Full paper

1-Methyl-2-undecyl-4(1H)-quinolone, a derivative of quinolone alkaloid evocarpine, attenuates high phosphate-induced calcification of human aortic valve interstitial cells by inhibiting phosphate cotransporter PiT-1

CrossMark

Kazuhiko Seya [a], Ken-Ichi Furukawa [a, *], Mari Chiyoya [a, b], Zaiqiang Yu [a, b], Haruhisa Kikuchi [c], Kazuyuki Daitoku [b], Shigeru Motomura [a], Manabu Murakami [a], Yoshiteru Oshima [c], Ikuo Fukuda [b]

[a] Department of Pharmacology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan
[b] Department of Thoracic and Cardiovascular Surgery, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan
[c] Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba-yama, Aoba-ku, Sendai 980-8578, Japan

ARTICLE INFO

ABSTRACT

An abnormally high serum phosphate level induces calcific aortic stenosis (CAS), which is characterized by ectopic valve calcification and stenosis of the orifice area. Inhibition of ectopic calcification is a critical function of any internal medical therapy for CAS disease. The aim of the present study was to investigate the inhibitory effects of several derivatives of evocarpine, methanolic extracts from the fruits of *Evodia rutaecarpa* Bentham (Japanese name: Go-Shu-Yu) on the high phosphate-induced calcification of human aortic valve interstitial cells (HAVICs) obtained from patients with CAS. High phosphate (3.2 mM) concentrations significantly increased the calcification of HAVICs after 7 days of culture. This calcification was completely inhibited in the presence of sodium phosphonoformate (PFA), a selective inhibitor of the type III sodium-dependent phosphate cotransporter (PiT-1). PiT-1 contributes to phosphate uptake, resulting in calcification. 1-Methyl-2-undecyl-4(1H)-quinolone (MUQ; 30–300 nM), but not evocarpine or its derivatives dihydroevocarpine and 1-methyl-2-nonyl-4(1H)-quinolone, inhibited the high phosphate-induced HAVICs calcification in a concentration-dependent manner. Although all of the evocarpine derivatives attenuated alkaline phosphatase activity, only MUQ also decreased PiT-1 gene expression with cellular PiT-1 protein diminution. These results suggest that MUQ mitigated high phosphate-induced HAVICs calcification by inhibiting PiT-1 gene expression.

1. Introduction

Calcific aortic valve stenosis (CAS) is the most frequent heart valve disease in the elderly (1). Aortic valves from patients with CAS are characterized by massive fibrotic thickening of the valve leaflets and extensive focal ectopic calcification (2). Because the irreversible aortic valve calcification that occurs in CAS limits the internal treatment options, the most viable treatment is surgical aortic valve replacement, which is an extremely invasive procedure (3). Although several research groups have examined the mechanism of aortic valve calcification in CAS (4,5), no effective drug target for preventing the disease has been found, and no effective non-invasive treatment has been established.

CAS is an active process that may be correlated with several inflammatory factors (6). Inflammation is a prominent feature of aortic valve ectopic calcification and can be caused by endothelial dysfunction induced by atherosclerotic risk factors (7). Macrophages and T lymphocytes (8,9) release cytokines, such as transforming growth factor-β1 (10) and interleukin-1 (11), both of

which contribute to extracellular matrix formation, remodeling, and ectopic calcification. Recently, we demonstrated that tumor necrosis factor-α (TNF-α) also accelerates the calcification of aortic valves via the bone morphogenetic protein 2 (BMP2)-Dlx5 axis (12).

In addition to cytokines, abnormally high phosphate levels also appear to play an essential role in the process of ectopic calcification (13). Patients with chronic kidney disease (CKD), who have a hugely elevated risk of cardiovascular mortality compared with age-matched controls, show accelerated medial and intimal calcification, and this calcification rapidly progresses in patients on dialysis. The development of calcification in CKD patients is strongly linked to their dysregulated mineral metabolism, which is characterized by the long-term elevation of serum phosphate levels, as well as transient bouts of hypercalcemia (14). Recently, we demonstrated that high phosphate (3.2 mM) concentrations induced calcification in human aortic valve interstitial cells (HAVICs) at the cellular level (15). In addition to aging, hyperphosphatemia in CKD and dialyzed patients is a major risk factor for CAS. Thus, the development of new medical drugs that inhibit the calcification induced by hyperphosphatemia is eagerly awaited (16).

The quinolone alkaloid evocarpine, 1-methyl-2-[(Z)-8-tridecene-1-yl]-quinoline-4(1H)-one (Fig. 1), was first isolated from the fruits of the *Evodia rutaecarpa* plant (Japanese name: Go-Shu-Yu) (17). The dried fruits of *E. rutaecarpa* have been used for a long time in traditional Chinese medical practices for abdominal pain, dysentery, headache, and amenorrhea (18). This fruit has also been reported to have a transient hypertensive effect (19) and positive inotropic and chronotropic effects (20). Although there are several reports detailing the cardiovascular effects of evocarpine, the pharmacological effects of evocarpine and its derivatives on aortic valve ectopic calcification are still unclear.

The aim of the present study was to determine whether the methanolic extracts, evocarpine and its derivatives, from the fruits of *E. rutaecarpa* inhibit high phosphate-induced HAVIC calcification as a first step in the development of effective inhibitors for hyperphosphatemia-induced ectopic calcification of the aortic valve. The results show that 1-methyl-2-undecyl-4(1H)-quinolone (MUQ), but not evocarpine or the other derivatives, successfully inhibited HAVICs calcification by decreasing the gene expression of the type III sodium-dependent phosphate cotransporter (PiT-1).

2. Materials and methods

2.1. Materials

Penicillin, α-minimal essential medium (α-MEM), and streptomycin were purchased from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Nichirei Biosciences (Tokyo, Japan). The other analytical grade chemicals were obtained from Wako Pure Chemicals (Osaka, Japan). All of the chemicals used were of the highest purity commercially available. All of the chemicals were made fresh at sufficiently high concentrations such that only very small aliquots were added to the assay tubes or culture media. The final concentration of DMSO in the experimental tubes never exceeded 0.1% and had no effect on the cells or assays.

