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Original Article

Pretreatment with tadalafil attenuates cardiotoxicity induced by combretastatin A4 disodium phosphate in rats

Yoshiyasu Nagashima[1], Ryota Tochinai[1–3]*, Shin-ichi Sekizawa[1], Daiki Kato[4], Takayuki Nakagawa[4], Yoshiharu Tsuru[5], Yasuko Tatewaki[2], Tatsushi Mutoh[2,3], Yasuyuki Taki[2], and Masayoshi Kuwahara[1]

[1] Department of Veterinary Pathophysiology and Animal Health, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[2] Department of Aging Research and Geriatric Medicine, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryocho, Aobaku, Sendai 980-8575, Japan
[3] Research Institute for Brain and Blood Vessels, Akita Cerebrospinal and Cardiovascular Center, 6-10 Sensyu-Kubota-machi, Akita 010-0874, Japan
[4] Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-8657, Japan
[5] Primetech Corp. Life Science Laboratory, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-8657, Japan

Abstract: Combretastatin A4 disodium phosphate (CA4DP) is a prodrug of combretastatin A4 (CA4), a microtubule-disassembling agent that exhibits antitumor effects by inhibiting tumor cell proliferation and inducing morphological changes and apoptosis in vascular endothelial cells in tumors. However, cardiotoxicity induced by ischemia and hypertension is a severe adverse event. In this study, we focused on the fact that phosphodiesterase (PDE) 5 inhibitors dilate the heart and peripheral blood vessels and aimed to investigate whether co-administration of tadalafil, a PDE5 inhibitor, can attenuate cardiotoxicity without altering the antitumor effect of CA4DP. To investigate cardiotoxicity, CA4DP and/or tadalafil were administered to rats, and blood pressure, echocardiography, histopathology, and cGMP concentration in the myocardium were examined. Administration of CA4DP increased systolic blood pressure, decreased cardiac function, lowered cGMP levels in the myocardium, and led to necrosis of myocardial cells. Co-administration of tadalafil attenuated these CA4DP-induced changes. To investigate the antitumor effect, canine mammary carcinoma cell lines (CHMp-13a) and human umbilical vein endothelial cells were cultured with CA4 and/or tadalafil, and cell proliferation and endothelial vascular tube disruption were examined. CHMp-13a cells were transplanted into nude mice and treated with CA4DP and/or tadalafil. CA4-induced inhibition of cell proliferation and disruption of the endothelial vascular tube were not affected by co-treatment with tadalafil, and the antitumor effects of CA4DP in xenograft mice were not reduced by co-administration of tadalafil. These results revealed that myocardial damage induced by CA4DP was attenuated by co-administration of tadalafil while maintaining antitumor efficacy. (DOI: 10.1293/tox.2022-0143; J Toxicol Pathol 2023; 36: 151–158)

Key words: combretastatin A4, cardiotoxicity, blood pressure, cardiac necrosis, phosphodiesterase 5, tadalafil

Introduction

Combretastatin A4 disodium phosphate (CA4DP) [cis-1-(3,4,5,-trimethoxy-phenyl)-2-(4'-me-thoxyphenyl) ethane-3′-0-phosphate, disodium salt] is a prodrug of combretastatin A4 (CA4), a microtubule-disassembling agent[1]. CA4 exhibits antitumor effects by inhibiting spindle fiber formation during tumor cell mitosis or disrupting the microtubule, which acts as a cytoskeleton required to maintain endothelial cell morphology and proliferation of the tumor vasculature[2]. Therefore, CA4DP is expected to be used as an anticancer drug in both veterinary and human medicine[3, 4]. However, CA4 has been shown to cause cardiovascular toxicity in clinical trials, including hypertensive and ischemic myocardial injury[3–5]. While CA4 also causes gastrointestinal toxicity, peripheral neurotoxicity, and testicular toxicity, cardiotoxicity is life-threatening and is reported to be the dose-limiting factor in clinical trials[5]. Therefore, attenuation of cardiotoxicity is a primary challenge in CA4. Although the mechanism by which CA4 induces cardiotoxicity remains unclear, it is thought that CA4 causes morphological changes in endothelial cells and consequently induces

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*Corresponding author: R Tochinai
(e-mail: ar-tochinai@g.ecc.u-tokyo.ac.jp)

myocardial ischemia[6, 7]. In addition, because hypertension is known to increase resistance in the coronary artery and decrease coronary blood flow[8], it is thought that hypertension furthers the progression of CA4DP-induced cardiotoxicity. Thus, reducing hypertension and increasing coronary blood flow are required to attenuate CA4DP-induced cardiotoxicity.

