

4'-O-methylbavachalcone inhibits succinate induced cardiomyocyte hypertrophy via the NFATc4 pathway

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Abstract. Pathological cardiac hypertrophy is an independent risk factor for complications such as arrhythmia, myocardial infarction, sudden mortality and heart failure. Succinate, an intermediate product of the Krebs cycle, is released into the bloodstream by cells; its levels increase with exacerbations of hypertension, myocardial and other tissue damage and metabolic disease. Succinate may also be involved in several metabolic pathways and mediates numerous pathological effects through its receptor, succinate receptor 1 (SUCNR1; previously known as GPR91). Succinate-induced activation of SUCNR1 has been reported to be related to cardiac hypertrophy, making SUCNR1 a potential target for treating cardiac hypertrophy. Traditional Chinese medicine (TCM) and its active ingredients have served important roles in improving cardiac functions and treating heart failure. The present study investigated whether 4'-O-methylbavachadone (MeBavaC), an active ingredient of the herbal remedy Fructus Psoraleae, which is often used in TCM and has protective effect on myocardial injury and hypertrophy induced by adriamycin, ischemia-reperfusion and sepsis, could ameliorate succinate-induced cardiomyocyte hypertrophy by inhibiting the NFATc4 pathway. Using immunofluorescence staining,

reverse transcription-quantitative PCR, western blotting and molecular docking analysis, it was determined that succinate activated the calcineurin/NFATc4 and ERK1/2 pathways to promote cardiomyocyte hypertrophy. MeBavaC inhibited cardiomyocyte hypertrophy, nuclear translocation of NFATc4 and ERK1/2 signaling activation in succinate-induced cardiomyocytes. Molecular docking analysis revealed that MeBavaC interacts with SUCNR1 to form a relatively stable binding and inhibits the succinate-SUCNR1 interaction. The results demonstrated that MeBavaC suppressed cardiomyocyte hypertrophy by blocking SUCNR1 receptor activity and inhibiting NFATc4 and ERK1/2 signaling, which will contribute to the preclinical development of this compound.

Introduction

Pathological cardiac hypertrophy is an independent risk factor for arrhythmia, myocardial infarction, sudden death and heart failure. Concentric ventricular hypertrophy results in systolic or diastolic dysfunction and can be caused by chronic hypertension, aortic stenosis, myocardial infarction, hereditary cardiomyopathy, obesity and diabetes (1-3). Pathological cardiac hypertrophy is characterized by numerous decompensations, such as cardiomyocyte death, fibrosis, Ca²⁺-regulated protein dysregulation, mitochondrial dysfunction, metabolic reprogramming, reactivation of fetal gene expression, altered sarcomere structure and insufficient angiogenesis leading to microvascular sparseness (4-8).

Calcineurin is a cytoplasmic Ca²⁺/calmodulin-dependent protein phosphatase that contributes to pathological cardiac hypertrophy. The calcineurin/nuclear factor-activated T-cell (NFATc) pathway affects cardiac structure under pathological conditions and serves a role in cardiac hypertrophy (9,10). In the adult heart, NFATc activity is continuously upregulated during pathological cardiac hypertrophy caused by pressure overload or hypertension and is more pronounced after myocardial infarction-induced heart failure (11,12). NFATc4 is required for calcineurin-mediated cardiomyocyte hypertrophy (13,14). Angiotensin II (Ang II) or phenylephrine can activate calcineurin/NFATc4 to promote cardiomyocyte hypertrophy (15).

The tricarboxylic acid (TAC) cycle in the mitochondrial matrix is considered a core process in cellular metabolism and energy balance (16-18). Succinate is an intermediate of

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Abbreviations: Ang II, angiotensin II; ERK1/2, extracellular signal-regulated kinases 1/2; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinases; MeBavaC, 4'-O-methylbavachalcone; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; NFATc, nuclear factor activated T cell; PBS, phosphate-buffered saline; PLCβ, Phospholipase C beta; SUCNR1/GPR91, succinate receptor 1; TAC, tricarboxylic acid; TCM, Traditional Chinese Medicine