2.2. Isolation of evocarpine and its derivatives

The fruits (5 kg) of an *E. rutaecarpa*, which was purchased from Kinokuniya-kanyakkyoku Co., Ltd. (Tokyo, Japan), were extracted three times with methanol (20 L) at room temperature. The extract was then partitioned with ethyl acetate and water to obtain the ethyl acetate solubles (135 g). The ethyl acetate solubles were applied to a silica gel column for chromatography and were eluted with hexane-ethyl acetate and ethyl acetate-methanol solutions of increasing polarity to create fraction A (hexane-ethyl acetate (1:1) eluent, 6.34 g). Fraction A was further separated on a silica gel column using the chloroform−methanol solvent system to produce evocarpine (205 mg) and fraction B (chloroform−methanol (39:1) eluent, 162 mg). Fraction B was separated on an octadecyl-silica column using the water-acetonitrile solvent system to give 1-methyl-2-nonyl-4(1H)-quinolone (MNQ, 22 mg), 1-methyl-2-undecyl-4(1H)-quinolone (MUQ; 63 mg), and dihydroevocarpine (32 mg). The structures of these isolated compounds are shown in Fig. 1.

Data for evocarpine: colorless amorphous solid; ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.44 (1H, dd, $J = 8.0$, 1.7 Hz), 7.64 (1H, ddd, $J = 8.7$, 7.0, 1.7 Hz), 7.49 (1H, dd, $J = 8.7$, 0.9 Hz), 7.25 (1H, ddd, $J = 8.0$, 7.0, 0.9 Hz), 6.21 (1H, s), 5.30−5.40 (2H, m), 3.72 (3H, s), 2.69 (2H, t, $J = 7.8$ Hz), 2.01−2.05 (4H, m), 1.67 (2H, quint, $J = 7.9$ Hz), 1.40−1.45 (2H, m), 1.25−1.36 (10H, m), 0.89 (3H, t, $J = 7.1$ Hz); EIMS m/z 339 [M]$^+$ (29%), 310 (36%), 186 (96%), 173 (100%).

Data for 1-methyl-2-undecyl-4(1H)-quinolone (MUQ): colorless amorphous solid; ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.45 (1H, dd, $J = 8.0$,
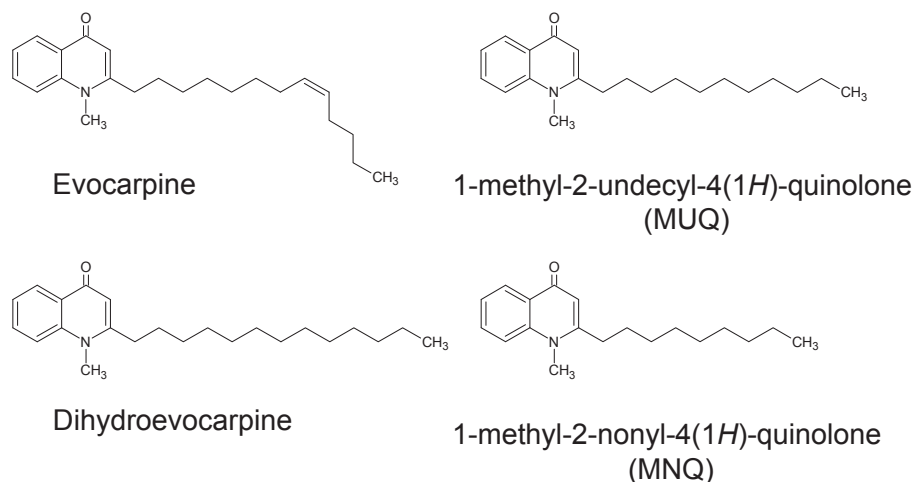

Fig. 1. Chemical structure of evocarpine and its derivatives. All of the compounds have a quinolone structure. Evocarpine includes a 2-tridecylidene group, dihydroevocarpine includes a 2-tridecyl group, 1-methyl-2-undecyl-4(1H)-quinolone (MUQ) includes a 2-undecyl group, and 1-methyl-2-nonyl-4(1H)-quinolone (MNQ) includes a 2-nonyl group.

1.7 Hz), 7.66 (1H, ddd, $J = 8.7$, 7.0, 1.7 Hz), 7.49 (1H, dd, $J = 8.7$, 0.9 Hz), 7.35 (1H, ddd, $J = 8.0$, 7.0, 0.9 Hz), 6.24 (1H, s), 3.74 (3H, s), 2.71 (2H, t, $J = 7.8$ Hz), 1.68 (2H, quint, $J = 7.7$ Hz), 1.39–1.44 (2H, m), 1.20–1.36 (14H, m), 0.88 (3H, t, $J = 7.1$ Hz); EIMS m/z 313 $[M]^+$ (17%), 186 (75%), 173 (100%).

Data for dihydroevocarpine: colorless amorphous solid; ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.45 (1H, dd, $J = 8.0$, 1.7 Hz), 7.66 (1H, ddd, $J = 8.7$, 7.0, 1.7 Hz), 7.50 (1H, dd, $J = 8.7$, 0.9 Hz), 7.35 (1H, ddd, $J = 8.0$, 7.0, 0.9 Hz), 6.24 (1H, s), 3.74 (3H, s), 2.71 (2H, t, $J = 7.8$ Hz), 1.68 (2H, quint, $J = 7.8$ Hz), 1.39–1.46 (2H, m), 1.20–1.37 (18H, m), 0.88 (3H, t, $J = 7.0$ Hz); EIMS m/z 341 $[M]^+$ (17%), 186 (89%), 173 (100%).

Data for 1-methyl-2-nonyl-4(1H)-quinolone (MNQ): colorless amorphous solid; ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.45 (1H, dd, $J = 8.0$, 1.7 Hz), 7.67 (1H, ddd, $J = 8.7$, 7.0, 1.7 Hz), 7.50 (1H, dd, $J = 8.7$, 0.9 Hz), 7.35 (1H, ddd, $J = 8.0$, 7.0, 0.9 Hz), 6.24 (1H, s), 3.73 (3H, s), 2.71 (2H, t, $J = 7.8$ Hz), 1.68 (2H, quint, $J = 7.8$ Hz), 1.39–1.46 (2H, m), 1.22–1.35 (10H, m), 0.88 (3H, t, $J = 7.0$ Hz); EIMS m/z 285 $[M]^+$ (16%), 186 (62%), 173 (100%).

