Dihydromyricetin ameliorates vascular calcification in chronic kidney disease by targeting AKT signaling

Liyun Feng\textsuperscript{a,b,c,1}, Dongdong Que\textsuperscript{a,b,c,1}, Zehua Li\textsuperscript{a,b,c,1}, Xinglong Zhong\textsuperscript{a,b,c}, Jing Yan\textsuperscript{a,b,c}, Jintao Wei\textsuperscript{a,b,c}, Xiuli Zhang\textsuperscript{a,b,c}, Pingzhen Yang\textsuperscript{a,b,c}, Caiwen Ou\textsuperscript{a,b,c,*}, and Minsheng Chen\textsuperscript{a,b,c,*}

\textsuperscript{a}Department of Cardiology, Laboratory of Heart Center, Zhujiang Hospital, Southern Medical University, Guangzhou, PR China

\textsuperscript{b}Guangdong Provincial Biomedical Engineering Technology Research Center for Cardiovascular Disease, Guangzhou, PR China

\textsuperscript{c}Sino-Japanese Cooperation Platform for Translational Research in Heart Failure, Zhujiang Hospital, Southern Medical University, Guangzhou, PR China

\textsuperscript{*}Corresponding author: Minsheng Chen and Caiwen Ou

Department of Cardiology, Laboratory of Heart Center, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong 510280, PR China.

E-mail address: minshengsmu@163.com (Minsheng Chen); oucaiwen@smu.edu.cn (Caiwen Ou)

\textsuperscript{1}These authors contributed equally to this work.

Key words: Dihydromyricetin; Vascular calcification; Chronic kidney disease; AKT
Abstract

Vascular calcification is highly prevalent in chronic kidney disease (CKD), and characterized by trans-differentiation from contractile vascular smooth muscle cells (VSMCs) into an osteogenic phenotype. However, no effective and therapeutic option to prevent vascular calcification is yet available. Dihydromyricetin (DMY), a bioactive flavonoid isolated from Ampelopsis grossedentata, has been found to inhibit VSMCs proliferation and the injury-induced neointimal formation. However, whether DMY has an effect on osteogenic differentiation of VSMCs and vascular calcification is still unclear. In this study, we sought to investigate the effect of DMY on vascular calcification in CKD and the underlying mechanism. DMY treatment significantly attenuated calcium/phosphate-induced calcification of rat and human VSMCs in a dose-dependent manner, as shown by alizarin red S staining and calcium content assay, associated with down-regulation of osteogenic markers including type I collagen (COL I), RUNX2, BMP2 and osteocalcin (OCN). These results were further confirmed in aortic rings ex vivo. Moreover, DMY ameliorated vascular calcification in rats with CKD. Additionally, we found that AKT signaling was activated during vascular calcification, whereas significantly inhibited by DMY administration. DMY treatment significantly reversed AKT activator-induced vascular calcification. Furthermore, inhibition of AKT signaling efficiently attenuated calcification, which was similar to that after treatment with DMY alone, and DMY had a better inhibitory effect on calcification as compared to AKT inhibitor. The present study demonstrated that DMY has a potent inhibitory role in vascular calcification partially by inhibiting
AKT activation, suggesting that DMY may act as a promising therapeutic candidate for patients suffering from vascular calcification.

**Introduction**

Vascular calcification is a process of hydroxyapatite crystals deposits in the medial or intimal layers of arteries [1]. It is an important risk factor for cardiovascular disease and mostly occurs among the elderly, atherosclerosis, diabetes mellitus, and chronic kidney disease (CKD) patients [2,3]. Initially, vascular calcification was regarded as a passive process of calcium and phosphate deposition. However, accumulating evidence has suggested that vascular calcification is an active cell-mediated regulated process sharing similarities with bone formation [4,5]. Vascular smooth muscle cells (VSMCs) play a pivotal role in mediating vascular calcification by switching from contractile to osteogenic phenotype [6]. This phenotype switch is recognized as osteogenic differentiation. Molecules including runt-related transcription factor 2 (RUNX2), bone morphogenetic protein 2 (BMP2), osteocalcin (OCN), type I collagen (COL I), Osterix and alkaline phosphatase (ALP) are widely used as markers of osteogenic differentiation [7-9]. A variety of triggers and signaling pathways have been identified being involved in regulation of vascular calcification, but the exact molecular mechanisms of vascular calcification still remain elusive [10]. Consequently, no effective and convincing therapeutic strategies to prevent the progression of vascular calcification are yet available [11].

Dihydromyricetin (DMY), a bioactive flavonoid isolated from *Ampelopsis*
*grosedentata*, has been found to possess broad pharmacological activities, including anti-inflammatory, anti-oxidant, anti-apoptotic, antibacterial, anti-tumor, anti-alcohol and hepatoprotective properties [12-16]. A study reported that DMY could markedly inhibit VSMCs proliferation and the injury-induced neointimal formation via induction of TR3 [17]. A randomized controlled trial showed that DMY improved glucose and lipid metabolism and relieved inflammation in patients afflicted by nonalcoholic fatty liver disease [18]. In addition, DMY has been demonstrated to ameliorate atherosclerosis in LDL receptor deficient mice [19]. These findings suggest the potential role of DMY in vascular diseases. However, the effect and mechanism of DMY on vascular calcification are still unclear. In this study, we aimed to investigate the effect of DMY on vascular calcification using *in vitro, ex vivo, in vivo* models, and explore the underlying mechanisms.

**Materials and methods**

**Cell culture**

Primary aortic VSMCs were isolated from 2-month-old male Sprague-Dawley (SD) rats (200-220g) as described previously [20]. Briefly, the rats were intraperitoneally euthanized with sodium pentobarbital (150 mg/kg), and the descending thoracic aorta was removed. After removal of the external connective tissue, the thoracic aorta was longitudinally cut open and the intima and adventitia were peeled off. Then, the medial layer of vessel was cut into small pieces and cultured in the high glucose (4.5 g/L) Dulbecco’s modified eagle medium (DMEM;
Thermo Fisher, USA, C11995500BT) supplemented with 10% FBS (Gibco, USA, #1099-141), 100 U/mL penicillin and 100 mg/mL streptomycin (Thermo Fisher, USA) at 37°C under 5% CO₂ humidified atmosphere. Rat VSMCs migrating from explants were maintained in growth medium (GM; DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin). Human aortic VSMCs were purchased from ATCC (Manassas, USA, CRL-1999). VSMCs from passages 3 to 8 were used for the following experiments. Calcification of VSMCs was induced by calcifying medium (CM) containing 10 mmol/L β-glycerophosphate (Sigma, USA, G9422) and 3 mmol/L CaCl₂ (Sigma, USA, C5670). DMY was obtained from Hunan E.K. HERB Co. Ltd. (Hunan, China, 27200-12-0) and dissolved in dimethyl sulfoxide (DMSO; Sigma, USA, D2650). To investigate the role of DMY in vascular calcification, different concentrations of DMY (20, 40 or 80 μmol/L) were used to treat rat and human VSMCs in the presence of CM for 3-7 days. SC79 (10 μmol/L, MCE, HY-18749), a specific AKT activator and MK2206 (5 μmol/L, MCE, HY-10358), a potent AKT inhibitor were used to treat VSMCs in the presence of CM in some experiments. At day 3, cells were harvested for RT-PCR analysis of RUNX2 and BMP2 mRNA expression and for western blot analysis of p-AKT, AKT, p-AKT1, AKT1, p-AKT2 and AKT2 protein expression. In some experiments, cells were harvested at day 7 and western blot were performed to analyze the protein expression of RUNX2, BMP2, COL I, OCN and PCNA. All in vitro experiments were independently performed for at least 4 times.