Key words: succinate, succinate receptor 1, cardiomyocyte hypertrophy, nuclear factor activated T cell, 4'-O-methylbavachalcone

this cycle and its concentrations are significantly increased in hypertensive, obese and diabetic animal models (19). It is also the only metabolite whose levels are significantly increased in the coronary sinus of patients with ST-segment elevation myocardial infarction (STEMI) (20). Succinate produces broad pathophysiological effects by binding to the G-protein-coupled receptor 91 (GPR91)/Succinate receptor 1 (SUCNR1). SUCNR1 is expressed in the kidney, liver, heart, retinal cells and other tissues and is involved in regulating blood pressure, inhibiting lipolysis of white fat, forming retinal blood vessels, promoting cardiac hypertrophy and activating hepatic stellate cells (21,22). In a pulmonary arterial hypertension model of pressure overload-induced right ventricular hypertrophy in Sprague-Dawley rats, succinate treatment further aggravated right ventricular hypertrophy, upregulated the right ventricular hypertrophy-related gene ANP and activated PI3K/Akt axis signaling (23). Succinate was found to cause cardiac hypertrophy in SUCNR1-null mice in a SUCNR1-dependent manner. Activation of SUCNR1 triggers signals of cardiac hypertrophy such as phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) and expression of calmodulin-dependent protein kinase II delta (CaMKII δ) (24).

Fructus Psoraleae (the dried fruits of *Psoralea corylifolia* L.) is a common herb in traditional Chinese medicine (TCM) that has been included in Chinese Pharmacopoeia records (25). According to the theory of TCM, Fructus Psoraleae has the effect of warming and tonifying the kidney-Yang. It is widely used in the treatment of coronary artery disease, osteoporosis, vitiligo and psoriasis. Psoralea mainly contains coumarin, terpene phenol and prenylflavonoids, which are the material basis of drug treatment. Our team has summarized the pharmacological effects of five major psoralea prenylflavonoids, which have anti-inflammatory, cardiovascular protective, neuroprotective and antiosteoporosis effects (26). 4'-O-methylbavachalcone (MeBavaC) (Fig. 1B) is a prenylflavonoid that is abundantly present in Fructus Psoraleae (27). Few pharmacological studies have been conducted on MeBavaC; the only known report on the subject indicated that MeBavaC inhibits SARS-CoV papain-like protease PLpro (28). The present study investigated whether MeBavaC can ameliorate succinate-induced cardiomyocyte hypertrophy by inhibiting the calcineurin/NFATc4 pathway. The results revealed that succinate activated the calcineurin/NFATc4 and ERK1/2 pathways to promote cardiomyocyte hypertrophy and that MeBavaC treatment inhibited succinate-induced cardiomyocyte hypertrophy and related signaling; In addition, molecular docking analysis indicated that MeBavaC interacted with SUCNR1 to form a relatively stable binding and produced a certain inhibitory effect.

Materials and methods

Reagents and antibodies. Sodium succinate and Dulbecco's modified Eagle's medium (High Glucose) were purchased from MilliporeSigma. Fetal bovine serum was obtained from Gibco; Thermo Fisher Scientific, Inc. Penicillin-streptomycin solution, trypsin cell digestion solution (containing 0.25% trypsin and phenol red), cDNA first-strand synthesis kit (cat. no. D7178L), RIPA lysis solution (cat. no. P0013B) and

BCA protein concentration assay kit (cat. no. P0012) were purchased from Beyotime Institute of Biotechnology. Dimethyl sulfoxide, Triton X-100, paraformaldehyde and anhydrous ethanol were purchased from Shanghai Titan Scientific Co., Ltd. Trichloromethane and isopropanol were purchased from Sinopharm Chemical Reagent Co., Ltd. Tetramethylrhodamine (TRITC)-phalloidin (cat. no. MX4405-300T) was purchased from Shanghai Maokang Biotechnology Co., Ltd. Power SYBR Green PCR master mix (cat. no. 4367659) was purchased from Thermo Fisher Scientific, Inc. Anti-GAPDH (cat. no. 2118), anti-phospho-p38 MAPK (cat. no. 4511), anti-phospho-SAPK/JNK (cat. no. 4668), anti-SAPK/JNK (cat. no. 9252), anti-phospho-p44/42 MAPK (cat. no. 4370), anti-p44/42 MAPK (cat. no. 4695), anti-NFAT3/NFATc4 (23E6) rabbit mAb (cat. no. 2183) and antimouse IgG (cat. no. 7076) were purchased from Cell Signaling Technology, Inc. Anti-p38 α/β (sc-7149) was obtained from Santa Cruz Biotechnology, Inc. Donkey anti-rabbit IgG H&L (Alexa Fluor 488; cat. no. ab150073) was obtained from Abcam. Antirabbit IgG (cat. no. MR-R100) was purchased from Shanghai Mingrui Biotech Co., Ltd. PD98059 (cat. no. S1177), SP600125 (cat. no. S1640) and SB203580 (cat. no. S1076) were purchased from Selleck Chemicals. VIVIT (cat. no. HY-P1026) was obtained from MedChemExpress. Cyclosporin A (cat. no. A600352-0001) was purchased from Sangon Biotech Co., Ltd.