Phosphodiesterase (PDE) is an enzyme that disassembles intracellular second messengers, such as cAMP or cGMP. Eleven subtypes of PDEs have been reported[9], and inhibitors of these subtypes are being used clinically[10–13]. Among the multiple subtypes of PDEs, PDE5 is expressed in the cardiovascular system[14]. PDE5 inhibitors are known to induce vasodilation of the coronary artery[4, 15] or decrease ischemia-reperfusion infarct size in the heart[16]. Because of these effects, it is possible that PDE5 inhibitors could reduce cardiotoxicity by suppressing the CA4DP-induced increase in blood pressure and decrease in coronary blood flow. Therefore, the present study aimed to investigate whether the co-administration of tadalafil, a PDE5 inhibitor, can attenuate cardiotoxicity without altering the antitumor effect of CA4DP.

Materials and Methods

Male Sprague Dawley rats (Crl:CD(SD)) aged 5–6 weeks and nude mice (BALB/cAJcl-*nu/nu*) aged 8–9 weeks were used in this study. Animals were housed in plastic or stainless-steel mesh cages in a controlled environment (12 h/12 h light-dark cycle, temperature 22 ± 2°C) with *ad libitum* access to laboratory basal feed and tap water. All experiments using rats and mice were conducted in accordance with the Animal Experimentation Guidelines of the University of Tokyo and approved by the Institutional Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences at the University of Tokyo. CA4DP was purchased from MedKoo Biosciences (Chapel Hill, NC, USA). CA4 was obtained from the Tokyo Chemical Industry (Tokyo, Japan). Tadalafil was purchased from Combi-Blocks (San Diego, CA, USA).

Evaluation of cardiotoxicity using rats

Eighteen rats (vehicle [n=5], tadalafil [n=5], CA4DP [n=4], and CA4DP+tadalafil [n=4]) were used to evaluate systolic blood pressure, echocardiography, and histopathology of the heart, and 16 rats (vehicle [n=4], tadalafil [n=4], CA4DP [n=4], and CA4DP+tadalafil [n=4]) were used to measure cGMP concentration in the left ventricular free wall.

At six weeks of age, tadalafil (a single dose of 5 mg/kg, vehicle: 0.5% methylcellulose) or 0.5% methylcellulose was administered orally. Two hours after oral administration, CA4DP (a single dose of 120 mg/kg, vehicle: saline) or saline (a single dose) was administered via the caudal vein by bolus infusion. The dose of CA4DP was reported to induce cardiotoxicity[17] and the dose of tadalafil was an effective dose extrapolated from humans[18]. The dosing interval between tadalafil and CA4DP was determined based on the time required for the tadalafil blood concentration to reach the peak blood concentration[19].

Systolic blood pressure was measured using the tail-cuff method (BP-98AL; Softron, Tokyo, Japan) before the administration of tadalafil or 0.5% methylcellulose and 30 min after intravenous injection of CA4DP or saline, and the ratio between the two measurements was calculated. Seventy-two hours after the administration of CA4DP, echocardiography was performed under isoflurane anesthesia using a preclinical imaging system (Vevo 3100; FUJIFILM VisualSonics, Toronto, Canada). Stroke volume and cardiac output were calculated using an analysis software (Vevo LAB; FUJIFILM VisualSonics). Stroke volume and cardiac output were normalized by body surface area (S [cm^2]), and Stroke Index (SI) and Cardiac Index (CI) were calculated. Body surface area was calculated using Body Weight (BW [g]) and the following formula[20]:

$$S = 9.83 \times (BW)^{2/3}$$

After echocardiography examination, the rats were anesthetized with isoflurane, and necropsy was performed. After exsanguination, the hearts were removed and immediately fixed in 10% neutral phosphate-buffered formalin. The fixed hearts were cross-sectioned in two planes through the ventricles as previously described[21]. They were then embedded in paraffin and sectioned at a thickness of 4 μm. The specimens were stained with hematoxylin and eosin (HE) and observed under a light microscope. The specimen antigens were retrieved by microwave irradiation for 5 min, and immunostaining was performed using the macrophage marker anti-Iba-1 antigen (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan).