2.3. HAVICs isolation and culture

Calcified aortic valves were obtained from six patients with CAS undergoing surgical valve replacement at the Hirosaki University Hospital after obtaining informed consent. The present study was approved by the institutional review board of Hirosaki University Hospital. The human aortic valve specimens were gently cut into pieces 2 ± 1 mm long. The HAVICs were isolated by collagenase digestion of the aortic valve pieces as previously described (15,21). The cells were cultured on a plastic dish in α-MEM containing 10% fetal bovine serum (FBS). The medium was changed every 3 days.

To induce calcification, after the HAVICs reached confluency, they were further cultured in the presence or absence of high phosphate concentrations (3.2 mM) for 7 days.

2.4. Identification of calcification

Calcium precipitation in the HAVICs was assessed by Alizarin Red S staining (22). Briefly, the matrix was washed with saline and incubated with 10% buffered formalin for 15 min. After three washes with purified water, the cultures were treated with Alizarin Red S solution at room temperature for 5 min. Excess Alizarin Red S solution was completely washed away using purified water to allow color development. The stained cells were examined under a digital camera (Nikon, Tokyo, Japan). Subsequently, the Alizarin Red S dye was released from the extracellular matrix by incubation with 100 mM aqueous cetylpyridinium chloride solution, and the amount of released dye was quantified by spectrophotometry at 550 nm (23).

2.5. ALP activity

After the cells were washed twice with phosphate buffered saline, the cellular proteins were solubilized with 0.5% Triton X-100 in saline and centrifuged. The supernatants were assayed for ALP activity using a commercially available kit (Laboassay™ ALP; Wako, Tokyo, Japan). One unit was defined as the activity producing 1 nmol of p-nitrophenol over 30 min. The supernatant protein concentrations were determined by Bradford's method. The change in activity was expressed as a ratio to the basal value on Day 0.

2.6. Gene expression

The total RNA was isolated from the cells using a QuickGene RNA cultured cell kit S (Fujifilm, Tokyo, Japan). An aliquot of total RNA was reverse transcribed to obtain cDNA using random primers. For real-time polymerase chain reaction (PCR), the cDNA was amplified (ABI PRISM 7000, Life Technologies, Carlsbad, CA) under the following reaction conditions: 40 cycles of PCR (95 °C for 15 s and then 60 °C for 1 min) after an initial denaturation step (95 °C for 1 min). The reaction volume was adjusted to 20 μL containing 3 μL of a 1:4 dilution of the first-strand reaction product, 0.6 μL of 10 μM specific forward and reverses primers, 0.4 μL of 50× ROX reference dye, 5.4 μL of pure water, and 10 μL of SYBR qPCR. The primers used to detect the expression of ALP, BMP2, PiT-1, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were designed in Primer Express ver. 2.0$^®$ (Life Technologies) and their sequences are shown in Table 1. Amplification of the housekeeping gene G3PDH served as a normalization standard. The real-time PCR data were represented as cycle threshold (Ct) levels and normalized to the individual G3PDH control Ct values. The relative gene expression values were calculated using the 2(-delta delta C(T)) method (24).

2.7. Western blot analysis of PiT-1

After the HAVICs reached confluency, these cells were pre-treated with or without MUQ (300 nM) for 2 h and subsequently cultured in the presence or absence of high phosphate concentrations (3.2 mM) for 7 days. Cells were lysed in 20 mM Tris–HCl, pH 7.4. After a Bradford protein assay, proteins (each 3 μg) were resolved by SDS/polyacrylamide gel electrophoresis, separated on 10% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes, Immobilon-FL (Millipore Corporation, Billerica, MA). Membranes were incubated with primary antibody against PiT-1 (mouse monoclonal antibody 1:500, Abcam, UK) or β-actin (rabbit polyclonal antibody 1:1000, Abcam, UK) and Alexa Fluor 680 goat anti-mouse or anti-rabbit secondary antibody (1:1000, Abcam, UK). Proteins were detected using the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE).

2.8. Statistical analysis

All statistical analyses were performed in KyPlot 5.0 software (Kyenslab, Tokyo, Japan). Differences among groups were tested using ANOVAs with the Student–Newman–Keuls post hoc correction procedure. Values are presented as means ± S.E.M. p-values less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of 1-methyl-2-undecyl-4(1H)-quinolone (MUQ) on high phosphate-induced calcification of HAVICs

The HAVICs obtained from patients with calcific aortic valve stenosis (n = 6; age: 64.3 ± 5.6 years) were cultured in the presence or absence of high inorganic phosphate (3.2 mM) after reaching confluence. Marked calcification of the HAVICs occurred in the

Table 1
Primers used for the quantitative real-time PCR.

Gene symbol	Genbank accession no.	Sequences (5′-3′)
ALP	NM_000478	Forward: agaaccccaaaggcttcttc
		Reverse: cttggcttttccttcatggt
BMP2	NM_001200	Forward: cggactgcggtctcctaa
		Reverse: ggaagcagcaacgctagaag
PiT-1	NM_005415	Forward: gatgtcttggttcgtgtccc
		Reverse: ggaactggatctgccttatgga
G3PDH	NM_002046	Forward: tgcaccaccaactgcttagc
		Reverse: ggcatggactgtggtcatgag

presence of high inorganic phosphate for 7 days of culture. The magnitude of calcification was significantly mitigated by sodium phosphonoformate (PFA) (25), an inhibitor of PiT-1 (Fig. 2A).

Next, we investigated whether evocarpine and its derivatives inhibited high phosphate-induced HAVICs calcification. As shown in Figs. 2B and 3A, MUQ inhibited calcification in a concentration-dependent manner (30−300 nM), but evocarpine and the other derivatives did not inhibit the high phosphate-induced HAVICs calcification. Spectrophotometric quantification of the amount of released Alizarin Red S dye from the calcified areas confirmed that the high phosphate-induced HAVICs calcification was significantly inhibited by MUQ (300 nM, Fig. 3B).

