesthesia was performed, and blood and hearts were taken during necropsy. The hearts were fixed in 10% neutral phosphate-buffered formalin. The fixed hearts were cross-sectioned in two planes through the ventricles as described in a previous report[8]. The fixed hearts were embedded in paraffin and sectioned. The specimens were stained with hematoxylin and eosin and observed using a light microscope (BX53, Olympus Corporation, Tokyo, Japan). Blood samples were treated with heparin to obtain plasma for biomarker analysis (for myocardial injury markers, lactate dehydrogenase [LDH], LDH-1, creatine kinase [CK], CK-muscle/brain [CK-MB], cardiac troponin T [cTnT], cardiac troponin I [cTnI], fatty acid binding protein 3 [FABP3], and myosin light chain 3 [Myl3]; for cardiac failure markers, N-terminal pro-brain natriuretic peptide [NT-pro BNP] and brain natriuretic peptide [BNP]). Plasma levels of LDH, LDH-1, CK, and CK-MB were analyzed using an automatic analyzer (LABOSPECT 003, Hitachi High-Technologies, Tokyo, Japan, and Epalyzer 2 Plus, Helena Laboratories, Beaumont, TX, USA). Plasma levels of cTnT, cTnI, FABP3, Myl3, NT-pro BNP, and BNP were analyzed using an enzyme-linked immunosorbent assay kit (MSD MULTI-ARRAY Assay System, Meso Scale Discovery, Gaithersburg, MD, USA).

Table 2. Design of *Experiment 2*

Aim	Analysis of electrocardiography and echocardiography	
Test article	Saline	CA4DP
Dose (mg/10 mL/kg)	0	120
Time of examination after dosing (h)	0.5, 24, 72	0.5, 24, 72
Number of animals	2	3

In *Experiment 2* (Table 2), CA4DP (120 mg/10 mL/kg) or saline (10 mL/kg) was administered to rats (Crl:CD(SD), male, 6 weeks old; n=3/CA4DP group; n=2/saline group) via the caudal vein by bolus infusion. At 0.5, 24, and 72 h after administration, ECG (II induction) and echocardiography were repeatedly recorded under isoflurane anesthesia using a preclinical imaging system (Vevo 3100, FUJIFILM VisualSonics, Toronto, ON, Canada). Observation of ECG waveforms and analysis of left-ventricular short-axis, left-ventricular inflow waveform, and mitral valve septal tissue waveform images were performed. As parameters of cardiac function, heart rate (HR), ejection fraction (EF), cardiac output (CO), mitral valve early diastolic filling velocity/atrial filling velocity (MV E/A), and mitral valve early diastolic filling velocity/early diastolic mitral annular motion velocity (MV E/E′) were calculated using analysis software (Vevo LAB, FUJIFILM VisualSonics, Toronto, ON, Canada).

In *Experiment 1* (Figs. 1 and 2), increases in plasma levels of total CK, CK-MB, and FABP3 were detected at 0.5 h after CA4DP dosing. No histopathological changes were observed in the heart at 0.5 h. At 24 h after dosing, increases in plasma levels of LDH-1, CK, and CK-MB, a decrease in NT-proBNP, and BNP were detected. Multifocal vacuolar degeneration of myocardial cells in the inner layer of the apex of the left ventricular wall and pyknosis/fragmentation of capillary endothelial cell nuclei were observed. At 72 h after dosing, increases in plasma levels of LDH-1 were detected. An increase in Myl3 was also detected in one case (11.9 ng/mL) of the three cases in the group. Multifocal myocardial necrosis with the infiltration of inflammatory cells in the interventricular septum and apical subendocardial regions, pyknosis/fragmentation of capillary endothelial cell nuclei, and edema around the capillaries were observed. Increases in plasma levels of LDH, NT-proBNP, and BNP were not detected at 0.5, 24, or 72 h. Plasma cTnT and cTnI levels were lower than the limit of detection (cTnT, 0.49 ng/mL; cTnI, 0.098 ng/mL).