To investigate cGMP concentration in the left ventricular free wall, the hearts of rats were obtained 30 min after CA4DP or saline injection, under isoflurane anesthesia. Whole hearts were immediately frozen in liquid nitrogen and stored at −80°C until used for cGMP quantification. A Cyclic GMP ELISA Kit (Cayman Chemical, Ann Arbor, MI, USA) was used.

Evaluation of antitumor effects using cultured cells

A canine mammary carcinoma cell line (CHMp-13a)[22] and human umbilical vein endothelial cells (HUVECs) were used for the cell variability test, and HUVECs were used for the tube formation assay. CHMp-13a cells were grown in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corporation), and HUVECs were grown in complete human endothelial cell medium /w kit (Cell Biologics, Chicago, IL, USA).

PDE5 expression was confirmed by immunocytochemical staining with anti-PDE5/PDE5A antigen (rabbit; Abcam, Cambridge, UK). CHMp-13a cells and HUVECs were seeded into chamber slides (1.0×10^3 cells/500 μL). The cells were incubated at 37°C for 2 days, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton. The cells were blocked with 5% goat serum (NGS) and treated with primary antibodies against PDE5A/PDE5. They were

then diluted 1:1,000 with PBS containing 5% NGS, and the cells were incubated for 1 h at 20°C. After treatment with a fluorescence-labelled secondary antibody (anti-rabbit IgG and Alexa Fluor 488; Thermo Fisher Scientific, Waltham, MA, USA), the cells were incubated for 1 h at room temperature in the dark. PBS containing 0.1% 4',6-diamidino-2-phenylindole (DAPI) was added to the cells and incubated for an additional 5 min at room temperature in the dark. The cells were then sealed, observed, and imaged using a fluorescence microscope (LSM700; Zeiss, Oberkochen, Germany).

Cell variability was measured using the WST-8 assay. CHMp-13a cells (1.0×10^2 cells/100 μL) or HUVECs (1.0×10^3 cells/100 μL) were seeded into 96-well plates for 24 h. The cells were then incubated with CA4 at three different doses (1.0, 3.0, and 10 nM) and tadalafil at three different doses (1.0, 3.0, and 10 μM) for 72 h. Next, 2-(2-methoxy-4-nitrophenyl)-3(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8; DOJINDO, Kumamoto, Japan) was added according to the manufacturer's protocol and incubated for 1 h. Absorbance was then measured at 450 nM (620 nM as the reference) with a plate reader (iMark Microplate Reader; BIO-RAD, Hercules, CA, USA).

An *in vitro* assay of tube formation was performed to evaluate the disruption of existing blood vessels. ECM-gel (Endothelial Tube Formation Assay; Cell Biolabs Inc., San Diego, CA, USA) was added to a 96-well plate at 50 μL/well. After gelation at 37°C for 30 min, the gels were overlaid with medium containing HUVECs (2.0×10^5 cells/100 μL). Cells were then incubated for 18 h to create the capillary tube structure and then incubated with CA4 and tadalafil for 10 h. Images were taken before and after drugs were added, and the number of capillaries in the two images was counted to calculate the ratio after/before drug addition.

Evaluation of antitumor effects using xenograft mice was performed to confirm whether tadalafil did not diminish the antitumor effects of CA4DP *in vivo*. CHMp-13a cells were suspended in PBS (1.5×10^6 cells/100 μL), and the solution was subcutaneously injected into the dorsal lumbar region of nude mice. One week after injection, the mice were divided into four groups (vehicle [n=6], tadalafil [n=6], CA4DP [n=6], and CA4DP+tadalafil [n=6]). According to previous studies, the doses of CA4DP and tadalafil were 100 mg/kg and 5 mg/kg, respectively. Tadalafil and 0.5% methylcellulose were administered orally. Two hours later, CA4DP or saline was administered via the caudal vein by bolus infusion. These drugs were administered three times every other day (days 1, 3, and 5).

Each tumor volume was calculated just before the first dose of tadalafil and CA4DP (day 1) and the day after the final dose of CA4DP (day 6). The major and minor axes of the tumor were measured using calipers. Assuming the tumor to be ellipsoid, the tumor volume was calculated using the following formula[23]:

$V = a \times b^2 \times 0.52$ (mm^3) a: major axis, b: minor axis

The ratio between the tumor volumes on the first and final days of the experiment (relative tumor volume [RTV]) was calculated.