3.2. Gene expression of calcification markers show MUQ inhibition of calcification in HAVICs

We assessed the gene expression of several calcification markers to identify the intracellular signaling pathway leading to the inhibitory effect of MUQ on high phosphate-induced HAVICs calcification. The gene expression of PiT-1, but not BMP2, was significantly decreased in the presence of MUQ at Day 7 compared with those in the non-treatment group (Fig. 4A, B). Although MUQ did not affect the ALP gene expression, it significantly inhibited the
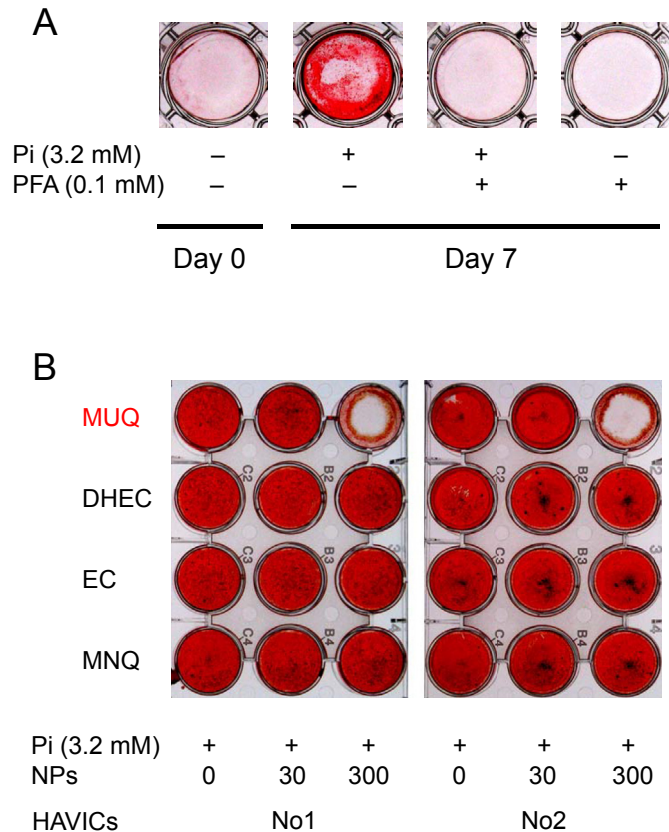

Fig. 2. High phosphate (3.2 mM)-induced calcification of HAVICs and the inhibitory effects of the evocarpine derivatives. HAVICs, obtained from patients with CAS, were cultured in α-MEM containing 10% FBS. After reaching confluence (Day 0), the HAVICs were further cultured for 7 days (Day 7). (A) Representative images of Alizarin Red S staining of the high phosphate (3.2 mM)-induced calcification of HAVICs. Sodium phosphonoformate (PFA) was used as a selective PiT-1 inhibitor. (B) Representative Alizarin Red S staining images from two different HAVIC populations. The inhibitory effect of MUQ, dihydroevocarpine (DHEC), evocarpine (EC), and MNQ on the high phosphate-induced HAVICs calcification were assessed.
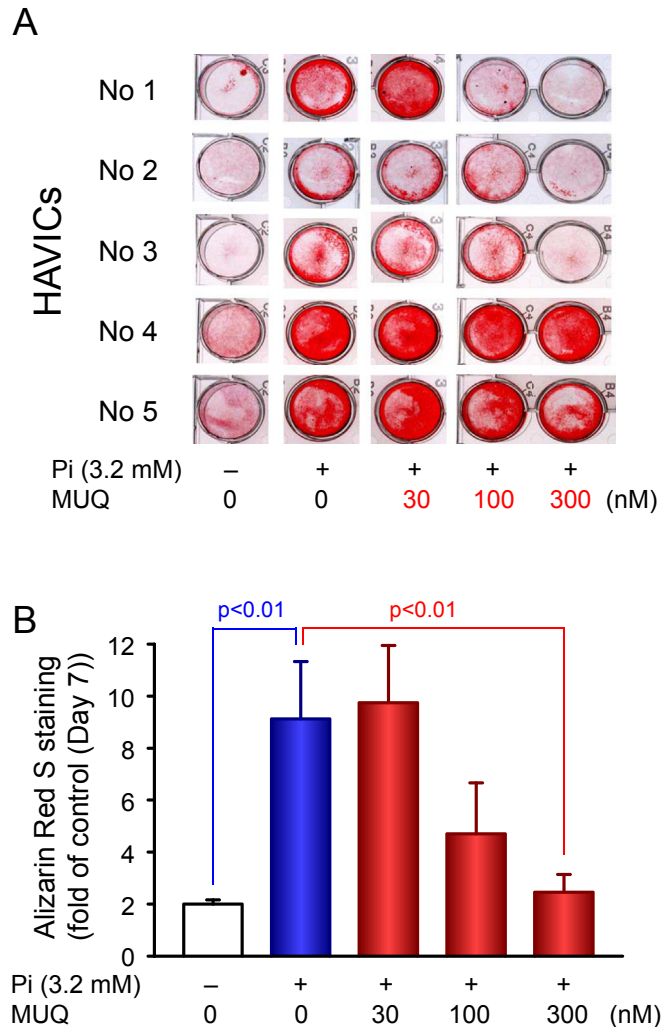

Fig. 3. Inhibitory effects of MUQ on high phosphate-induced calcification of HAVICs. HAVICs, obtained from patients with CAS, were cultured in α-MEM containing 10% FBS. After reaching confluence (Day 0), the HAVICs were further cultured for 7 days (Day 7). (A) Representative images of Alizarin Red S staining from five different HAVIC populations. (B) Quantification of the Alizarin Red S staining on Day 7 via extraction with cetylpyridinium chloride. The amount of released dye was quantified by spectrophotometry at 550 nm. All of the ratios were normalized to the control value on Day 7. White bar: high phosphate (−); blue bar: high phosphate (+); red bars: high phosphate (+) and MUQ (30−300 nM). Bars represent the mean ± S.E.M. (n = 5).

ALP activity, which was increased in the presence of high phosphate (Fig. 4C, D).