Cell culture. H9c2 cardiomyocytes (American Type Culture Collection) were cultured in DMEM containing 10% FBS supplemented with antibiotics (100 U/ml penicillin G and 100 μ g/ml streptomycin sulfate) at 37°C in a humidified atmosphere of 5% CO₂.

MTT assay. H9c2 cells were seeded into a 96-well plate (5,000 cells/well) and cultured overnight in a 37°C and 5% CO₂ cell incubator. On the second day, sodium succinate or MeBavaC with different final concentrations (diluted with serum- and antibiotic-free H-DMEM medium) was added, whereas for the control group, the same amount of H-DMEM medium was added. Then, six parallel wells were set up for each group. After 48 h, 10 μ l of MTT solution [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well and incubated for 4 h at 37°C and 5% CO₂. Next, 150 μ l of DMSO solution was mixed with the cell-MTT suspension and the formazan crystals were fully dissolved by shaking on a constant temperature microtiter plate with a fast shaker for 10 min. The OD value of each well was detected using a Varioskan Flash microplate spectrophotometer (Thermo Fisher Scientific, Inc.) at 570 nm.

Cell size analysis. The cell surface area was determined by staining the cells with TRITC-phalloidin. Briefly, the H9C2 cells were seeded at a density of 10,000 cells/plate in a 12-well plate. On the basis of the principle that phalloidin can combine with actin (29), when the confluence of H9c2 cells reached 50%, TRITC-phalloidin staining was performed to observe cardiomyocyte hypertrophy induced by succinate. Phalloidin staining of F-actin as a marker for measuring cell surface area has been used in numerous cell experiments (30,31). The cells were incubated with or without MeBavaC and then treated with

1 mM sodium succinate for 0-48 h. They were then washed with PBS and fixed with 4% formaldehyde solution for 10 min at room temperature, followed by washing twice with PBS. The cells were then treated with 0.5% Triton X-100 at room temperature for 5 min to increase permeability then washed twice with PBS. Finally, the cells were stained with 100 nM TRITC-Phalloidin staining solution for 30 min in the dark at room temperature with gently shaking. The cells were washed twice with PBS, which was followed by staining with DAPI for ~30 sec at room temperature. After washing, fluorescence microscopy images were recorded separately at excitation/emission wavelengths (Ex/Em=546/575 nm for TRITC and Ex/Em=364/454 nm for DAPI). A total of five images were taken from each plate of cells from different areas (each group $n=3$; counting 50 cells). Under an Olympus IX71 microscope (Olympus Corporation), the 'Count and Measure' facility in the imaging software CellSens 1.14 (Olympus Corporation) was used, the cell regions of interest were selected and defined, automatic image size measurement was implemented and the area test parameters were derived for statistical analysis.

Immunofluorescence. Following treatment, the H9c2 cells were fixed in 4% paraformaldehyde for 10 min at room temperature and then permeabilized with 1% Triton X-100 for 10 min at room temperature. After blocking with 5% bovine serum albumin (MilliporeSigma), the H9c2 cells were incubated with the anti-NFATc4 overnight at 4°C in a humidified chamber. This was followed by incubation with donkey antirabbit IgG H&L (Alexa Fluor® 488) for 2 h at 37°C. After washing, images were taken with an immunofluorescence microscope (Olympus IX71; Olympus Corporation).