Statistical analyses

Data were analyzed using the R software (ver. 4.1.1; R Foundation for Statistical Computing, Vienna, Austria). Tukey's test was used for the analysis of the systolic blood pressure ratio, CI, SI, cGMP concentration, and RTV. Two-way ANOVA was used to analyze cell variability and tube formation assays. A p-value <0.05 was considered statistically significant.

Results

Evaluation of cardiotoxicity using rats

An increase in systolic blood pressure was detected in the CA4DP group compared with that in the vehicle group (Fig. 1A). The CA4DP+tadalafil group showed a significantly lower systolic blood pressure than the CA4DP group.

Echocardiography revealed that the CA4DP group had significantly lower SI and CI than the vehicle group (Fig. 1B, 1C). The CA4DP+tadalafil group had a significantly higher CI than that of the CA4DP group.

Histopathological examination revealed necrosis of cardiomyocytes, infiltration of inflammatory cells (Fig. 2A, 2B), and edematous changes around capillaries, and pyknosis of capillary endothelial cells (Fig. 2C, 2D) were observed in both the CA4DP and CA4DP+tadalafil groups; these changes were milder in rats treated with CA4DP+adalafil compared to the CA4DP group. Iba-1-positive cells were detected in the CA4DP group, and fewer Iba-1-positive cells were detected in rats treated with CA4DP+tadalafil than in rats treated with CA4DP alone (Fig. 2E, 2F).

The cGMP concentration in the left ventricular free wall in rats treated with CA4DP alone showed a low tendency compared to those in rats treated with vehicle, whereas the cGMP concentration in rats treated with CA4DP+tadalafil was significantly higher than that in rats treated with CA4DP alone (Fig. 3).

Evaluation of antitumor effects using cultured cells

PDE5 expression was detected in the CHMp-13a cells (Fig. 4A) and HUVECs (Fig. 5A). The proliferation of CHMp-13a cells (Fig. 4B) and HUVECs (Fig. 5B) was inhibited by CA4 in the cell variability test. In the tube formation assay, disruption of the vascular tube induced by CA4 was observed, but tadalafil did not alter the effect of CA4 (Fig. 5C).

Evaluation of antitumor effects using xenograft mice

The RTV of the CA4DP and CA4DP+tadalafil groups were smaller than those of the vehicle or tadalafil groups, and the difference between the CA4DP and CA4DP+tadalafil groups was not noticeable (Fig. 6).

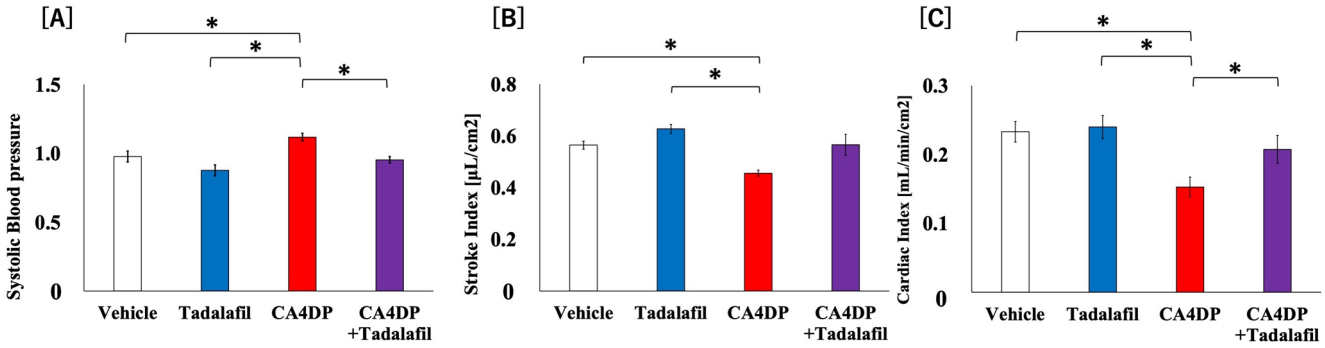

Fig. 1. Systolic blood pressure, Stroke Index, Cardiac Index, micrographs of cardiac tissue, and histopathology of CA4DP/CA4DP+tadalafil-treated rats. [A] Systolic blood pressure ratio between measured blood pressure before and 30 min after tadalafil/vehicle administration. Systolic blood pressure of rats treated with CA4DP was higher than that of rats treated with vehicle, tadalafil or CA4DP+tadalafil. [B, C] Stroke index (SI) and Cardiac Index (CI) were calculated from echo images in rats 72 h after CA4DP/vehicle administration. [B] SI of rats treated with CA4DP was lower than that of rats treated with vehicle or tadalafil. There was no significant difference between CA4DP and CA4DP+tadalafil-treated rats, but it was slightly higher in CA4DP+tadalafil-treated rats. [C] CI of rats treated with CA4DP was lower than that of rats treated with vehicle, tadalafil, or CA4DP+tadalafil. [A–C] *$p<0.05$.