3.3. The inhibitory effects of MUQ on PiT-1 gene expression and ALP activity compared with those of evocarpine and its other derivatives

To determine the mechanism of MUQ inhibition of high phosphate-induced HAVICs calcification, we compared the PiT-1 gene expression and ALP activity of HAVICs after treatment with MUQ, evocarpine, and the other derivatives. Evocarpine and its other derivatives in the presence of high phosphate significantly inhibited ALP activity in a similar manner as MUQ (Fig. 5A).

In the absence of high phosphate, MNQ significantly decreased the PiT-1 gene expression. However, in the presence of high phosphate, none of the other evocarpine derivatives decreased PiT-1 expression (Fig. 5B). MUQ alone has a tendency to decrease this gene expression in spite of non-significant difference ($p = 0.14$ vs.
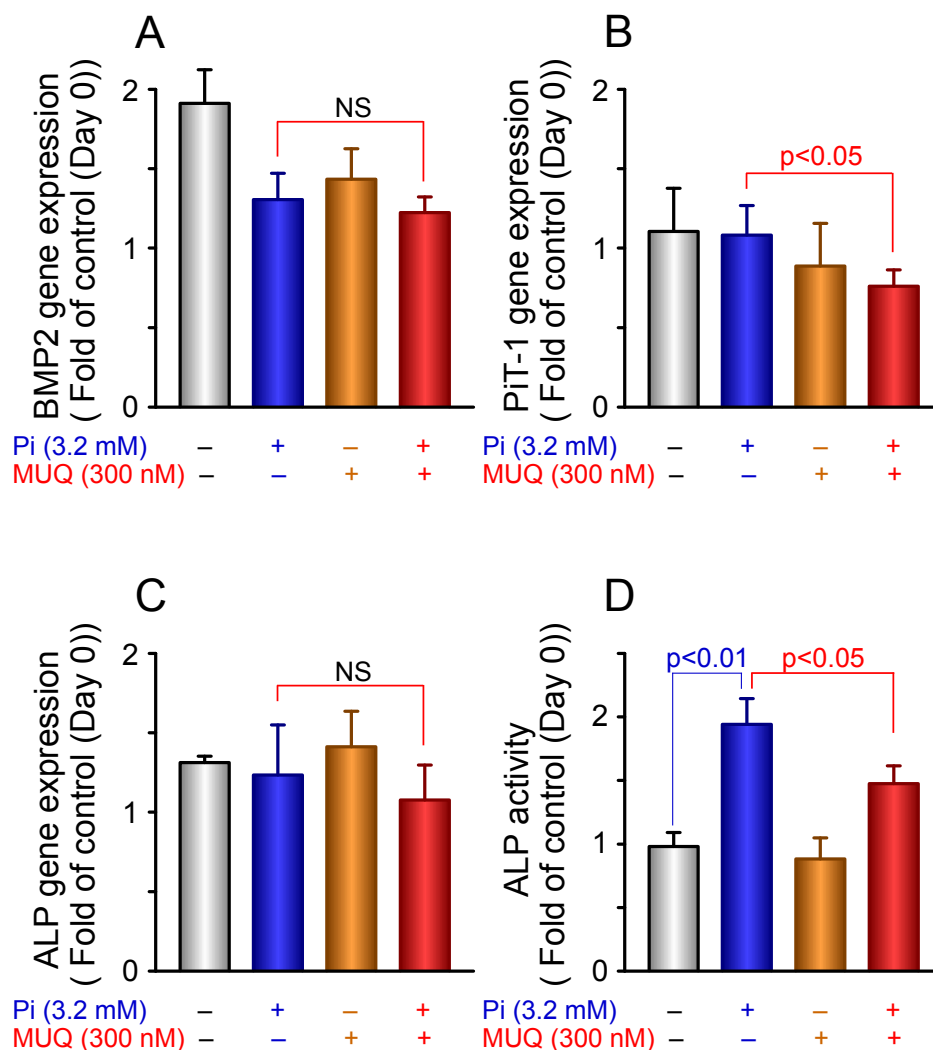
Fig. 4. Effects of MUQ on the mRNA expression of calcification-related genes and the ALP activity in HAVICs cultured in high phosphate medium. HAVICs, obtained from patients with CAS, were cultured in α-MEM containing 10% FBS. After reaching confluence (Day 0), the HAVICs were further cultured in high phosphate medium (3.2 mM) in the presence or absence of MUQ (300 nM) for 7 days (Day 7). BMP2 (A), PiT-1 (B), and ALP (C) gene expressions in HAVICs were measured on Day 7. All of the ratios were normalized to the control group on Day 0. The gene expression levels were normalized to the G3PDH gene expression. Bars represent the mean ± S.E.M. (n = 5). (D) ALP activity on Day 7. The cells were solubilized with 0.5% Triton X-100 in saline, and the supernatants were assayed for ALP activity using a commercially available kit (Laboassay™ ALP). One unit was defined as the activity producing 1 nmol of *p*-nitrophenol over 30 min. All of the ratios were normalized to the control group on Day 0. Bars represent the mean ± S.E.M. (n = 5).

high Pi). These results suggest that the inhibitory effect of MUQ on high phosphate-induced HAVICs calcification occurs by decreasing PiT-1 gene expression.

3.4. Western blot analysis of PiT-1 in the presence of MUQ in HAVICs

To prove the regulatory effect of MUQ on PiT-1 expression in HAVICs, we further measured the PiT-1 protein expression level on high phosphate-induced HAVICs calcification by Western blot analysis. MUQ significantly decreased in the PiT-1 levels in HAVICs in the presence or absence of high phosphate at Day 7 compared with that in the high phosphate group (Fig. 6).

4. Discussion

Ectopic calcification occurs frequently in the aortic valves of CAS patients. To develop a non-invasive treatment for such calcification, such as a drug, it is necessary to first elucidate the detailed mechanism of aortic valve calcification. Abnormally high serum

phosphate levels appear to play an important role in the calcification process (14). In the present study, exposure to high phosphate concentrations induced calcification of the HAVICs at the cellular level. This calcification was strongly inhibited by the presence of PFA, a PiT-1 inhibitor. Further, we confirmed that MUQ, isolated from the fruits of *E. rutaecarpa*, inhibited high phosphate-induced HAVICs calcification. Finally, we demonstrated that MUQ decreased not only the gene expression but also cellular protein level of PiT-1.