Small interfering RNA (siRNA) transfection
For RNAi knockdown, Rat VSMCs were seeded in a 6-well plate. When cells reached 70-80% confluence, AKT1 siRNA, AKT2 siRNA (30 nM; RiboBio, China) or Scrambled siRNA (Control siRNA; 30 nM; RiboBio, China) were transfected into cells using the Lipofectamine 3000 (Thermo Fisher, USA, L3000015) for 8 hours according to the manufacturer's instruction. To evaluate the efficiency of target gene knockdown, western blot analysis was performed at 48 hours after transfection.

**Aortic rings culture**

Thoracic aortas were dissected from 2-month-old male SD rats. Aortas were cut into 0.5 cm rings, and then incubated in GM, GM with DMY (80 μmol/L), CM or CM with DMY (80 μmol/L) for 7 days, with fresh medium renewed every 2 days. After 7 days incubation, aortic segments were harvested for further analysis. *Ex vivo* experiments were repeated for 4 times.

**CKD rat model**

All animal experiments were strictly performed to the US National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at Southern Medical University (Guangzhou, China). To exclude the influence of the hormonal changes (estrogen) on vascular calcification, only male rats were chosen for this study. Adult male SD rats aged 8 weeks weighing 220-250g were purchased from Guangdong Medical Lab Animal Center (Guangzhou, China). They were kept under a 12 h dark/light cycle with free access to standard rat chow and tap water in a temperature- and humidity-controlled room. All animal experiments were conducted at Laboratory Animal Center, Zhujiang Hospital of Southern Medical
University. CKD rat model was used in this experiment and was induced by subtotal 5/6 nephrectomy (extirpation of right kidney and ligation of arteries supplying two-thirds of left kidney) as previously described [21,22]. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg, Sigma-Aldrich, USA, #57-33-0). After shaving and disinfection with iodophor, a right ventrolateral incision was performed to expose the right kidney. The right kidney was ligated with non-absorbable thread and cut between the hilum and the ligated portion to remove the kidney. Similarly, the left renal artery was exposed and two-thirds of the arterial supply to the left kidney was ligated and verified by the subsequent homogeneous discoloration. Sham operated-controls were subjected to a sham operation, undergoing a simple laparotomy. Two weeks after the surgery, blood samples were taken to evaluate serum creatinine (CRE) by using Creatinine Assay Kit (Jiancheng Bio, Nanjing, China, C011-2). At the same time, the rats subjected to the 5/6 nephrectomy were fed with a high calcium and phosphate diet (4% calcium and 1.8% phosphate), and supplemented with 1,25-dihydroxy vitamin D3 by gavage (1 μg/kg, three times per week, Aladdin, Shanghai, China, C120126) for 4 weeks (Ca/P/VitD3).

Rats were randomly divided into three groups: (1) Sham group (Sham, n = 6), oral administration of a normal chow diet and sham-operation without 5/6 nephrectomy; (2) Model group (Model, n = 6), oral administration of a Ca/P/VitD3 diet combined with equivalent saline; (3) Model+DMY group (DMY, n = 6), oral administration of a Ca/P/VitD3 diet combined with DMY (200 mg/kg; grinded and dissolved in distilled water) daily by gavage. The dose of DMY used in this study was based on a previous
experimental study [23]. In some experiments, CKD rats were treated with SC79 (5 mg/kg; MCE, HY-18749) by intraperitoneal injection every 3 days for 4 weeks. At the end of the experiment, the rats were sacrificed under anesthesia by pentobarbital sodium (40 mg/kg, i.p.) and aortas (dissected from the ascending aortic root to the iliac bifurcation) were collected for further analysis.

**MTS Assay**

The viability of VSMCs was measured using MTS assay kit (Promega, Madison, WI, USA, G3580). Briefly, VSMCs were inoculated into 96-well plates at a density of 5×10^3 cells/well. Cells were then incubated with different concentrations of DMY (20, 40 or 80 μmol/L) for 1, 3 or 7 days. 10 μl of MTS solution was then added into each well and incubated for 2 hours at 37°C. The absorbance was measured by a microplate spectrophotometer at the wavelength of 490 nm.

**Micro-CT imaging of aortic calcification**

Micro-CT analysis was performed to analyze aortic calcification. Rat aortas were collected, and scanned in a micro-CT scanner (SCANCO Medical AG, Switzerland) at a resolution of 34.5 μm. Calcified arterial lesions were defined as CT attenuation equal to or greater than 130 HU in each section [24]. Images were analyzed by micro-CT Evaluation Program V6.6 software (SCANCO Medical AG, Switzerland).

**Alizarin red S Staining and quantification of calcium content**

Cells were harvested after 7 days incubation for alizarin red S staining and calcium content assay. Alizarin red S staining method was used to determine VSMCs and arterial calcification. To visualize VSMCs calcification, cells were washed 3
times with phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde for 10 minutes. After that, the cells were washed 3 times with PBS and exposed to 2% alizarin red S (pH 4.2; Solarbio, Beijing, China, G8550) for 10 minutes, and then washed 3 times with deionized water and observed under a Leica Microsystems microscope. Positively stained cells displayed a red color. To quantify the extent of calcification, alizarin red S dye was eluted with 10% formic acid and the absorbance at 405 nm was measured by a spectrophotometer. To visualize aortic tissue calcification, thoracic aortic segments, which were fixed in 4% paraformaldehyde and embedded in paraffin, were cut into 4 μm in thickness and deparaffinized. Aortic sections were stained with 2% alizarin red S for 5 minutes. The sections were visualized by an inverted microscope. Calcified areas of aortic rings were shown as red staining and were analyzed with Image J software. To visualize the whole-mount aorta calcification, aorta samples that dissected from the ascending aortic root to the iliac bifurcation were fixed in 95% ethanol overnight. Afterwards aortas were incubated with 0.004% alizarin red S dissolved in 1% KOH overnight, and then washed twice with 2% KOH before being photographed. The calcium content of cells and aortas was determined by Calcium Content Detection Kit (methyl thymol blue microplate method; Leagene, Beijing, China, TC1021) according to the manufacturer's protocol [25, 26]. The method is based on the mechanism that calcium reacts with methyl thymol blue (MTB) to form colored complexes monitored spectrophotometrically. Briefly, cells and aortic tissues were homogenized, and the supernatant was separated by centrifugation. Then, 2.5 μl of supernatant samples were
mixed with 200 μl of MTB solution and incubated for 10 min at room temperature. The calcium level was determined by absorbance measurement at 610 nm on a microplate reader (Thermo Scientific, USA). Protein concentration was quantified by BCA protein assay (Pierce, USA). Calcium content was normalized to total protein concentration and expressed as μg/mg protein, and the final results were normalized to control.

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was isolated from VSMCs by the Trizol method (AG, Hunan, China, AG21102) and was reverse-transcribed into cDNA with the Evo M-MLV RT Premix (AG, Hunan, China, AG11706) according to the manufacturer’s protocol. Quantitative real-time PCR (RT-qPCR) was performed on a 7500 FAST Real-Time PCR System (Applied Biosystems, USA) using SYBR Green Premix Pro Taq HS qPCR Kit (AG, Hunan, China, AG11719) and specific primers. Amplification reactions were set up in 20 μl reaction volumes. The specific polymerase chain reaction primers were listed as below: Rat: RUNX2, 5’-GCCGGGAATGATGAGAACTA-3’ and 5’-GGACCGTCCACTGTCACTTT-3’; BMP2, 5’-GTGTTGGCCTGAAGCAGAGAC-3’ and 5’-CTCGATGGCTTCTTCGTGAT-3’; GAPDH, 5’-GGCAAGGTCATCCCAGAGCT-3’ and 5’-CCCAGGATGCCCTTTAGTG-3’; Human: RUNX2, 5’-TCTTGAACAAATTCTGCTTCTTT-3’ and 5’-TGCTTTGGTCTTGAAATCACA-3’; GAPDH,
5′-GCGAGATCCCTCCAAAATCAA-3′ and 5′-GTTCACACCCATGACGAACAT-3′.