Reverse transcription-quantitative (RT-q) PCR. According to the manufacturer's user manuals, total RNA was extracted from $\sim 5 \times 10^6$ cultured cardiomyocytes per sample with NucleoSpin RNA Plus kit (Takara Bio, Inc.) and cDNA was synthesized using the BeyoR III cDNA Synthesis kit (Beyotime Institute of Biotechnology). Gene expression analysis was performed on an ABI 7500 Fast real-time PCR system using the Power SYBR Green PCR master mix according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). The primer sequences (synthesized by Sangon Biotech Co., Ltd.) were: rat GAPDH: forward primer 5'-GATCCCGCTAACATCAATG-3', reverse primer 5'-GAGGGAGTTGTCATATTTCTC-3'; rat α -actinin: forward primer 5'-AGAAGAGATCGTGGATGGCAATGC-3', reverse forward primer 5'-GATGCTTGGATGGCGAACCTGAG-3'; rat GATA4: forward primer 5'-CCTCCTTCTCTACCTGCCTGTCC-3'; and reverse primer 5'-TGTCTTGAAGCCTCGGTCCCTAC-3'. Gene expression was normalized to the reference gene GAPDH. The reaction conditions were: Denaturation (95°C for 10 min), annealing (60°C for 1 min) and extension (95°C for 15 sec) for 40 cycles. Three independent experiments were conducted. PCR results were calculated using the $2^{-\Delta\Delta C_q}$ method and statistically analyzed (32).

Western blotting analysis. The cultured cells were washed twice with cold PBS containing PMSF (1 mM), NaF (2 mM) and Na_3VO_4 (2 mM) and then lysed with 100 μl of RIPA lysis buffer containing PMSF, NaF and Na_3VO_4 at the same

final concentration. The cells at the bottom of the dish were collected with a scraper into a 1.5 ml microcentrifuge tubes, lysed on ice for 30 min and then centrifuged at $13,400 \times g$ in a microcentrifuge for 15 min at 4°C. The supernatant was taken into new prechilled 1.5 ml microcentrifuge tubes and employed for measuring the protein concentration at 562 nm by using a bicinchoninic acid protein assay kit. The protein samples were adjusted to a uniform concentration with SDS-PAGE protein loading buffer (5X) and PBS and then denatured in a water bath at 95°C for 10 min. Protein (30 μg) was separated by performing 10% SDS-PAGE and transferred to nitrocellulose membranes (Pall Filter Beijing Co. Ltd.). The membranes were blocked at room temperature for 2 h with 5% non-fat dried milk in a buffer containing 140 mmol/l NaCl, 20 mmol/l Tris-HCl (pH 7.5) and 0.1% Tween-20 and incubated overnight at 4°C with the primary antibodies (dilution ratio: 1:1,000). Finally, the membranes were incubated for 2 h with horseradish peroxidase-conjugated mouse monoclonal antibody (1:2,000) at room temperature with gentle shaking. The PVDF membrane was immersed in ECL working solution (Sangon Biotech Co., Ltd.) and incubated in the dark for 1 min. The membrane was placed in a chemiluminescence developer (Tanon Science and Technology Co., Ltd.) for gradient exposure and the images was captured with the Tanon-5200 Multi chemiluminescent imaging system (Tanon Science and Technology Co., Ltd.), which uses the Sony ICX694 sensor CCD chip. Quantitative analysis of band density was performed using Quantity One (version 25.0) software from Bio-Rad Laboratories, Inc.

Molecular docking simulations. Molecular docking simulations were performed to analyze the inhibitory mechanism of compounds against SUCNR1. Due to the absence of a crystal structure of human SUCNR1, the structure of a rat SUCNR1 (PDB Code: 6IBB) was acquired from the Protein Data Bank (<https://www.rcsb.org>) and was used as a receptor (33,34). Graphical user interface AutoDock (<https://ccsb.scripps.edu/>) Tools 1.5.6 software was used to format coordinate files of SUCNR1 and compounds by adding polar hydrogens, deriving Kollman charges and setting AutoDock 4 type of atoms. The SUCNR1 docking site was placed at the orthosteric site described in previous studies (34,35). The grid box was settled to encircle the orthosteric site and AutoDock Vina (1.1.2) was used as the docking and scoring program. Further analyses of the SUCNR1-inhibitor interactions based on the docking and scoring results were aided by BIOVIA Discovery Studio Visualizer (Dassault Systemes).