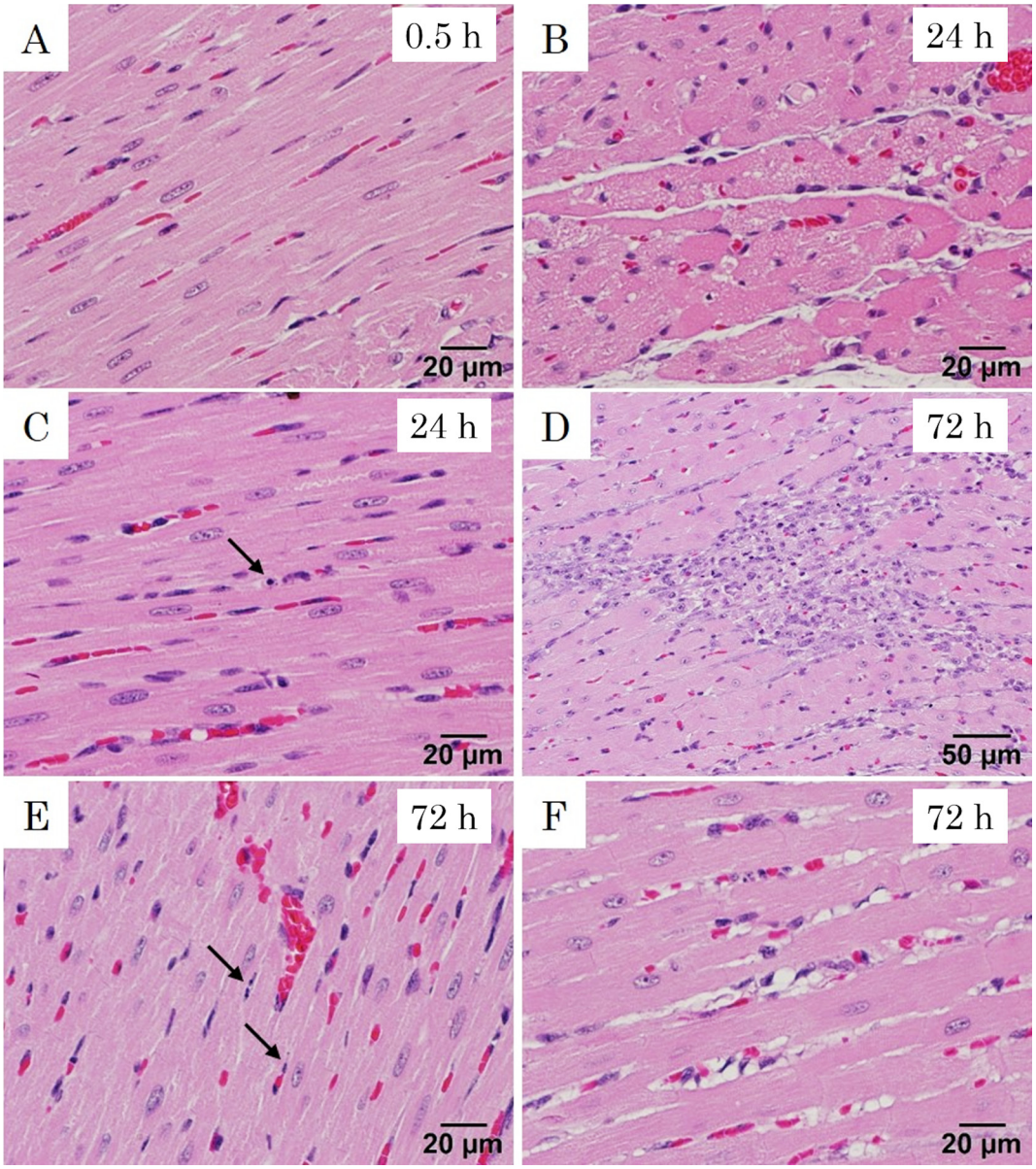

Fig. 1. Micrographs of the cardiac tissue of combretastatin A4 disodium phosphate (CA4DP)-treated rats. A: No obvious changes were observed in the hearts of rats at 0.5 h after the administration of CA4DP 120 mg/kg. B: Multifocal vacuolar degeneration of myocardial cells was observed in the hearts of rats at 24 h after the administration of CA4DP 120 mg/kg. This lesion was prominent in the inner layer of the apex of the left ventricular wall. C: Pyknosis and fragmentation of the capillary endothelial cell nuclei (arrow) were observed in the hearts of rats at 24 h after the administration of CA4DP 120 mg/kg. D: Multifocal necrosis of myocardial cells and infiltration of inflammatory cells were observed in the hearts of rats at 72 h after the administration of CA4DP 120 mg/kg. These lesions were prominent in the interventricular septum and inner layer of the apex of the left ventricular wall. E: Pyknosis and fragmentation of capillary endothelial cell nuclei (arrows) were observed in the hearts of rats at 72 h after the administration of CA4DP 120 mg/kg. F: Edema around the capillaries was observed in the hearts of rats at 72 h after the administration of CA4DP 120 mg/kg. A–E: hematoxylin and eosin staining.