GAPDH was used as an internal control. Data were processed by using the comparative 2^−ΔΔCT method for relative quantification.

**Western blot analysis**

Rat aortic tissues and rat/human VSMCs were lysed with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China, P0013B) supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, USA, 78440), and the supernatants were harvested to extract proteins. Protein concentration was measured using a BCA protein assay kit (Pierce, USA). Equal amounts of proteins were loaded to each well and were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then proteins were transferred onto PVDF membranes (Millipore, USA). The membranes were then blocked with 5% nonfat dried milk for 2h at room temperature, followed by incubation with primary antibodies against RUNX2 (1:1000, Cell signaling technology, #12556), BMP2 (1:1000, Abcam, USA, ab14933), OCN (1:500; Thermo Fisher, USA, PA5-86886), COL I (1:1000; Affinity, China, AF7001), PCNA (1:5000, Abcam, USA, ab92552), p-AKT1 (1:1000; Cell Signaling Technology, #9018), AKT1 (1:1000; Cell Signaling Technology, #2938), p-AKT2 (1:1000; Cell Signaling Technology, #8599), AKT2 (1:1000; Cell Signaling Technology, #3063), p-AKT (1:2000; Cell Signaling Technology, #4060), AKT (1:1000; Cell Signaling Technology, #4685) and GAPDH (1:5000; Bioworld, St. Louis Park, MN, USA, AP0063) overnight at 4°C. After being washed 3 times with Tris Buffered Saline Tween (TBST) for 10 min, the membranes
were incubated with the secondary antibody (Boster, China, BA1054) and then visualized with the Imaging System (GE, Amersham Imager 600, GE, Piscataway, NJ, USA). The gray scale value of the target protein band was quantified using Image J software and normalized to GAPDH.

**Immunofluorescence assay**

Rat VSMCs grown on confocal dishes were fixed with 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100 for 20min at room temperature. Next VSMCs were blocked with 5% BSA in PBS at room temperature for 30 min and incubated overnight at 4°C with anti-p-AKT antibody (1:400; Cell Signaling Technology, #4060) or AKT antibody (1:100; Proteintech, 60203-2-lg) followed by labeling with Cy3 goat anti-rabbit IgG (1:400; Bioworld, BS10007) or goat anti-mouse IgG 488 (1:400; Bioworld, BS10015) for 2 h at room temperature. After being washed with PBS three times, the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology, Shanghai, China, C1006) for 15min. Images were visualized by using a microscope (Leica Microsystem, Wetzlar, Germany).

**Statistical Analysis**

The SPSS software was used for statistical analysis. One-way analysis of variance followed by the Tukey post hoc test were used to analyze the data. Data were expressed as mean ± standard error of the mean (SEM). $P<0.05$ was considered significant differences.
Results

DMY inhibits calcification of rat VSMCs

To determine the role of DMY on calcification of rat VSMCs, VSMCs were exposed to different concentrations of DMY (20, 40 or 80 μmol/L) in the presence of CM for 7 days. As shown in Fig. 1A and B, DMY treatment significantly attenuated calcification of rat VSMCs in a dose dependent manner in the presence of CM, but this effect was not observed in GM or GM together with DMY. It indicates DMY has no effect on VSMCs calcification under the condition of GM. MTS assay showed that DMY did not impair cell viability at concentrations of 20, 40 or 80 μmol/L at day 3, indicating that DMY has no cytotoxicity on VSMCs. However, at day 7, DMY treatment at the concentration of 80 μmol/L decreased cell viability of VSMCs (Fig. 1C). This may be due to the inhibitory role of DMY in VSMCs proliferation. Moreover, western blot analysis demonstrated that PCNA was downregulated by DMY treatment in comparison to CM-treated cells (Fig. S1). These data revealed that DMY inhibits the proliferation ability of VSMCs at day 7. Calcium content assay further confirmed that DMY reduced CM-induced VSMCs calcification with strongest effects at a concentration of 80 μmol/L (Fig. 1D). Therefore, the concentration of 80 μmol/L of DMY was selected for the subsequent experiments. Additionally, RT-qPCR analysis revealed that DMY decreased the mRNA expression of osteogenic differentiation related genes including RUNX2 and BMP2 (Fig. 1E). Concomitantly, Western blot analysis exhibited decreased RUNX2, BMP2, COLI, OCN expression by DMY treatment (Fig. 1F). Taken together, these results indicated
that DMY effectively attenuated osteogenic differentiation and calcification of rat VSMCs.

DMY inhibits calcification of human VSMCs

To further verify the effect of DMY on VSMCs calcification, human VSMCs were cultured in different concentrations of DMY (20, 40 or 80 μmol/L) in the presence of CM for 7 days. Alizarin red staining and calcium content assay further confirmed the inhibitory role of DMY in human VSMCs calcification at day 7 (Fig. 2A-C). Furthermore, RT-qPCR analysis revealed that DMY treatment markedly reduced RUNX2 expression (Fig. 2D). Western blot analysis revealed that DMY markedly decreased osteogenic differentiation markers such as COL I, RUNX2 and OCN (Fig. 2E). Collectively, these data demonstrated that DMY inhibited osteogenic differentiation and calcification of human VSMCs.

DMY attenuates calcification of aortic rings

Next, ex vivo aortic ring culture assay was performed to confirm the role of DMY in vascular calcification. Rat aortic rings were incubated in GM, GM supplemented with DMY (80 μmol/L), CM or CM supplemented with DMY (80 μmol/L) for 7 days. Alizarin red S staining demonstrated that DMY reduced mineral deposits in rat aortic rings (Fig. 3A and B), and DMY has no effect on mineralization in rat aortic rings under the condition of GM. Calcium content assay also revealed that 80 μmol/L of DMY attenuated calcium content in aortic rings (Fig. 3C). Moreover, as illustrated in Fig. 3D, DMY treatment significantly down-regulated COL I, RUNX2 and BMP2 expression compared with aortic rings cultured in CM alone. Accordingly,
these results suggested that DMY inhibited vascular calcification \textit{ex vivo}.

**DMY ameliorates aortic artery calcification of CKD rats**

To explore the effects of DMY on vascular calcification \textit{in vivo}, DMY (200 mg/day) was used to treat CKD rats for 4 weeks. The rats were randomly divided into three groups: sham, model, DMY. Rats from model group and DMY group were subjected to 5/6 nephrectomy, while rats from sham group were given laparotomy without 5/6 nephrectomy. The concentration of CRE levels were dramatically elevated 2 weeks after surgery in model group and DMY group rats compared with sham group rats (Fig. 4A), which implied successfully established CKD model rats. Alizarin red S staining of whole aortas and sections confirmed that DMY ameliorated aortic calcification (Fig. 4B, E and F). Similarly, micro-CT showed that the DMY treatment significantly decreased the mineral density in CKD rats (Fig. 4C), which indicating that DMY ameliorated aortic calcification. In accordance with \textit{ex vivo} experiments, calcium content analysis demonstrated decline of calcium deposits in DMY-treated aortic arteries (Fig. 4D). In addition, western blot analysis showed that the expression of COL I, RUNX2, BMP2 and OCN was up-regulated in calcified arteries, and DMY markedly decreased the expression of these osteogenic differentiation markers (Fig. 4G). In addition, to investigate the effect of DMY on rat vascular calcification under normal conditions, SD rats were randomly divided into sham group and sham+DMY group. Alizarin red S staining of whole aortas and sections and western blot analysis showed that DMY has no effect of vascular calcification under normal conditions (Fig. S2 A-D). These findings confirmed the effect of DMY on ameliorating vascular
calcification of CKD rats.