Discussion

CA4DP-induced hypertension can cause an increase in cardiac workload and a decrease in blood flow in the coronary artery[6, 8, 24]. In humans, intravenous administration of CA4DP leads to an increase in blood pressure within 30 min to 1 h and continues for 3–4 h, whereas ischemia of the muscles is seen immediately after administration up to 24 h afterward[4]. It is possible that CA4DP induces myocardial damage in the acute phase immediately after administration, and that this effect may continue for up to 24 h. The blood half-lives of typical PDE5 inhibitors are 3–5 h for sildenafil, 4–5 h for vardenafil, and 17.5 h for tadalafil[19]. Therefore, in the current study, we investigated the effect of reducing myocardial injury by using tadalafil, which has the longest half-life in blood and is presumably able to maintain its PDE5 inhibitory effect during the period when the risk of CA4DP-induced myocardial injury is high. The results of the evaluation of cardiotoxicity in rats revealed that CA4DP-induced cardiotoxicity was attenuated by tadalafil, and adalafil attenuated the CA4DP-induced increase in peripheral systolic blood pressure. This effect of tadalafil on peripheral blood pressure may decrease the afterload in the heart. Tadalafil also prevented a decrease in cGMP levels in the left ventricular free wall. Because cGMP is one of the main factors of vasodilation, it is thought that the coronary artery of the left ventricular free wall expands, and blood flow remains after tadalafil administration. Thus, the mechanism by which tadalafil reduces CA4DP-induced cardiotoxicity can be explained by its effects on the peripheral and myocardial circulation. The reduction in edematous changes around capillaries in the CA4DP+tadalafil group also indirectly suggests that the concomitant use of tadalafil improved cardiac blood flow. The pyknosis of capillary endothelial cells is thought to be a result of both the direct effect of CA4DP on vascular endothelial cells and the secondary effect of ischemia. Our *in vitro* experiments using HUVECs suggested that the direct effect of CA4DP on vascular endothelial cells in the CA4DP+tadalafil group was not altered. There are differences in cardiac anatomy and pharmacokinetics between rats, dogs, and humans, and a direct assessment of the changes in myocardial blood flow was not performed in the present study. However, the results indicate the possibility that tadalafil can reduce cardiotoxicity, which has been an issue in clinical trials. Future translational research using larger animals such as rabbits, dogs, and swine is expected to evaluate blood flow through imaging of the coronary arteries.

To develop cancer chemotherapy with a low risk of cardiotoxicity, it is essential to discuss the margin between the effective and toxic doses of anticancer drugs. Therefore, we conducted both *in vitro* and *in vivo* drug efficacy evaluations using tumor cells and xenograft mice, respectfully. The cytotoxic activity of CA4 against tumor cells and vascular endothelial cells *in vitro* and the antitumor effects of CA4DP *in vivo* did not decrease, even in the presence of tadalafil. These results suggest that the co-administration of tadalafil does not increase the effective dose. It is generally known that tadalafil preserves tissue blood flow through vasodilation[13]. Therefore, we hypothesized that the antitumor effects of CA4 were hindered by tadalafil while maintaining tumor blood flow. However, tadalafil did not affect the efficacy of CA4DP in the current study. Since tumor vessels are known to be immature and have underdeveloped vascular smooth muscle cells[7, 25], tadalafil may be less effective in maintaining blood flow in tumor vessels. In a xenograft model in which heterologous tumor cells were transplanted, the development of tumor blood vessels was insufficient. To fully discuss the effects of tadalafil on tumor blood flow, further experiments using chemical carcinogenesis models are necessary.