In *Experiment 2* (Figs. 3 and 4), decreases in HR, EF, and CO and an increase in MV E/A were detected 0.5 h after CA4DP dosing. At 24 h after dosing, decreases in CO were detected. At 72 h after dosing, decreases in HR, EF, and CO were detected. Moreover, depression of the T-wave amplitude and progression of the R-wave and S-wave amplitudes were observed.

In single-dose CA4DP-treated rats, histopathological changes were induced in the hearts. It is generally thought that multifocal necrosis in the interventricular septum and inner layer of the apex of the left ventricular wall is induced ischemically when regional blood flow decreases relative to the workload of the myocardium[11]. In addition, histopathological changes in the capillaries were induced by CA4DP. Therefore, it seems reasonable that one of the main cause of CA4DP-induced myocardial lesions observed in *Experi-*

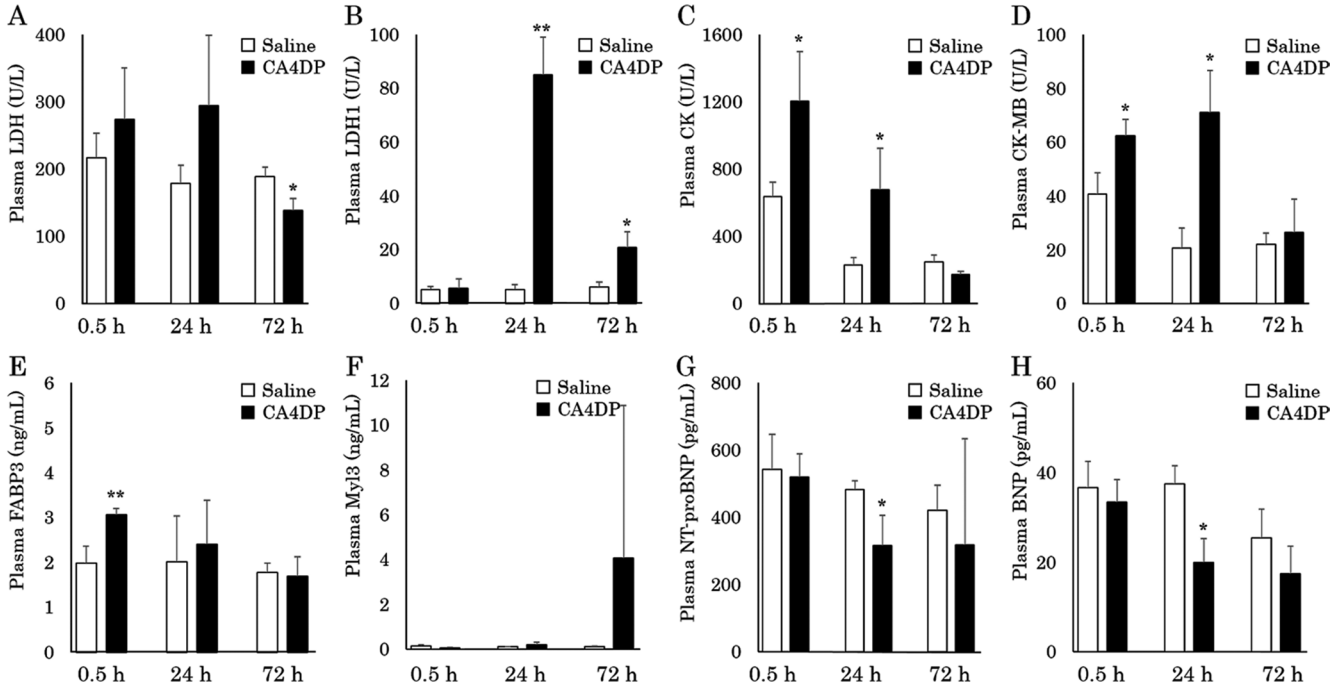

Fig. 2. Plasma lactate dehydrogenase (LDH), LDH-1, creatine kinase (CK), CK-muscle/brain (CK-MB), fatty acid binding protein 3 (FABP3), myosin light chain 3 (Myl3), N-terminal pro-brain natriuretic peptide (NT-proBNP), and brain natriuretic peptide (BNP) levels in saline- or CA4DP-treated rats. A: Plasma LDH levels. Plasma LDH levels of CA4DP-treated rats were not higher than those of saline-treated rats. B: Plasma LDH-1 levels. Plasma LDH-1 levels of CA4DP-treated rats at 24 and 72 h after the administration of CA4DP 120 mg/kg were higher than those of saline-treated rats. C: Plasma CK levels. Plasma