**DMY inhibits AKT signaling in vascular calcification both in vitro and in vivo**

Previous studies have illustrated that the AKT signaling pathway plays a pivotal role in vascular calcification [27,28]. In line with these findings, we have previously reported that AKT signaling was up-regulated during vascular calcification [29]. In order to investigate whether AKT signaling is involved in inhibition of vascular calcification by DMY, we performed western blot analysis and immunofluorescence assay for p-AKT and AKT expression after 3-day incubation. Western blot analysis revealed that the protein level of p-AKT was significantly elevated in CM-treated VSMCs, while DMY dramatically down-regulated p-AKT protein expression compared with CM-treated cells (Fig. 5A). Then we examined the effect of DMY on p-AKT expression in vivo. As shown by Fig. 5B, the protein expression of p-AKT was significantly decreased in DMY group compared with the model group. In addition, immunofluorescence analysis showed increased p-AKT expression in VSMCs cultured in CM, which was drastically inhibited by DMY treatment (Fig. 5C). As showed in supplementary figure S3, immunostaining showed that DMY treatment has no effect on total AKT expression in VSMCs.

**AKT signaling mediates the inhibitory effects of DMY on VSMCs calcification**

To explore the role of AKT signaling in mediating DMY-induced inhibition of VSMCs calcification, SC79, a potent AKT activator was used. Alizarin red S staining showed that SC79 enhanced VSMCs calcification compared with CM-treated cells at day 7. However, this effect was abrogated by DMY treatment (Fig. 6A).
Quantification analysis of alizarin red S staining further demonstrated the inhibitory role of DMY in SC79-enhanced VSMCs calcification (Fig. 6B). The similar findings were also demonstrated by calcium content assay (Fig. 6C). Additionally, we evaluated the protein expression of p-AKT and osteogenic markers RUNX2 and BMP2. The results showed that the ratio of p-AKT/total AKT, RUNX2 and BMP2 were increased by SC79 compared with CM. However, this effect was blocked by DMY, indicating the inhibitory effect of DMY on AKT signaling and VSMCs calcification (Fig. 6D and E). We concluded that DMY ameliorated VSMCs calcification probably by inhibiting AKT signaling. To further confirm the relationship between DMY and AKT on calcification, AKT inhibitor MK2206 was used to treat VSMCs. As shown by Fig.7A and B, cells in MK2206 group, DMY group, and MK2206+DMY group all showed reduced calcium deposition. Interestingly, DMY exerts a stronger inhibitory effect on calcium deposition than MK2206, and MK2206+DMY group showed a reduced calcification in comparison to MK2206 group. Meanwhile, MK2206+DMY group did not show more reduction in calcium deposition than DMY group alone, suggesting that inhibition of AKT signal could not enhance the inhibitory effect of DMY on vascular calcification. Furthermore, calcium content assay demonstrated similar results (Fig. 7C). Western blot analysis revealed that inhibition of AKT signaling by MK2206 or DMY significantly decreased protein expression of RUNX2 and BMP2, compared with CM-treated VSMCs (Fig. 7D and E). Accordingly, DMY may have other mechanisms to inhibit VSMCs calcification. Altogether, these findings indicate that AKT signaling plays an
important role in mediating CM-induced VSMCs calcification, and DMY inhibits VSMCs calcification at least partly via AKT signaling.

**AKT signaling pathway plays a role in DMY-mediated amelioration of vascular calcification in CKD rats**

The role of AKT signaling in DMY-inhibited vascular calcification in CKD rats was further evaluated with the AKT activator SC79. As evidenced by micro-CT analysis, more aortic calcification was observed in SC79 group than the model group, whereas the pro-calcific effect of SC79 was significantly retarded by DMY (Fig. 8A). Simultaneously, Alizarin red S staining of the whole mount aortas and aortic sections revealed that SC79 promoted the aortic calcification of SD rats, but this effect was abolished by DMY treatment (Fig. 8B-D, Figure S4). Moreover, calcium content analysis showed a similar pattern (Figure 8E). Furthermore, the protein level of p-AKT and osteogenic markers RUNX2 and BMP2 were up-regulated in the SC79 group compared with model group, while this up-regulation was abrogated by DMY treatment (Fig. 8F). According to these data, it suggests that DMY attenuates vascular calcification in CKD rats, at least partly by blocking the AKT signaling pathway.

**Knockdown of AKT1 and AKT2 suppressed calcification of VSMCs**

AKT has three isoforms: AKT1, AKT2, and AKT3. AKT1 and AKT2 are widely expressed in VSMCs, whereas AKT3 is mainly expressed in brain and testis [30,31]. During osteogenic differentiation, both protein expression ratio of p-AKT1/AKT1 and p-AKT2/AKT2 were elevated in CM-treated cells, whereas this upregulation was abolished by DMY (Fig. S5 A and B). These results suggested that both AKT1 and
AKT2 may play a role in osteogenic differentiation and calcification of VSMCs. We then investigated the effect of AKT1 or AKT2 knockdown on VSMC calcification. Western blot analysis confirmed the successful knockdown of AKT1 and AKT2 by siRNA in VSMCs (Fig. S6 A and B). At day 7, alizarin red S staining revealed that both AKT1 and AKT2 knockdown reduced mineralization of VSMCs, DMY+AKT1 siRNA group showed reduced VSMCs mineralization in comparison with CM+AKT1 siRNA group, and the same results existed between DMY+AKT2 siRNA group and CM+AKT2 siRNA group (Fig. 9A), and these results were further confirmed by quantification of alizarin red S staining (Fig. 9B). Consistently, calcium content assay showed similar results (Fig. 9C). In addition, knockdown of AKT1 or AKT2 by siRNA significantly decreased RUNX2 and BMP2 expression in comparison to Control siRNA-treated cells in the presence of CM (Fig. 9D). Altogether, these data indicate that both AKT1 knockdown and AKT2 knockdown suppress calcification of VSMCs, confirming a role for AKT signal in osteogenic differentiation and calcification.

Discussion

Vascular calcification decreases vessel elasticity and compliance, thus impairing cardiovascular hemodynamics, resulting in substantial cardiovascular morbidity and mortality [2,32]. Given the huge medical costs, it is urgent to find effective therapeutic strategies to prevent and treat vascular calcification. DMY, as the most abundant natural flavonoid in vine tea, can also be found in traditional medical plants
including *Hovenia dulcis* and *Cedrus deodara* and plant-based fruits including grapes and red bayberry [33-35]. Several studies showed that DMY had multiple cardiovascular protective effects. DMY has been found to attenuate angiotensin II-induced cardiomyocyte hypertrophy and myocardial hypertrophy induced by transverse aortic constriction via oxidative stress inhibition [36,37]. Recent studies have shown that DMY inhibits rat VSMCs proliferation and migration and angiotensin II-induced cardiac fibroblast proliferation [17,38]. Besides, DMY is capable of protecting vascular endothelial cells, alleviating lipid accumulation, enhancing cholesterol efflux and inhibiting foam cell formation during the process of atherosclerosis [19,39]. Moreover, DMY also regulates glucose metabolism, improves insulin resistance and protects against diabetic cardiomyopathy [40]. It suggests DMY could be an effective therapeutic agent in cardiovascular disease. However, it is unknown whether and how DMY plays a role in vascular calcification.