There is a report that MUQ inhibits monoamine oxidase B (IC_{50} value: 15.3 μM) in mouse brain responsible for the oxidative catabolism of catecholamine which act as neurotransmitters in the nervous system (26). However, the concentration of MUQ we used was very low (up to 300 nM) and may not affect the activities of these monoamine oxidases.

Serum phosphorus levels are normally maintained in the range of 2.5–4.5 mg/dl. However, in the present study, the mean serum phosphorus level of the CAS patients (n = 6) was high (4.8 ± 0.4 mg/dL). Inorganic phosphorus is a critical mineral that is mostly found in bone (85% of total body phosphorus) and is required for high-

A

ALP activity (Fold of control (Day 0))

B
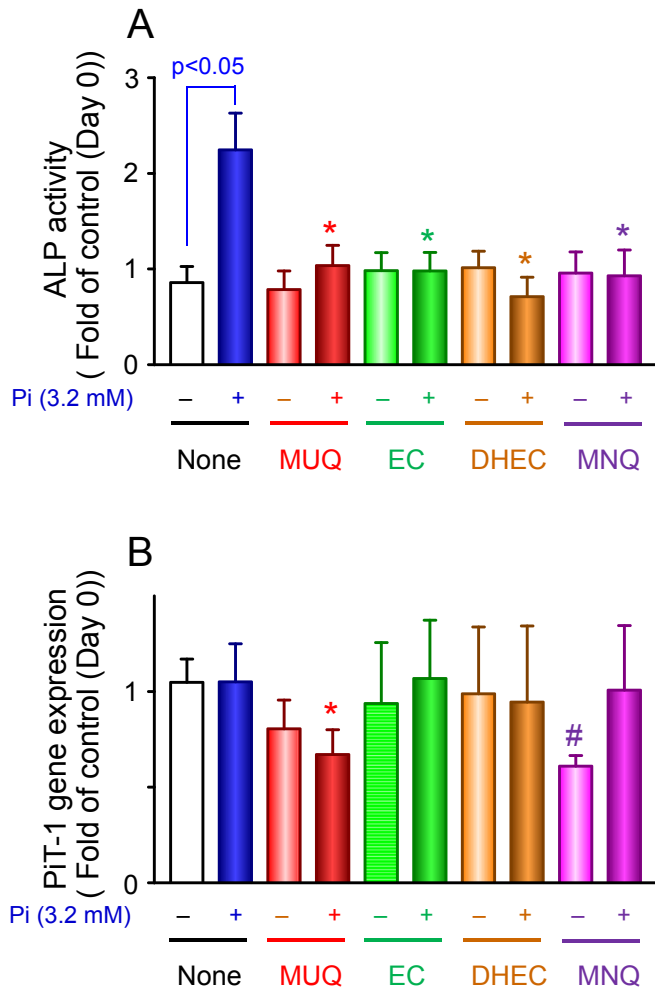
PiT-1 gene expression (Fold of control (Day 0))

Fig. 5. Effects of MUQ and other evocarpine derivatives on the mRNA expression of calcification-related genes and the ALP activity in HAVICs cultured in high phosphate medium. HAVICs, obtained from patients with CAS, were cultured in α-MEM containing 10% FBS. After reaching confluence (Day 0), the HAVICs were further cultured in high phosphate medium (3.2 mM) in the presence or absence of MUQ and other evocarpine derivatives (300 nM) for 7 days (Day 7). (A) ALP activity on Day 7. The cells were solubilized with 0.5% Triton X-100 in saline, and the supernatants were assayed for ALP activity using a commercially available kit (Laboassay™ ALP). One unit was defined as the activity producing 1 nmol of p-nitrophenol over 30 min. All of the ratios were normalized to the control group on Day 0. Bars represent the mean ± S.E.M. (n = 5). *: p < 0.05 vs. high phosphate (+) on Day 7. (B) PiT-1 gene expression in HAVICs was measured on Day 7. All of the ratios were normalized to the control group on Day 0. The gene expression levels were normalized to the G3PDH gene expression. Bars represent the mean ± S.E.M. (n = 5). *: $p < 0.05$ vs. high phosphate (+) at Day 7. #: P < 0.05 vs. control at Day 7.

energy phosphate bonds, intracellular signaling, and pH buffering (27). The kidney is the major regulator of phosphate homeostasis, whereby 70% of phosphate reabsorption in the proximal convoluted tubule occurs via a sodium-dependent phosphate cotransporter. Two other phosphate transporters, PiT-1 and PiT-2, also contribute to renal phosphate reabsorption (28). In general, high phosphorus levels mainly induce cell calcification via the activation of Pit-1 (29), which is also supported by our present results. Further, we confirmed that MUQ did not affect the PiT-2 gene expression (K. Seya et al., unpublished data). Thus, the inhibition of PiT-1 activity is useful for preventing ectopic calcification caused by hyperphosphatemia.

The pharmacological effects of MUQ on HAVICs include not only the inhibition of PiT-1, but also of ALP activity. ALP is well-known
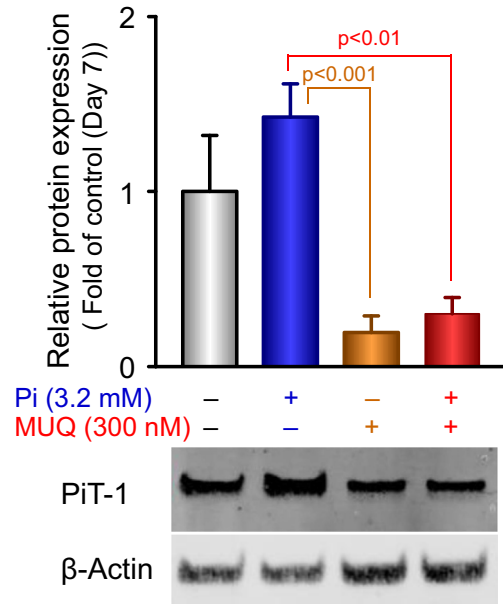
Fig. 6. Effects of MUQ on the PiT-1 protein expression of calcification-related genes in HAVICs cultured in high phosphate medium. HAVICs, obtained from patients with CAS, were cultured in α-MEM containing 10% FBS. After reaching confluence, the HAVICs were further cultured in high phosphate medium (3.2 mM) in the presence or absence of MUQ (300 nM) for 7 days (Day 7). PiT-1 protein expression in HAVICs was measured on Day 7. All of the ratios were normalized to the control group on Day 7. The protein expression levels were normalized to the β-actin protein expression. Lane 1, non-treatment (control); lane 2, high phosphate (3.2 mM); lane 3, MUQ (300 nM); lane 4, high phosphate (3.2 mM) in the presence of MUQ (300 nM). Bars represent the mean ± S.E.M. (n = 5).