Statistical analysis. GraphPad Prism 7 software (Dotmatics) was used for data analysis and statistics and the data were expressed as means \pm standard deviations. Comparisons between groups were analyzed using one-way analysis of variance followed by Dunnett's multiple comparison test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Succinate induces cardiomyocyte hypertrophy and activates nuclear translocation of NFATc4. First, MTT assay was used to evaluate the effects of sodium succinate and MeBavaC on the survival of H9C2 cardiomyocytes. Sodium succinate was

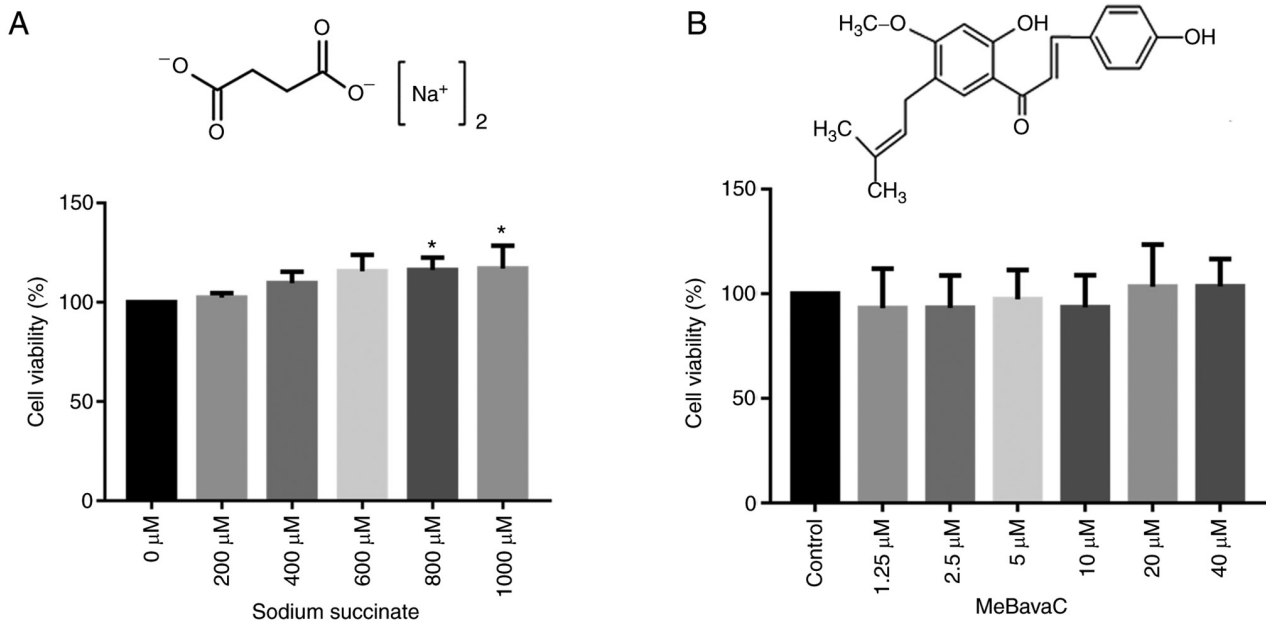


Figure 1. Effects of sodium succinate and MeBavaC on the viability of H9c2 cardiomyocytes. After H9c2 cells were treated with the indicated concentrations of (A) sodium succinate or (B) MeBavaC for 48 h, an MTT test was performed (six wells per test; $n=3$ tests). Comparisons between the groups were analyzed completed using Dunnett's multiple comparison test after one-way ANOVA. Data are expressed as mean \pm standard deviation. * $P<0.05$ vs. 0 μM group. MeBavaC, 4'-O-methylbavachalcone.