In this study, we demonstrated for the first time that DMY inhibited vascular calcification by using *in vitro*, *ex vivo* and *in vivo* models. Firstly, we observed DMY treatment reduced calcium deposition of VSMCs under CM culture in a dose-dependent manner, suggesting the inhibitory role of DMY in VSMCs calcification. Reduced expression of osteogenic markers COL I, RUNX2, BMP2 and OCN in VSMCs further supported that DMY inhibited osteogenic differentiation and VSMCs calcification. Secondly, DMY significantly attenuated calcification of rat aortic rings. Moreover, DMY remarkably ameliorated aortic calcification in rats with CKD. Finally, we demonstrate that AKT signaling is participated in the process of
vascular calcification, and mediates the inhibitory effects of DMY on calcification. According to our knowledge, this is the first report delineating that DMY suppresses vascular calcification partially by inhibiting AKT signaling.

AKT signaling plays a vital role in regulating VSMCs function, including proliferation, differentiation, cell survival, cell death by regulating various downstream signaling effectors [41-43]. There are convincing evidence supporting that AKT signaling is deeply involved in osteogenic differentiation and calcification, and acts as an upstream signaling to directly activate RUNX2 [28,44]. A recent publication has showed that AKT signaling drives H$_2$O$_2$-induced VSMCs calcification by upregulating RUNX2 activity [45]. Inhibition of the AKT signaling resulted in decreased osteogenic differentiation of VSMCs [46]. Additionally, activated AKT was proved sufficiently to promote vascular calcification in animals in vivo [27]. Consistent with these findings, we found that AKT signaling was activated in calcified VSMCs and aortas. DMY has been reported to inhibit AKT phosphorylation in vitro [47,48]. Consistently, our study also showed that DMY down-regulated phosphorylation of AKT both in vitro and in vivo. Therefore, we postulate that AKT signaling mediates the inhibitory effects of DMY on vascular calcification. The role of AKT signaling in DMY-attenuated vascular calcification was further evaluated with the specific AKT activator SC79 and inhibitor MK2206. We identified that AKT activation led to increased RUNX2 activation in VSMCs, as measured by western blot, and AKT deactivation decreased RUNX2 protein expression level in VSMCs. This indicated a potential correlation between AKT signaling and RUNX2 in vascular
calcification. Congruently, we observed that activation of AKT signaling with its specific activator SC79 enhanced CM-induced osteogenic differentiation and calcification of VSMCs, which characterized by elevated protein expression level of osteogenic differentiation markers RUNX2 and BMP2, but this up-regulation was abrogated by DMY. In vivo experiment, we found that SC79 stimulation increased the p-AKT and enhanced calcium density level of aortas in CKD rats, which is agreement with the previous studies that AKT has a pro-calcific effect on the vascular calcification. Interestingly, DMY relieved the SC79-induced activation of AKT signal and vascular calcification in CKD rats. Additionally, we further confirmed that inhibition of AKT by MK2206 significantly reduced CM-induced osteogenic differentiation and calcification of VSMCs, suggesting an essential role of AKT signaling in osteogenic differentiation and vascular calcification. Moreover, we found DMY is much more effective in inhibiting osteogenic differentiation and calcification of VSMCs than MK2206, implying that additional signaling pathways independent of AKT are involved during the process. Furthermore, as shown by alizarin red S staining and calcium content assay, MK2206+DMY group and DMY group cells showed similar reduction in calcium deposition, suggesting that inhibition of AKT signal could not enhance the inhibitory effect of DMY on vascular calcification. On the basis of these data, we conclude that DMY attenuates vascular calcification at least partly through inhibiting AKT signaling.

Emerging evidence suggests that there are distinct functions for AKT isoforms [49,50]. It is unclear whether AKT isoforms exhibit distinct functions in CM-induced
calcification. Therefore, we examined the effect of AKT1 and AKT2 on vascular calcification. Interestingly, knockdown of AKT1 and AKT2 both significantly reduced VSMCs calcification. Notably, when AKT1 or AKT2 silencing combined with DMY treatment, the inhibitory effect on VSMCs calcification was enhanced in comparison to that without DMY treatment. Given that AKT signal is important for cell proliferation, we also examined the effect of DMY on cell proliferation. Interestingly, we found DMY at 80 μmol/L decreased cell viability of VSMCs and PCNA expression at day 7. Therefore, we cannot rule out the possibility that the anti-calcification effect of DMY is related to its capability of anti-proliferation, and this need for future investigation.

DMY may become a promising agent for preventing initiation and progression of vascular calcification. DMY has some advantages. Firstly, since DMY is a natural flavonoid extracted from vine tea that is widely distributed in southern China, the acquisition of DMY is very convenient. Secondly, DMY is efficacious and safe and has been sold as capsules in US for nutraceutical supplement to prevent alcoholic hangovers. However, our study has several limitations. Firstly, the precise mechanism by which DMY inhibits AKT signaling is not clear. It has been reported that DMY inhibits TGF-β1 signaling, and TGF-β1 participates in activation of the PI3K/AKT pathway [51,52]. We postulate DMY may suppress TGF-β1-induced activation of PI3K/AKT signaling, thus abolishing AKT-mediated vascular calcification, but it needs further investigation. Therefore, it will be worth investigating the direct molecular target of DMY in inhibiting vascular calcification. Secondly, since vascular
Calcification is a complex process triggered by various factors including oxidative stress, high calcium and phosphate levels, inflammation, and apoptosis [53-55], and DMY can inhibit oxidative stress, inflammation and apoptosis, we cannot exclude the possibility that DMY inhibits vascular calcification through other mechanisms such as inhibition of oxidative stress, inflammation and apoptosis. Therefore, additional mechanisms underlying DMY-mediated inhibition of vascular calcification require further investigation.

In summary, our study indicates that DMY effectively ameliorates vascular calcification in CKD partially through inhibition of AKT signaling. DMY may become a preventive agent for vascular calcification in CKD and other cardiovascular diseases.

**Data availability Statement**

The data underlying this study will be shared on request to the corresponding author.

**Clinical perspectives**

- Vascular calcification is highly prevalent in CKD and is associated with increased cardiovascular morbidity and mortality. However, no effective and therapeutic option to prevent vascular calcification is yet available.

- This work demonstrated for the first time that DMY inhibited vascular calcification by using *in vitro, ex vivo* and *in vivo* models. Moreover, we have found that AKT signaling is participated in the process of vascular calcification,
and mediates the inhibitory effects of DMY on calcification.

- DMY may become an effective agent for preventing progression of vascular calcification in CKD.

**Competing Interests**

None.

**Founding**

This work was supported by National Natural Science Foundation of China (No. 81971765, No. 31771060, No. 31771099 and No. 81871504).
References

1. Tóth A, Balogh E and Jeney V. (2020) Regulation of Vascular Calcification by Reactive Oxygen Species. Antioxidants (Basel). 9 (10).

2. Demer LL and Tintut Y. (2008) Vascular calcification: pathobiology of a multifaceted disease. Circulation. 117, 2938-2948.

3. Shanahan CM, Crouthamel MH, Kapustin A and Giachelli CM. (2011) Arterial calcification in chronic kidney disease: key roles for calcium and phosphate. Circ Res. 109, 697-711.