CK levels of CA4DP-treated rats at 0.5 and 24 h after the administration of CA4DP 120 mg/kg were higher than those of saline-treated rats. D: Plasma CK-MB levels. Plasma CK-MB levels of CA4DP-treated rats at 0.5 and 24 h after the administration of CA4DP 120 mg/kg were higher than those of saline-treated rats. E: Plasma FABP3 levels. Plasma FABP3 levels of CA4DP-treated rats at 0.5 h after the administration of CA4DP 120 mg/kg were higher than those of saline-treated rats. F: Plasma Myl3 levels. Plasma Myl3 levels of CA4DP-treated rats at 0.5, 24, and 72 h after the administration of CA4DP 120 mg/kg were not significantly different from those of saline-treated rats. However, one (11.9 ng/mL) of the three cases in the 72 h group showed a high level of Myl3. G: Plasma NT-proBNP levels. Plasma NT-proBNP levels of CA4DP-treated rats at 24 h after the administration of CA4DP 120 mg/kg were lower than those of saline-treated rats. H: Plasma BNP levels. Plasma BNP levels of CA4DP-treated rats at 24 h after the administration of CA4DP 120 mg/kg were lower than those of saline-treated rats. A-H: Each bar represents the mean ± SD. *$p<0.05$, **$p<0.01$ compared with values of saline-treated rats (*t*-test).

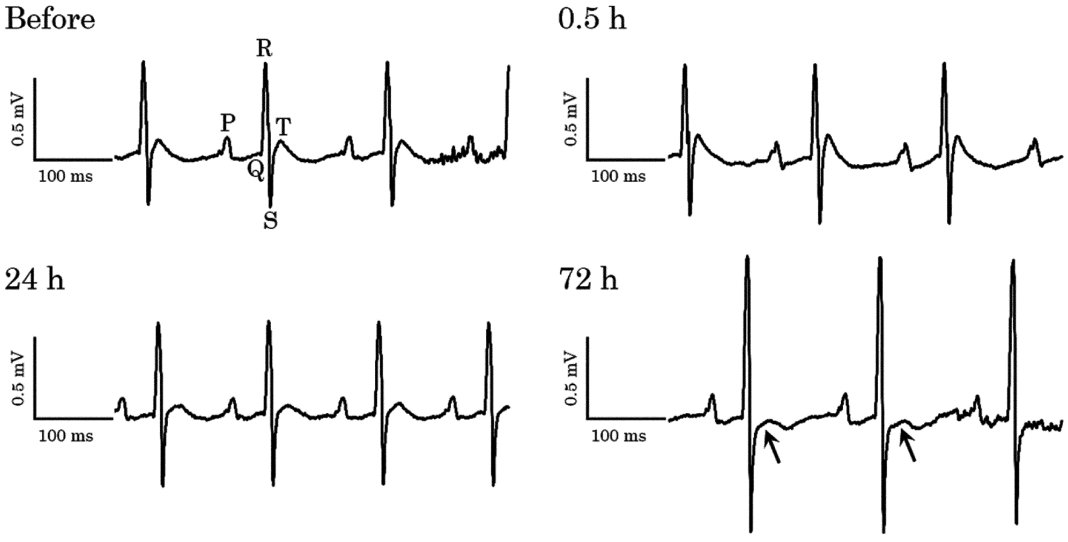

Fig. 3. Electrocardiography traces before and after combretastatin A4 disodium phosphate (CA4DP) administration. Depression of the T-wave amplitude (arrows) and progression of the R-wave and S-wave amplitudes were observed 72 h after the administration of CA4DP 120 mg/kg.