In conclusion, our non-clinical study revealed the possibility that co-administration of tadalafil with CA4DP can attenuate CA4DP-indicated cardiotoxicity, and that tadalafil

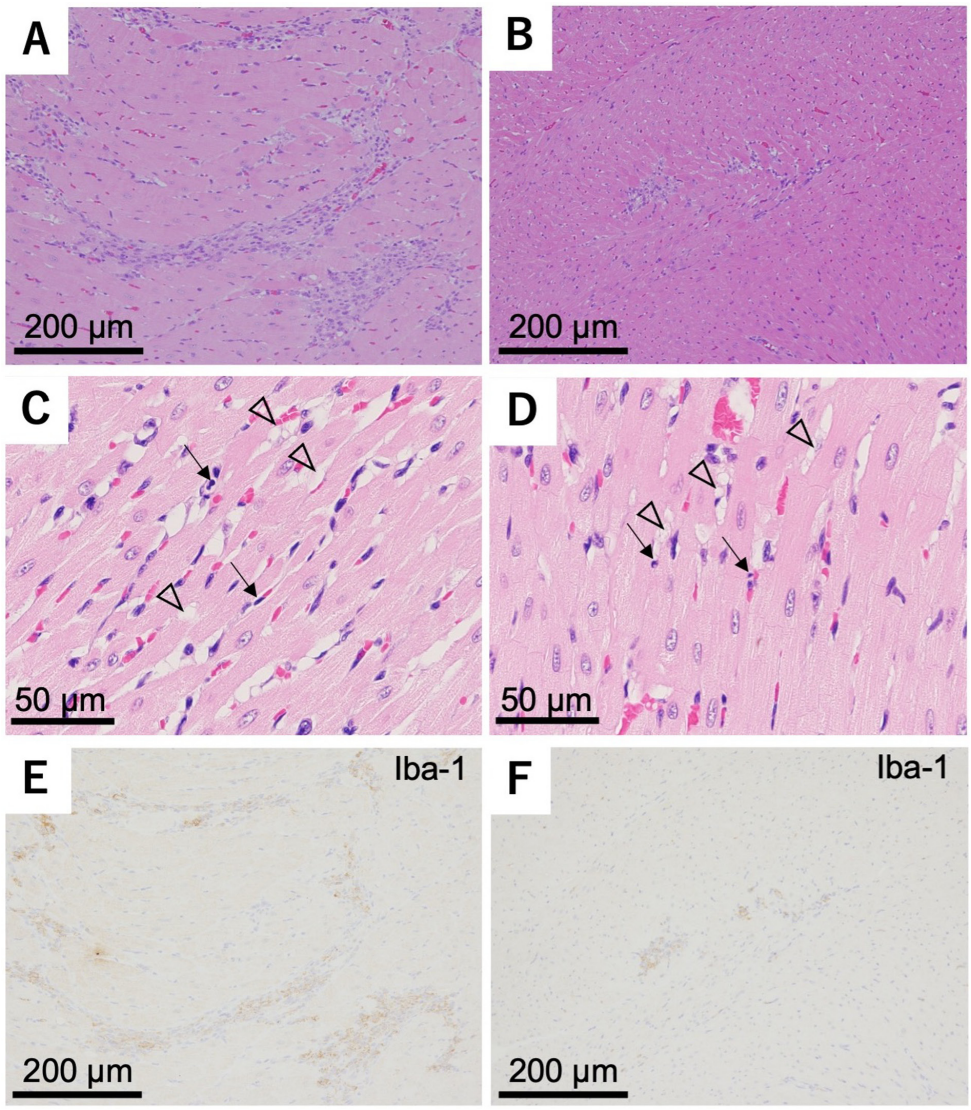

Fig. 2. Micrographs of myocardial lesions in rats 72 h after administration of CA4DP or CA4DP+tadalafil. [A] Multifocal necrosis of myocardial cells and infiltration of inflammatory cells were observed in hearts of rats administered CA4DP. [B] Necrosis of myocardial cells and infiltration of inflammatory cells were milder in rats that were administered CA4DP+tadalafil than in rats administered CA4DP alone. [C] Edema (arrowheads) around capillaries and pyknosis of capillary endothelial cells (arrow) were observed in hearts of rats administered CA4DP. [D] Edema (arrowheads) around capillaries and pyknosis of capillary endothelial cells (arrow) were milder in rats that were administered CA4DP+tadalafil than in rats that were administered CA4DP alone. [E] Macrophages were identified in myocardium of rats administered CA4DP. [F] Fewer macrophages were observed in myocardium of rats administered CA4DP+tadalafil than in rats administered CA4DP alone.