4. Johnson RC, Leopold JA and Locasalo J. (2006) Vascular calcification: pathobiological mechanisms and clinical implications. Circ Res. 99, 1044-1059.

5. Lanzer P, Boehm M, Sorribas V, Thiriet M, Janzen J, Zeller T, St HC and Shanahan C. (2014) Medial vascular calcification revisited: review and perspectives. Eur Heart J. 35, 1515-1525.

6. Speer MY, Yang HY, Brabb T, Leaf E, Look A, Lin WL, Frutkin A, Dichek D and Giachelli CM. (2009) Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries. Circ Res. 104, 733-741.

7. Li X, Yang HY and Giachelli CM. (2008) BMP-2 promotes phosphate uptake, phenotypic modulation, and calcification of human vascular smooth muscle cells. Atherosclerosis. 199, 271-277.

8. Steitz SA, Speer MY, Curinga G, Yang HY, Haynes P, Aebersold R, Schinke T, Karsenty G and Giachelli CM. (2001) Smooth muscle cell phenotypic transition associated with calcification: upregulation of Cbfa1 and downregulation of smooth muscle lineage markers. Circ Res. 89, 1147-1154.

9. Wang C, Xu W, An J, Liang M, Li Y, Zhang F, Tong Q and Huang K. (2019) Poly(ADP-ribose) polymerase 1 accelerates vascular calcification by upregulating Runx2. Nat Commun. 10, 1203.

10. Boström K, Watson KE, Horn S, Wortham C, Herman IM and Demer LL. (1993) Bone morphogenetic protein expression in human atherosclerotic lesions. J Clin Invest. 91, 1800-1809.

11. Hénaut L, Chillon JM, Kamel S and Massy ZA. (2018) Updates on the Mechanisms and the Care of Cardiovascular Calcification in Chronic Kidney Disease. Semin Nephrol. 38, 233-250.

12. Tang N, Ma J, Wang KS, Mi C, Lv Y, Piao LX, Xu GH, Li X, Lee JJ and Jin X. (2016) Dihydromyricetin suppresses TNF-α-induced NF-kB activation and target gene expression. Mol Cell Biochem. 422, 11-20.

13. Zhang J, Chen Y, Luo H, Sun L, Xu M, Yu J, Zhou Q, Meng G and Yang S. (2018) Recent Update on the Pharmacological Effects and Mechanisms of Dihydromyricetin. Front Pharmacol. 9, 1204.

14. Ye L, Wang H, Duncan SE, Eigel WN and O’Keefe SF. (2015) Antioxidant activities of Vine Tea (Ampelopsis grossedentata) extract and its major component dihydromyricetin in soybean oil and
cooked ground beef. Food Chem. 172, 416-422.

15 Zeng Y, Hua YQ, Wang W, Zhang H and Xu XL. (2020) Modulation of SIRT1-mediated signaling cascades in the liver contributes to the amelioration of nonalcoholic steatohepatitis in high fat fed middle-aged LDL receptor knockout mice by dihydromyricetin. Biochem Pharmacol. 175, 113927.

16 Liang J, Shen Y, Shao XM, Scott MB, Ly E, Wong S, Nguyen A, Tan K, Kwon B, Olsen RW and Spigelman I. (2014) Dihydromyricetin prevents fetal alcohol exposure-induced behavioral and physiological deficits: the roles of GABAA receptors in adolescence. Neurochem Res. 39, 1147-1161.

17 Huang B, Li Y, Yao Y, Shu W and Chen M. (2018) Dihydromyricetin from ampelopsis grossedentata protects against vascular neointimal formation via induction of TR3. Eur J Pharmacol. 838, 23-31.

18 Chen S, Zhao X, Wan J, Ran L, Qin Y, Wang X, Gao Y, Shu F, Zhang Y, Liu P, Zhang Q, Zhu J and Mi M. (2015) Dihydromyricetin improves glucose and lipid metabolism and exerts anti-inflammatory effects in nonalcoholic fatty liver disease: A randomized controlled trial. Pharmacol Res. 99, 74-81.

19 Liu TT, Zeng Y, Tang K, Chen X, Zhang W and Xu XL. (2017) Dihydromyricetin ameliorates atherosclerosis in LDL receptor deficient mice. Atherosclerosis. 262, 39-50.

20 Hou M, Song Y, Li Z, Luo C, Ou JS, Yu H, Yan J and Lu L. (2016) Curcumin attenuates osteogenic differentiation and calcification of rat vascular smooth muscle cells. Mol Cell Biochem. 420, 151-160.

21 Zhang X, Li Y, Yang P, Liu X, Lu L, Chen Y, Zhong X, Li Z, Liu H, Ou C, Yan J and Chen M. (2020) Trimethylamine-N-Oxide Promotes Vascular Calcification Through Activation of NLRP3 (Nucleotide-Binding Domain, Leucine-Rich-Containing Family, Pyrin Domain-Containing-3) Inflammasome and NF-kB (Nuclear Factor kB) Signals. Arterioscler Thromb Vasc Biol. 40, 751-765.

22 Liu H, Zhang X, Zhong X, Li Z, Cai S, Yang P, Ou C and Chen M. (2019) Puerarin inhibits vascular calcification of uremic rats. Eur J Pharmacol. 855, 235-243.

23 Sun Z, Lu W, Lin N, Lin H, Zhang J, Ni T, Meng L, Zhang C and Guo H. (2020) Dihydromyricetin alleviates doxorubicin-induced cardiotoxicity by inhibiting NLRP3 inflammasome through activation of SIRT1. Biochem Pharmacol. 175, 113888.

24 Shemesh J, Evron R, Koren-Morag N, Apter S, Rozenman J, Shaham D, Itzchak Y and Motro M. (2005) Coronary artery calcium measurement with multi-detector row CT and low radiation dose: comparison between 55 and 165 mAs. Radiology. 236, 810-814.

25 Liu X, Chen A, Liang Q, Yang X, Dong Q, Fu M, Wang S, Li Y, Ye Y, Lan Z, Chen Y, Ou JS,
Yang P, Lu L and Yan J. (2021) Spermidine inhibits vascular calcification in chronic kidney disease through modulation of SIRT1 signaling pathway. Aging Cell. 20, e13377.

26 Yang X, Chen A, Liang Q, Dong Q, Fu M, Liu X, Wang S, Li Y, Lan Z, Ou JS, Lu L and Yan J. (2021) Up-regulation of heme oxygenase-1 by celastrol alleviates oxidative stress and vascular calcification in chronic kidney disease. Free Radic Biol Med. 172, 530-540.

27 Heath JM, Sun Y, Yuan K, Bradley WE, Litovsky S, Dell'Italia LJ, Chatham JC, Wu H and Chen Y. (2014) Activation of AKT by O-linked N-acetylglucosamine induces vascular calcification in diabetes mellitus. Circ Res. 114, 1094-1102.

28 Lino M, Wan MH, Rocca AS, Ngai D, Shobeiri N, Hou G, Ge C, Franceschi RT and Bendeck MP. (2018) Diabetic Vascular Calcification Mediated by the Collagen Receptor Discoidin Domain Receptor 1 via the Phosphoinositide 3-Kinase/Akt/Runt-Related Transcription Factor 2 Signaling Axis. Arterioscler Thromb Vasc Biol. 38, 1878-1889.

29 Li Z, Wu J, Zhang X, Ou C, Zhong X, Chen Y, Lu L, Liu H, Li Y, Liu X, Wu B, Wang Y, Yang P, Yan J and Chen M. (2019) CDC42 promotes vascular calcification in chronic kidney disease. J Pathol. 249, 461-471.