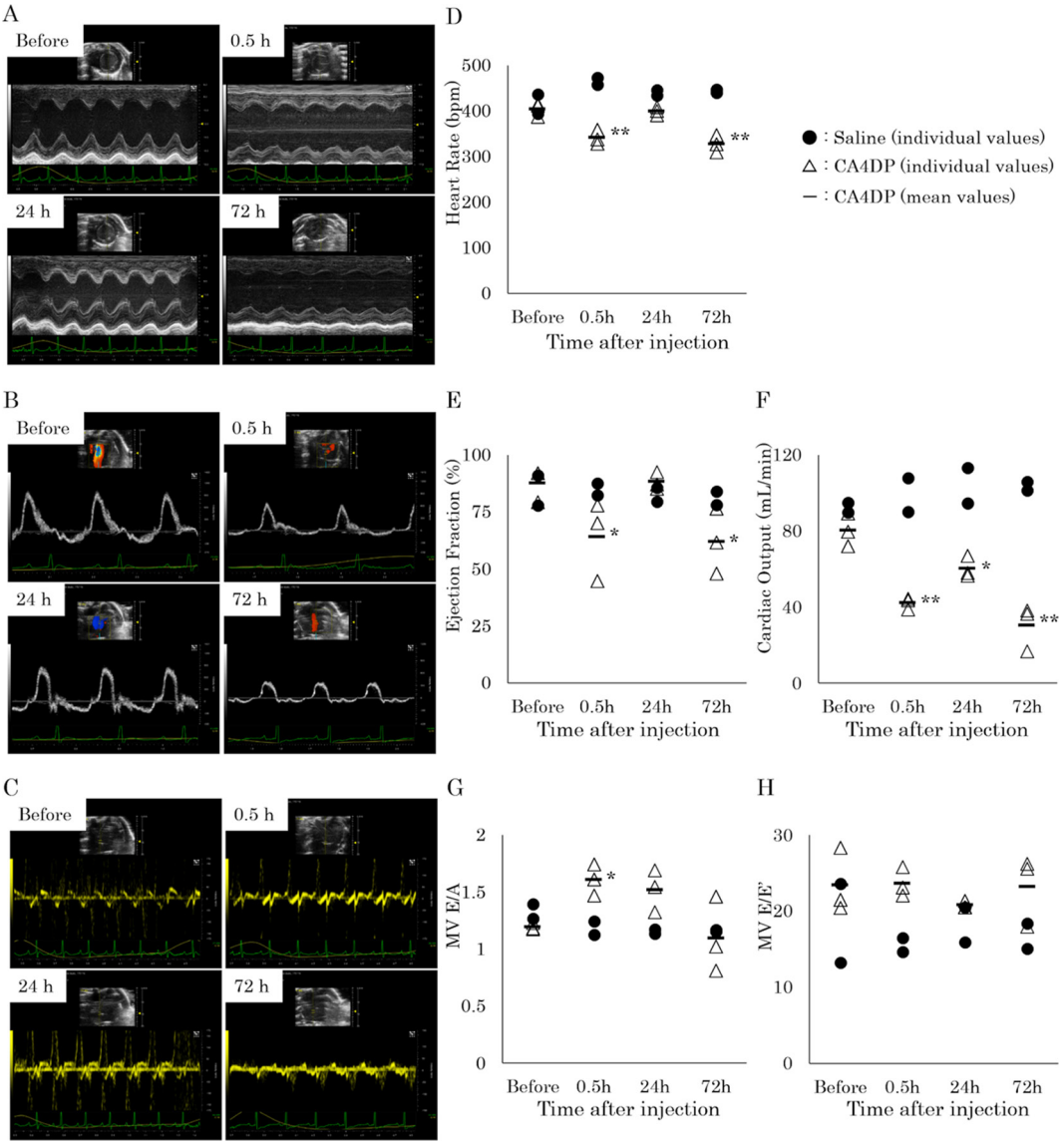

Fig. 4. Results of the echocardiographic examinations. A: Left ventricular short-axis image of a CA4DP-treated rat. B: Left ventricular inflow waveform image of a CA4DP-treated rat. C: Mitral valve septal tissue waveform image of a CA4DP-treated rat. D: Heart rate (HR) decreased 0.5 and 72 h after the administration of CA4DP 120 mg/kg. E: Ejection fraction (EF) decreased 0.5 and 72 h after the administration of CA4DP 120 mg/kg. F: Cardiac output (CO) decreased 0.5, 24, and 72 h after the administration of CA4DP 120 mg/kg. G: Mitral valve early diastolic filling velocity/atrial filling velocity (MV E/A) increased 0.5 h after the administration of CA4DP 120 mg/kg. H: Mitral valve early diastolic filling velocity/early diastolic mitral annular motion velocity (MV E/E′) did not change after the administration of CA4DP 120 mg/kg. D–H) black circles and white triangles represent individual values of saline- and CA4DP-treated rats, respectively, while - represents the mean values of CA4DP-treated rats. *p<0.05, **p<0.01 compared with values obtained before the administration of CA4DP (Dunnett's test).

ment 1 was ischemia due to microcirculatory collapse. This ischemic damage and direct toxic effect on cardiomyocytes caused myocardial lesions in a coordinated manner, as our previous study suggested that CA4DP also has direct effects on myocardial cells[10].