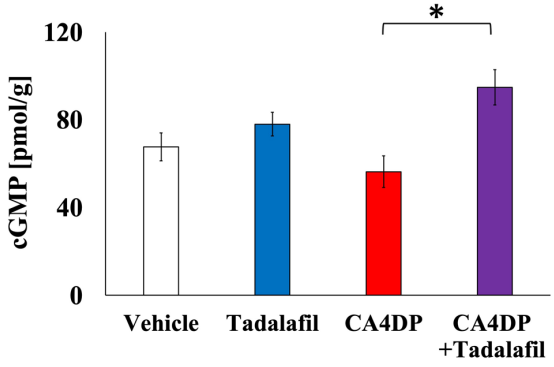

Fig. 3. cGMP concentration in left ventricular free wall of CA4DP/CA4DP+tadalafil-treated rats. cGMP concentration in rat ventricular free wall. cGMP concentration was lower in rats treated with CA4DP, but the difference was not significant when compared to rats treated with vehicle. Higher cGMP concentrations were observed in rats treated with tadalafil. Rats treated with CA4DP+tadalafil showed significantly higher cGMP concentrations than those treated with CA4DP alone. *$p<0.05$.

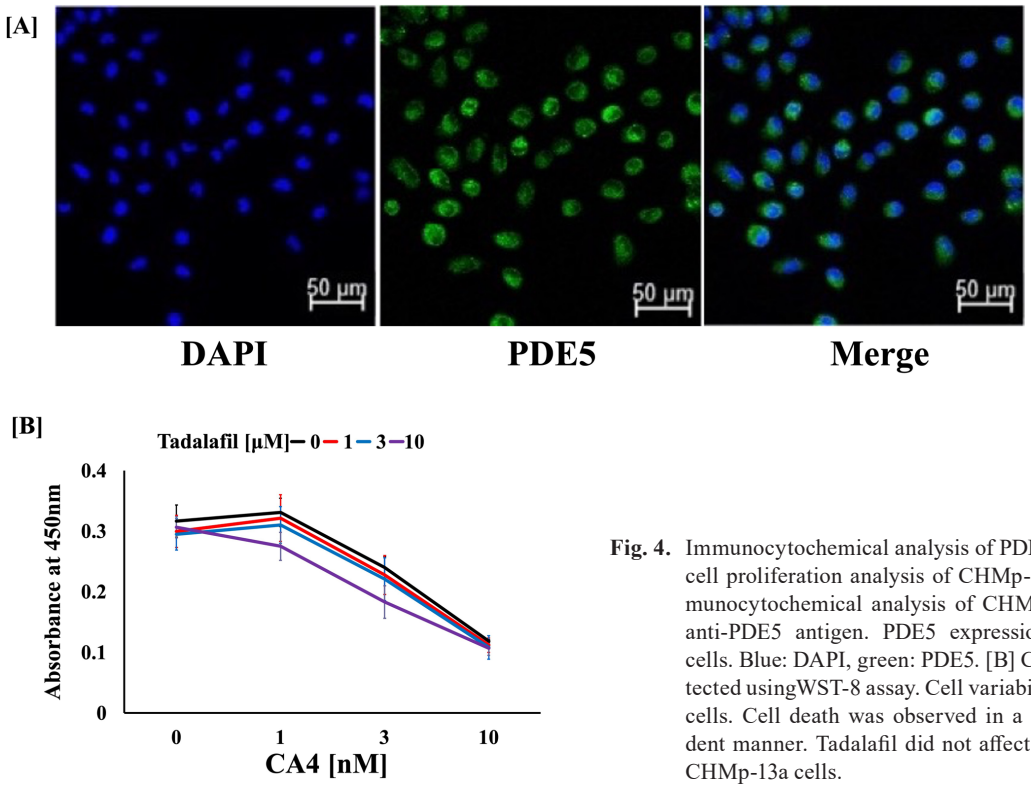

Fig. 4. Immunocytochemical analysis of PDE5 expression and cell proliferation analysis of CHMp-13a cells. [A] Immunocytochemical analysis of CHMp-13a cells using anti-PDE5 antigen. PDE5 expression in CHMp-13a cells. Blue: DAPI, green: PDE5. [B] Cell variability detected usingWST-8 assay. Cell variability of CHMp-13a cells. Cell death was observed in a CA4 dose-dependent manner. Tadalafil did not affect the variability of CHMp-13a cells.