30 Chen J, Somanath PR, Razorenova O, Chen WS, Hay N, Bornstein P and Byzova TV. (2005) Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. Nat Med. 11, 1188-1196.

31 Cohen MJ. (2013) The AKT genes and their roles in various disorders. Am J Med Genet A. 161A, 2931-2937.

32 Wayhs R, Zelinger A and Raggi P. (2002) High coronary artery calcium scores pose an extremely elevated risk for hard events. J Am Coll Cardiol. 39, 225-230.

33 Sferrazza G, Brusotti G, Zonfrillo M, Temporini C, Tengattini S, Bononi M, Tateo F, Calleri E and Pierimarchi P. (2021) Hovenia dulcis Thunberg: Phytochemistry, Pharmacology, Toxicology and Regulatory Framework for Its Use in the European Union. Molecules. 26.

34 Wu Y, Bai J, Zhong K, Huang Y and Gao H. (2017) A dual antibacterial mechanism involved in membrane disruption and DNA binding of 2R,3R-dihydromyricetin from pine needles of Cedrus deodara against Staphylococcus aureus. Food Chem. 218, 463-470.

35 Wu P, Ma G, Li N, Deng Q, Yin Y and Huang R. (2015) Investigation of in vitro and in vivo antioxidant activities of flavonoids rich extract from the berries of Rhodomyrtus tomentosa(Ait.) Hassk. Food Chem. 173, 194-202.

36 Meng G, Yang S, Chen Y, Yao W, Zhu H and Zhang W. (2015) Attenuating effects of dihydromyricetin on angiotensin II-induced rat cardiomyocyte hypertrophy related to antioxidative activity in a NO-dependent manner. Pharm Biol. 53, 904-912.

37 Chen Y, Luo HQ, Sun LL, Xu MT, Yu J, Liu LL, Zhang JY, Wang YQ, Wang HX, Bao XF and
Meng GL. (2018) Dihydromyricetin Attenuates Myocardial Hypertrophy Induced by Transverse Aortic Constriction via Oxidative Stress Inhibition and SIRT3 Pathway Enhancement. Int J Mol Sci. 19.

38 Song Q, Liu L, Yu J, Zhang J, Xu M, Sun L, Luo H, Feng Z and Meng G. (2017) Dihydromyricetin attenuated Ang II induced cardiac fibroblasts proliferation related to inhibitory of oxidative stress. Eur J Pharmacol. 807, 159-167.

39 Hu Q, Zhang T, Yi L, Zhou X and Mi M. (2018) Dihydromyricetin inhibits NLRP3 inflammasome-dependent pyroptosis by activating the Nrf2 signaling pathway in vascular endothelial cells. Biofactors. 44, 123-136.

40 Wu B, Lin J, Luo J, Han D, Fan M, Guo T, Tao L, Yuan M and Yi F. (2017) Dihydromyricetin Protects against Diabetic Cardiomyopathy in Streptozotocin-Induced Diabetic Mice. Biomed Res Int. 2017, 3764370.

41 Abeyrathna P and Su Y. (2015) The critical role of Akt in cardiovascular function. Vascul Pharmacol. 74, 38-48.

42 Manning BD and Cantley LC. (2007) AKT/PKB signaling: navigating downstream. Cell. 129, 1261-1274.

43 Mathew OP, Ranganna K, Mathew J, Zhu M, Yousefi-pour Z, Selvam C and Milton SG. (2019) Cellular Effects of Butyrate on Vascular Smooth Muscle Cells are Mediated through Disparate Actions on Dual Targets, Histone Deacetylase (HDAC) Activity and PI3K/Akt Signaling Network. Int J Mol Sci. 20.

44 Deng L, Huang L, Sun Y, Heath JM, Wu H and Chen Y. (2015) Inhibition of FOXO1/3 promotes vascular calcification. Arterioscler Thromb Vasc Biol. 35, 175-183.

45 Byon CH, Javed A, Dai Q, Kappes JC, Clemens TL, Darley-Usmar VM, McDonald JM and Chen Y. (2008) Oxidative stress induces vascular calcification through modulation of the osteogenic transcription factor Runx2 by AKT signaling. J Biol Chem. 283, 15319-15327.

46 He X, Jiang H, Gao F, Liang S, Wei M and Chen L. (2019) Indoxyl sulfate-induced calcification of vascular smooth muscle cells via the PI3K/Akt/NF-κB signaling pathway. Microsc Res Tech. 82, 2000-2006.

47 Xia J, Guo S, Fang T, Feng D, Zhang X, Zhang Q, Liu J, Liu B, Li M and Zhu R. (2014) Dihydromyricetin induces autophagy in HepG2 cells involved in inhibition of mTOR and regulating its upstream pathways. Food Chem Toxicol. 66, 7-13.

48 Zhang Z, Zhang H, Chen S, Xu Y, Yao A, Liao Q, Han L, Zou Z and Zhang X. (2017) Dihydromyricetin induces mitochondria-mediated apoptosis in HepG2 cells through down-regulation of the Akt/Bad pathway. Nutr Res. 38, 27-33.

49 Martin KA, Merenick BL, Ding M, Fetalvero KM, Rzucidlo EM, Kozul CD, Brown DJ, Chiu HY,
Shyu M, Drapeau BL, Wagner RJ and Powell RJ. (2007) Rapamycin promotes vascular smooth muscle cell differentiation through insulin receptor substrate-1/phosphatidylinositol 3-kinase/Akt2 feedback signaling. J Biol Chem. 282, 36112-36120.

Jin Y, Xie Y, Ostriker AC, Zhang X, Liu R, Lee MY, Leslie KL, Tang W, Du J, Lee SH, Wang Y, Sessa WC, Hwa J, Yu J and Martin KA. (2017) Opposing Actions of AKT (Protein Kinase B) Isoforms in Vascular Smooth Muscle Injury and Therapeutic Response. Arterioscler Thromb Vasc Biol. 37, 2311-2321.

Liu B, Zhou W, Chen X, Xu F, Chen Y, Liu J, Zhang Q, Bao S, Chen N, Li M and Zhu R. (2015) Dihydromyricetin induces mouse hepatoma Hepal-6 cell apoptosis via the transforming growth factor-β pathway. Mol Med Rep. 11, 1609-1614.

Runyan CE, Schnaper HW and Poncelet AC. (2004) The phosphatidylinositol 3-kinase/Akt pathway enhances Smad3-stimulated mesangial cell collagen I expression in response to transforming growth factor-beta1. J Biol Chem. 279, 2632-2639.

Giachelli CM. (2009) The emerging role of phosphate in vascular calcification. Kidney Int. 75, 890-897.

Proudfoot D, Skepper JN, Hegyi L, Bennett MR, Shanahan CM and Weissberg PL. (2000) Apoptosis regulates human vascular calcification in vitro: evidence for initiation of vascular calcification by apoptotic bodies. Circ Res. 87, 1055-1062.

Demer LL and Tintut Y. (2014) Inflammatory, metabolic, and genetic mechanisms of vascular calcification. Arterioscler Thromb Vasc Biol. 34, 715-723.
Figure legends

**Fig. 1. Effect of DMY on rat VSMCs calcification.**

Rat VSMCs were cultured in GM, GM supplemented with DMY (80 μmol/L), CM or CM supplemented with DMY (20, 40 or 80μmol/L) for 7 days (n = 5). (A) Calcium deposition in VSMCs was detected by alizarin red S staining at day 7 (scale bar = 500 μm). (B) Alizarin red dye was eluted with 10% formic acid and quantified by a microplate reader. (C) Effects of DMY on the viability of VSMCs by MTS assay. (D) Quantitative measurement of calcium content at day 7. (E) RUNX2 and BMP2 mRNA expression levels in VSMCs were determined by RT-qPCR at day 3. (F) Western blot analysis and quantification of COL I, RUNX2, BMP2 and OCN by densitometry at day 7. GM indicates growth medium; and CM, calcifying medium. *P <0.05, **P <0.01.