for its contributions to aortic valve calcification. However, ALP gene expression was not affected by MUQ, and neither was the expression of BMP2, an important upstream factor for ALP. Further, although evocarpine and its derivatives except MUQ also inhibited the high phosphate-induced increase in ALP activity, they did not mitigate the high phosphate-induced HAVICs calcification. In another study, MUQ did not inhibit the TNF-α-induced calcification of HAVICs via BMP2-ALP signaling (data not shown). Thus, the high phosphate-induced ALP activation may be caused by a compensatory reaction and may not contribute to the HAVICs calcification. On the other hand, in the high phosphate condition, none of these compounds besides MUQ altered the PiT-1 gene expression. These results suggest that the inhibitory effects of MUQ on high phosphate-induced HAVICs calcification are driven by the suppression of inorganic phosphate uptake into HAVICs via PiT-1. Although MNQ alone decreased the PiT-1 gene expression, this effect may be weak in the presence of high phosphate.

Unlike MUQ, the gene expression of PiT-1 by HAVICs was not decreased by evocarpine or its other derivatives. However, these observations do not explain why MUQ, but not the other compounds, decreases the PiT-1 gene expression in HAVICs. The structural differences between MUQ and the other evocarpine derivatives are limited to functional groups at the 2-position. Imramovsky et al. reported that the acetylcholinesterase-inhibiting activity of salicylanilide N-alkylcarbamates was largely varied depending on the side chain (30). In the present study, the undecyl group of the MUQ at the 2-position may affect the expression of the PiT-1 gene. However, further study of the role of the undecyl group at the 2-position of MUQ is needed to clarify the molecular mechanism of the inhibition of high phosphate-induced HAVICs calcification.

No effective drugs have been identified that inhibit the ectopic calcification of aortic valve (31). Son et al. reported that statins

protected human aortic smooth muscle cells and HAVICs from phosphate-induced calcification by inhibiting apoptosis via restoration of the Gas6-Axl pathway (32). However, a critique of the results of the "JUPITER trial" (REF) stated that the results of the trial did not support the use of statins for the primary prevention of cardiovascular diseases and raised troubling questions concerning the role of commercial sponsors (33). Further, simvastatin and ezetimibe did not reduce the composite outcome of combined aortic valve events and ischemic events in patients with aortic stenosis. These therapies reduced the incidence of ischemic cardiovascular events, but not the incidence of events related to aortic valve stenosis (34). The results of the present study suggest that MUQ may be a candidate for development as a new medical entity that can inhibit ectopic calcification in aortic stenosis caused by hyperphosphatemia.

One limitation of the present study is that the detailed molecular mechanism of the action of MUQ was not elucidated. Although PFA inhibits PiT-1 activity at the experimental level, its molecular mechanism has also not yet been elucidated (35). In future studies, we should analyze the structure-activity relationships among MUQ and other evocarpine derivatives and the biophysical interactions between MUQ and PiT-1. That information will allow us to develop a more active compound through chemical modification of the quinolone alkyl group of MUQ.

In conclusion, we demonstrated that MUQ (30–300 nM), isolated from the fruits of *E. rutaecarpa*, inhibited high phosphate (3.2 mM)-induced HAVICs calcification in a concentration-dependent manner. We also showed that the inhibitory effect of MUQ is caused by the attenuation of both gene expression and cellular protein level of PiT-1. We hope that these results will help to clarify the molecular mechanism of the inhibitory effect of MUQ and assist the development of new therapies for ectopic calcification in aortic stenosis.

Conflicts of interest

The authors have no financial conflicts of interest to declare.

Acknowledgments

References

(1) Chikwe J, Walther A, Pepper J. The surgical management of aortic valve disease. Br J Cardiol. 2003;10:453–461.

(2) Mohler III ER. Mechanisms of aortic valve calcification. Am J Cardiol. 2004;94: 1396–1402.

(3) Iung B, Baron G, Butchart BG, Delahaye F, Gohlke-Bärwolf C, Levang OW, et al. A prospective survey of patients with valvular heart disease in Europe: the Euro Heart Survey on valvular heart disease. Eur Heart J. 2003;24:1231–1243.

(4) Rajamannan NM, Subramaniam M, Rickard D, Stock SR, Donovan J, Springett M, et al. Human aortic valve calcification is associated with an osteoblast phenotype. Circulation. 2003;107:2181–2184.

(5) Clark-Greuel JN, Connolly JM, Sorichillo E, Narula NR, Rapoport HS, Mohler III ER, et al. Transforming growth factor-β1 mechanisms in aortic valve calcification: increased alkaline phosphatase and related events. Ann Thorac Surg. 2007;83:946–953.

(6) Mohler 3rd ER, Chawla MK, Chang AW, Vyavahare N, Levy RJ, Graham L, et al. Identification and characterization of calcifying valve cells from human and canine aortic valves. J Heart Valve Dis. 1999;8:254–260.

(7) Goldbarg SH, Elmariah S, Miller MA, Fuster V. Insights into degenerative aortic valve disease. J Am Coll Cardiol. 2007;50:1205–1213.

(8) Freeman RV, Otto CM. Spectrum of calcific aortic valve disease, pathogenesis, disease progression, and treatment strategies. Circulation. 2005;111: 3316–3326.

(9) Wallby L, Janerot-Sjoberg B, Steffensen T, Broqvist M. T lymphocyte infiltration in non-rheumatic aortic stenosis: a comparative descriptive study between tricuspid and bicuspid aortic valves. Heart. 2002;88:348–351.

(10) Jian B, Narula N, Li QY, Mohler III ER, Levy RJ. Progression of aortic valve stenosis: TGF-β1 is present in calcified aortic valve cusps and promotes aortic valve interstitial cell calcification via apoptosis. Ann Thorac Surg. 2003;75: 457–466.