dissolved in DMEM medium and MeBavaC was dissolved in DMSO as a stock solution and diluted with the medium to the final concentration. The final concentration of DMSO was $<0.1\%$, which ensured it was safe for the cells. As presented in Fig. 1A, no cytotoxicity was detected in the H9C2 cells, indicating that the concentrations of sodium succinate within 1,000 μM and stimulation for 48 h were safe (six wells per test, $n=3$ tests). In addition, the effects of 1, 2, 5, 10, 25 and 50 mM sodium succinate on the H9C2 cardiomyocytes was measured and no toxicity found (data not shown). When the cardiomyocytes were treated with 800 and 1,000 μM of sodium succinate, a slight increase in MTT was observed (116.4 ± 5.5 and 117.1 ± 5.5 , respectively). The effect of MeBavaC within 40 μM on H9C2 cells was also tested and no cytotoxicity was discovered (six wells per test, $n=3$ tests, Fig. 1B). Given that no cytotoxicity was noted with 1 mM sodium succinate (Fig. 1A) and that this concentration has also been used in a number of studies (24,36), 1 mM sodium succinate was used in all subsequent experiments.

To explore the molecular mechanisms of MeBavaC-mediated protection against cardiac hypertrophy, a model of cardiomyocyte hypertrophy was first established by exposing H9C2 cells to sodium succinate (1 mM) for 12, 24 and 48 h, respectively. TRITC-phalloidin staining revealed a time-dependent increase in cardiomyocyte size in the group that underwent sodium succinate treatment compared with the nontreatment group. In addition, sodium succinate treatment increased the relative cell cross-sectional area from $4,601 \pm 124 \mu\text{m}^2$ at 0 h to $6,049 \pm 120 \mu\text{m}^2$ at 48 h ($n=3$, counting 50 cells, Fig. 2A and B). Stimulation with sodium succinate for 6 h also promoted a nearly three-fold increase in cardiomyopathy-associated α -actinin mRNA expression without altering GATA4 expression ($n=3$, Fig. 2C and D). In addition, H9c2 cardiomyocytes were treated with sodium succinate for

60 min and immunofluorescence staining indicated a significant greater two-fold increase in NFATc4 content related to its nuclear translocation ($n=3$; counting 50 cells; Fig. 3A). However, treatment with the NFAT inhibitor VIVIT (2 μM) or the calcineurin inhibitor cyclosporine A (CsA, 5 nM) for 30 min prior to sodium succinate significantly blocked the nuclear translocation of NFATc4 induced by sodium succinate ($n=3$; counting 50 cells; Fig. 3B), indicating that succinate activates the calcineurin-NFATc4 pathway in cardiomyocytes. Additionally, the induction of cardiomyocyte hypertrophy for 48 h by sodium succinate was blocked by VIVIT or CsA, with the relative cell cross-sectional area decreasing from $6,211 \pm 122 \mu\text{m}^2$ in the succinate-stimulated model group to $4,529 \pm 90 \mu\text{m}^2$ in the VIVIT-treated group or to $5,064 \pm 605 \mu\text{m}^2$ in the CsA-treated group ($n=3$; counting 50 cells; Fig. 3B). The results demonstrated that succinate induced cardiomyocyte hypertrophy and activated NFATc4 nuclear translocation.

4'-O-methylbavachalcone prevents succinate-induced cardiomyocyte hypertrophy via NFATc4 signaling. To determine whether MeBavaC prevent cardiomyocyte hypertrophy, the present study investigated the effect of MeBavaC on succinate-induced cardiomyocyte hypertrophy. H9c2 cardiomyocytes were pretreated with MeBavaC at the concentrations of 1.25, 2.5 and 5 μM for 30 min. MeBavaC was found to inhibit NFATc4 nuclear translocation in a concentration-dependent manner. This inhibition was induced by sodium succinate for 60 min. NFATc4 nuclear translocation nearly returned to the prestimulation levels after 5 μM MeBavaC treatment ($n=3$; counting 50 cells; Fig. 4A). The inhibitory effect of MeBavaC on cardiomyocyte hypertrophy was determined by TRITC-phalloidin staining. Pretreatment with MeBavaC for 30 min effectively prevented cardiomyocyte hypertrophy induced by sodium succinate for 48 h, with an inhibition of