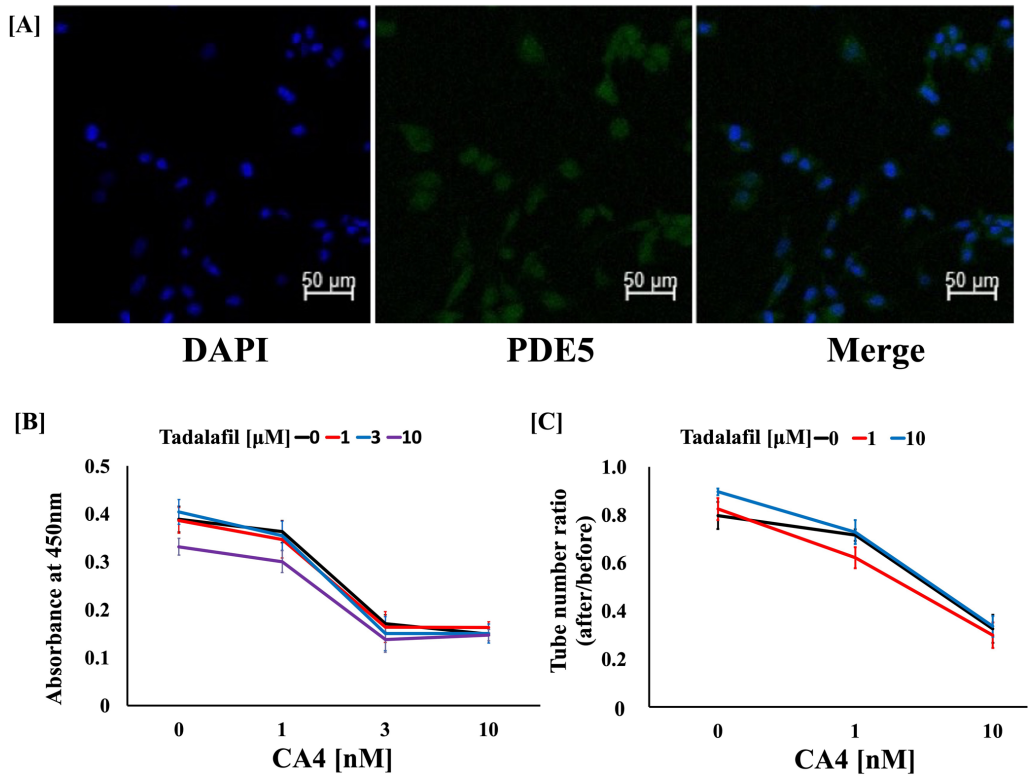

Fig. 5. Immunocytochemical analysis of PDE5 expression, cell proliferation analysis, and endothelial vascular formation analysis of HUVECs. [A] Immunocytochemical analysis of HUVECs. PDE5 expression was detected in HUVECs. Blue: DAPI, green: PDE5. [B] Cell variability in HUVEC. Cell variability decreased in an escalating CA4 dose-dependent manner. [C] Tube number ratio before and after addition of drugs to capillary-like structures formed by HUVECs. Disruption of capillary-like structures was observed in an escalating CA4 dose-dependent manner. No correlation was observed between tadalafil dose and tube number.

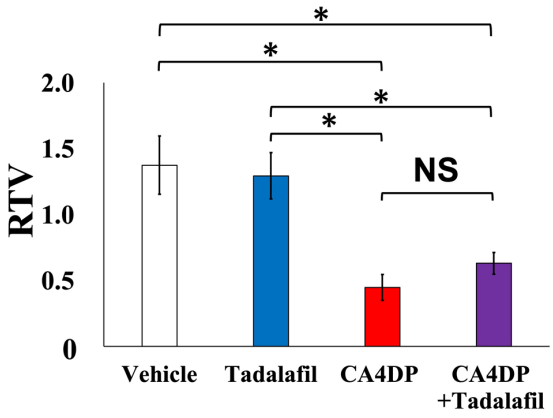

Fig. 6. Relative tumor volume in xenograft mice. Relative tumor volume (RTV) of subcutaneous tumors using CHMp-13a cells. Nude mice treated with CA4DP or CA4DP+tadalafil had lower RTVs than those treated with the vehicle or tadalafil alone. *p<0.05. NS: no significance.

did not affect the antitumor effect of CA4DP. These results may lead to the development of cancer chemotherapy with a low cardiotoxicity risk.

Disclosure of Potential Conflicts of Interest: Yoshiharu Tsuru is a part of Primetech Corp., which is the vendor of the instruments used for echocardiography in this study.

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