**Fig. 2. Effect of DMY on human VSMCs calcification.**

Human VSMCs were incubated with GM with or without DMY (80 μmol/L), or CM with or without (20, 40 or 80μmol/L) for 7 days (n = 5). (A) Calcium deposition was detected by alizarin red S staining at day 7 (scale bar = 500 μm). (B) Alizarin red dye was quantified. (C) Calcium content was measured. (D) Quantification of RUNX2 mRNA expression. (E) Representative western blots for COL I, RUNX2 and OCN, and quantification of COL I, RUNX2 and OCN protein expression. GM indicates growth medium; and CM, calcifying medium. *P <0.05, **P <0.01.
Fig. 3. Effect of DMY on calcification of rat aortic rings.

Rat aortic rings were cultured in GM with or without DMY (80 μmol/L), CM with or without DMY (80 μmol/L) for 7 days (n = 4). (A) Calcium deposition of aortic rings was detected by alizarin red S staining. Scale bar = 500 μm (high panel), Scale bar = 100 μm (low panel). (B) Quantification of Alizarin red positive area was analyzed by Image J software. (C) Quantitative measurement of calcium content. (D) Protein expression of COL I, RUNX2 and BMP2 was determined by western blot analysis. *P <0.05, **p <0.01.

Fig. 4. Effect of DMY on aortic artery calcification of CKD rats.

Two weeks after subtotal (5/6) kidney nephrectomy, SD rats of sham group were treated with a normal chow diet, model group were treated with a high calcium and phosphate diet (4% calcium and 1.8% phosphate) combined with 1,25-dihydroxyvitamin D3 by gavage, with DMY (200 mg/kg/day) added by gavage of DMY group for 4 weeks (n = 6). (A) Levels of CRE were measured after 2 weeks after surgery. (B) Alizarin red S staining showed mineral deposition of aortic arteries (black arrow). (C) Calcification of aortic arteries (red arrow) was scanned by micro-CT. (D) Calcium content of aortas was measured by the methyl thymol blue microplate method. (E) Representative images of aortic sections were detected by alizarin red S staining. Scale bar = 500 μm. Higher magnifications are shown to the below. Scale bar = 100 μm. (F) Quantification of Alizarin red positive area was analyzed by Image J software. (G) Protein expression of COL I, RUNX2, BMP2 and
OCN was determined by western blot. NS indicates no significance. *P <0.01.

Fig. 5. DMY suppressed AKT signaling in vascular calcification.

(A) Rat VSMCs were incubated with GM, CM, or CM with DMY (80 μmol/L) for 3 days (n = 5). Western blot analysis and quantification of p-AKT protein expression in VSMCs. (B) Western blot analysis and quantification of p-AKT expression of calcified arteries in CKD rats (n = 5). (C) Immunostaining for p-AKT expression in VSMCs (n = 5). Scale bar = 100 μm. GM indicates growth medium; and CM, calcifying medium. *P <0.01.

Fig. 6. AKT signaling is involved in DMY-inhibited VSMCs calcification.

VSMCs were incubated with GM, CM, DMY (80 μmol/L) in the presence of CM alone, and with or without AKT activator SC79 (10 μmol/L) for 7 days (n = 4). (A) Alizarin red S staining of VSMCs after treatment with DMY and/or SC79 at day 7 (scale bar = 500 μm). (B) Quantification of alizarin red S staining was measured by formic acid. (C) Calcium content assay was quantified. (D and E) p-AKT, AKT, RUNX2 and BMP2 protein expression was analyzed by western blot. The level of p-AKT was normalized to total AKT. GM indicates growth medium; and CM, calcifying medium. *P <0.05, **P <0.01.

Fig. 7. AKT signaling mediates the process of inhibition of VSMCs calcification by DMY.
VSMCs were incubated with GM, CM, DMY (80 μmol/L) in the presence of CM alone, and with or without AKT inhibitor MK2206 (5 μmol/L) for 7 days (n = 4). (A) Alizarin red S staining of VSMCs after treatment with DMY and/or MK2206 at day 7 (scale bar = 500 μm). (B) Quantification of alizarin red S staining was measured by formic acid. (C) Calcium content assay was quantified. (D and E) p-AKT, AKT, RUNX2 and BMP2 protein expression was analyzed by western blot. The level of p-AKT was normalized to total AKT. GM indicates growth medium; and CM, calcifying medium. *P <0.05 and **P <0.01

Fig. 8. AKT signaling mediates the inhibitory effects of DMY on vascular calcification.

Rats subjected to surgery were treated with DMY (200 mg/kg/day) by gavage alone, and/or SC79 (5 mg/kg) every 3 days by intraperitoneal injection for 4 weeks (n = 6). (A) Mineral density of aortas (red arrow) in CKD rats treated by DMY and/or SC79 was scanned by micro-CT. (B) Representative images showing alizarin red S staining of the whole mount of aortic arteries. (C) Representative images of aortic sections stained with alizarin red solution. Scale bar = 500 μm. Higher magnifications are shown to the below. Scale bar = 100 μm (D) Quantification of Alizarin red positive area was analyzed by Image J software. (E) Measurement of calcium content of aortas. (F) Protein expression levels of p-AKT, AKT, RUNX2, BMP2 was determined by western blot. The level of p-AKT was normalized to total AKT. *P <0.05, **P <0.01.
Fig. 9. Knockdown of AKT1 and AKT2 inhibited VSMCs calcification

Rat VSMCs were transfected with AKT1 siRNA, AKT2 siRNA or Control siRNA in the presence of CM with or without DMY (80 μmol/L) for 7 days (n = 4). (A) Representative images showing cells stained with alizarin red solution. Scale bar = 500 μm. (B) Quantification of alizarin red S staining was measured by a microplate reader. (C) Calcium content assay was quantified. (D) RUNX2 and BMP2 protein expression was analyzed by western blot. GM indicates growth medium; and CM, calcifying medium. *P <0.05, **P <0.01.
Figure A: Alizarin red staining images showing bone formation in different conditions.

Figure B: Alizarin red OD units vs. CM+DYM (µM) showing the effects of GM, GM+DYM, and CM treatments.

Figure C: Cell viability (OD value) over time (Day 1, Day 3, Day 7) for GM, CM, DYM 20, 40, 80.

Figure D: Relative calcium content vs. CM+DYM (µM) showing the effects of GM, GM+DYM, and CM treatments.

Figure E: Relative mRNA levels for RUNX2 and BMP2 comparing GM, CM, and DYM treatments.

Figure F: Western blot images and relative protein levels for COL1, RUNX2, BMP2, OCN, and GAPDH with corresponding molecular weights.
A

GM  CM  DMY  SC79  SC79+DMY

B

Allarzin red (OD units)  **  **  **

C

Relative calcium content  **  **  **

D

Normalised phospho-total AKT expression  **  **

E

| p-AKT | AKT | RUNX2 | BMP2 | GAPDH |
|-------|-----|-------|------|-------|
| 60 kDa| 60 kDa| 57 kDa| 45 kDa| 36 kDa |

*GM  CM  DMY  SC79  SC79+DMY

Relative protein levels

RUNX2  BMP2