ECG and echocardiographic changes were induced in single-dose CA4DP-treated rats in *Experiment 2*. The depressed T-wave amplitude in CA4DP-treated rats may be explained by ischemia, as ischemia, hypertrophy, and bundle branch blocks are general causes of a depressed T-wave amplitude and lesions suspected of ischemic change were observed in *Experiment 1*. The cause of R-wave and S-wave progression may be explained by a decrease in electric resistance due to decreased body mass. The echocardiographic analysis showed that a single dose of CA4DP induced bradycardia and reduced contractile function (manifesting as decreases in EF and CO) within 24 h. Meanwhile, increases in MV E/A were detected without changes in MV E/E′. This change in MV E/A was thought to be a result of systolic failure because the early diastolic filling velocity (E-wave)

increases when systolic function is impaired.

In human studies, myocardial injury, ischemia, apical hypokinesis, systolic failure, bradycardia, tachycardia, and QT prolongation were induced by MDA administration[4–7], and these cardiotoxic events emerged within 24 h[4, 5]. In this study, myocardial injuries with suspected ischemic changes, apical histopathological changes, systolic failure, and bradycardia were induced in rats. These findings are similar to MDA-induced cardiotoxicity in humans[4–7]. At 0.5 h after dosing, decreases in HR, EF, and CO and increases in plasma CK, CK-MB, and FABP3 were detected in CA4DP-treated rats. Meanwhile, slight histopathological changes in the myocardium and capillary were observed, but changes in HR and EF were not obvious at 24 h after dosing. At 72 h after dosing, severe histopathological changes in the myocardium were observed, and decreases in HR, EF, and CO recurred. These results suggested that CA4DP-induced cardiac events in rats had occurred within a few minutes and recovered temporarily at 24 h along with the decreases in blood level of CA4DP. Progression of necrosis and inflammation in addition to deterioration of general condition might induce decreases in HR, EF, and CO again. Our previous study showed that CA4DP induced an increase in blood pressure in rats and an increase in beating rate of human induced pluripotent stem cell-derived cardiomyocytes[10]. Therefore, it is thought that the baroreflex resulted in a decrease in HR at 0.5 after dosing in this study, although CA4DP has cardiostimulatory activity. This multiple participation of both direct cardiostimulation and the baroreflex following an increase in blood pressure may be one reason why both bradycardia and tachycardia were observed in human studies.

There are several reports about CA4DP-induced cTnI elevation in humans[5, 7], however, variations of the other biomarkers were not reported in detail. In this study, plasma cTnT and cTnI were not detected at any point. There is also a reports about CA4DP-induced chest pain and ST elevation in humans without elevation of serial cardiac enzymes including troponin[7]. So, it is thought that the brief half-lives of cTnT and cTnI[12] represent one reason for the lack of changes in cTnI in this study. However, further studies are needed to elucidate the reasons for these kinetic patterns. Moreover, increases in NT-proBNP and BNP were not detected in spite of the reduction of contractile function in *Experiment 2*, and decreases in NT-proBNP and BNP were detected. Myocardial injury may be the cause of these changes, because it has been reported that the BNP level decreased in myocardial stunning patients[13].

CA4DP 120 mg/kg in rats is comparable to approximately eight times the maximum dose in humans[6, 10]. It is possible that cardiotoxic changes would be induced by a lower single dose because 60 mg/kg of CA4DP in our previous 4-day repeated-dose study induced myocardial injury in rats[10]. The dose-response relationship is the subject of a future study. In addition, ECG wave components including QT interval were not analyzed in this study; however, QT prolongation was induced by CA4DP 50 mg/kg in our previous 3-day repeated-dose study in rats[10]. An analysis of detailed ECG components is another subject of a further study.

In conclusion, myocardial injury with apical predilection, systolic failure, and bradycardia were induced in rats by a single dose of CA4DP within 24 h. Moreover, the distribution of CA4DP-induced lesions and ECG changes suggested that myocardial injury was induced by ischemia. These findings are similar to MDA-induced cardiotoxicity in humans, and it is thought that the single-dose model of CA4DP-induced myocardial damage in rats mimics the pathological condition of MDA-induced cardiotoxicity in humans. Therefore, this rat model will be useful in studies of the molecular mechanisms of MDA-induced cardiotoxicity and preventive strategies for cardiotoxicity in humans.

Disclosure of Potential Conflicts of Interest: The authors declare that they have no conflicts of interest.

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