(11) Kaden JJ, Dempfle CE, Grobholz R, Tran HT, Kilic R, Sarikoc A, et al. Interleukin-1 beta promotes matrix metalloproteinase expression and cell proliferation in calcific aortic valve stenosis. Atherosclerosis. 2003;170:205–211.

(12) Yu Z, Seya K, Daitoku K, Motomura S, Fukuda I, Furukawa K-I. Tumor necrosis factor-α accelerates the calcification of human aortic valve interstitial cells obtained from patients with calcific aortic valve stenosis via the BMP2-Dlx5 pathway. J Pharmacol Exp Med. 2011;337:16–23.

(13) Linefsky JP, O'Brien KD, Katz R, de Boer IH, Barasch E, Jenny NS, et al. Association of serum phosphate levels with aortic valve sclerosis and annular calcification. J Am Coll Cardiol. 2011;58:291–297.

(14) Johnson RC, Leopold JA, Loscalzo J. Vascular calcification: pathobiological mechanisms and clinical implications. Circ Res. 2006;99:1044–1059.

(15) Nomura A, Seya K, Yu Z, Daitoku K, Motomura S, Murakami M, et al. CD34-negative mesenchymal stem-like cells may act as the cellular origin of human aortic valve calcification. Biochem Biophys Res Commun. 2013;440: 780–785.

(16) Ureña P, Malergue MC, Goldfarb B, Prieur P, Guédon-Rapoud C, Pétrover M. Evolutive aortic stenosis in hemodialysis patients: analysis of risk factors. Nephrologie. 1999;20:217–225.

(17) Tschesche R, Werner W. Evocarpine, a new alkaloid from *Evodia rutaecarpa*. Tetrahedron. 1967;23:1873–1881.

(18) Liao JF, Chen CF, Chow SY. Pharmacological studies of Chinese herbs. (9) Pharmacological effects of *Evodiae fructus*. J Formos Med Assoc. 1981;79: 30–38.

(19) Yamahara J, Kobayashi G, Matsuda H, Fujimura H. The vasorelaxant effect of evocarpine in isolated aortic strips: mode of action. Eur J Pharmacol. 1988;155:139–143.

(20) Chen CF, Chen SM, Lin MT, Chow SY. In vivo and in vitro studies on the mechanism of the cardiovascular effect of Wu-Chu-Yu (*Evodiae furctus*). Am J Chin Med. 1981;1:39–47.

(21) Seya K, Yu Z, Kanemaru K, Daitoku K, Akemoto Y, Shibuya H, et al. Contribution of bone morphogenetic protein-2 to aortic valve calcification in aged rat. J Pharmacol Sci. 2011;115:8–14.

(22) Puchtler H, Meloan SN, Terry MS. On the history and mechanism of alizarin and alizarin red S stains for calcium. J Histochem Cytochem. 1969;17: 110–124.

(23) Stanford CM, Jacobson PA, Eanes ED, Lembke LA, Midura RJ. Rapidly forming apatitic mineral in an osteoblastic cell line (UMR 106-01 BSP). J Biol Chem. 1995;270:9420–9428.

(24) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2($-\Delta\Delta$C(T)) method. Methods. 2001;25: 402–408.

(25) Szczepanska-Konkel M, Yusufi AN, VanScoy M, Webster SK, Dousa TP. Phosphonocarboxylic acids as specific inhibitors of Na$^+$-dependent transport of phosphate across renal brush border membrane. J Biol Chem. 1986;261: 6375–6383.

(26) Lee ML, Hwang BY, Lee SA, Oh GB, Choi WH, Hong SS, et al. 1-Methyl-2-undecyl-4(1*H*)-quinolone as an irreversible and selective inhibitor of type B monoamine oxidase. Chem Pharm Bull. 2003;51:409–411.

(27) Lau WL, Festing MH, Giachelli CM. Phosphate and vascular calcification: emerging role of the sodium-dependent phosphate cotransporter PiT-1. Thromb Haemost. 2010;104:464–470.

(28) Villa-Bellosta R, Ravera S, Sorribas V, Stange G, Levi M, Murer H, et al. The Na$^+$-Pi cotransporter PiT-2 (SLC20A2) is expressed in the apical membrane of rat renal proximal tubules and regulated by dietary Pi. Am J Physiol Renal Physiol. 2009;296:F691–F699.

(29) Crouthamel MH, Lau WL, Leaf EM, Chavkin N, Wallingford MC, Peterson DF, et al. Sodium-dependent phosphate cotransporters and phosphate-induced calcification of vascular smooth muscle cells: redundant roles for PiT-1 and PiT-2. Arterioscler Thromb Vasc Biol. 2013;33:2625–2632.

(30) Imramovsky A, Stepankova S, Vanco J, Pauk K, Monreal-Ferriz J, Vinsova J, et al. Acetylcholinesterase-inhibiting activity of salicylanilide N-alkylcarbamates and their molecular docking. Molecules. 2012;17:10142–10158.

(31) Dweck MR, Boon NA, Newby DE. Calcific aortic stenosis. A disease of the valve and the myocardium. J Am Coll Cardiol. 2012;60:1854–1863.

(32) Son B-K, Kozaki K, Iijima K, Eto M, Kojima T, Ota H, et al. Statins protect human aortic smooth muscle cells from inorganic phosphate-induced calcification by restoring Gas6-Axl survival pathway. Circ Res. 2006;98: 1024–1031.

(33) Lorgeril M, Salen P, Abramson J, Dodin S, Hamazaki T, Kostucki W, et al. Cholesterol lowering, cardiovascular diseases, and the rosuvastatin-JUPITER controversy. Arch Intern Med. 2010;170:1032–1036.

(34) Rossebø AB, Pedersen TR, Boman K, Brudi P, Chambers JB, Egstrup K, et al. Intensive lipid lowering with simvastatin and ezetimibe in aortic stenosis. N Engl J Med. 2008;359:1343–1356.

(35) Villa-Bellosta R, Sorribas V. Response to 'On vascular calcification prevention with phosphonoformate and bisphosphonates'. Kidney Int. 2009;75: 1355–1356.