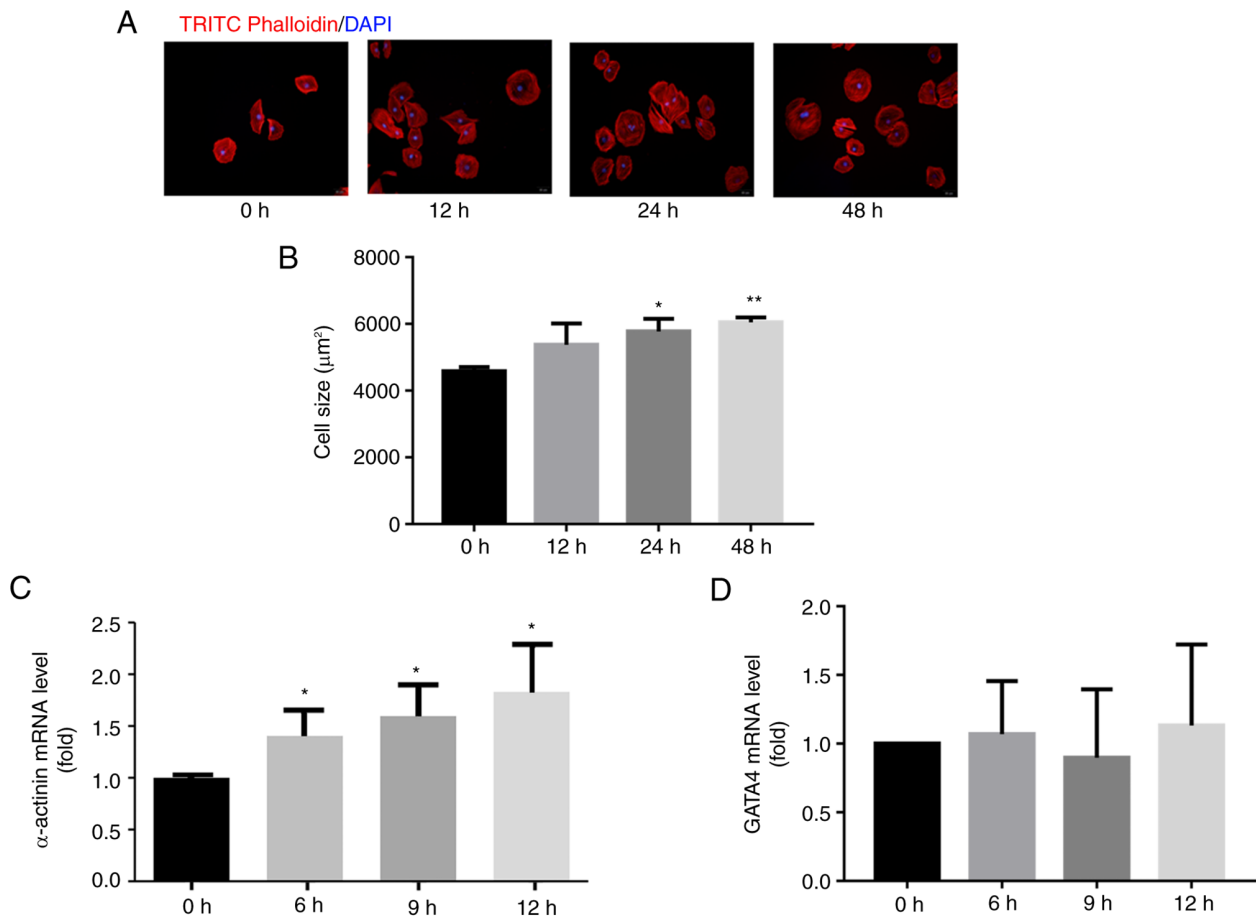


Figure 2. Succinate induced cardiomyocyte hypertrophy. (A and B) Time-dependent succinate-stimulated hypertrophy of H9c2 cells determined by TRITC-phalloidin staining (n=3 each, counting 50 cells). Magnification, x200. (C and D) Succinate stimulated mRNA expression of α -actinin and GATA4 measured using real-time quantitative PCR (n=3 each). Comparisons between groups were analyzed using Dunnett's multiple comparison test after one-way ANOVA. Data are expressed as mean \pm standard deviation. *P<0.05 and **P<0.01 vs. 0 h group. TRITC, tetramethylrhodamine.

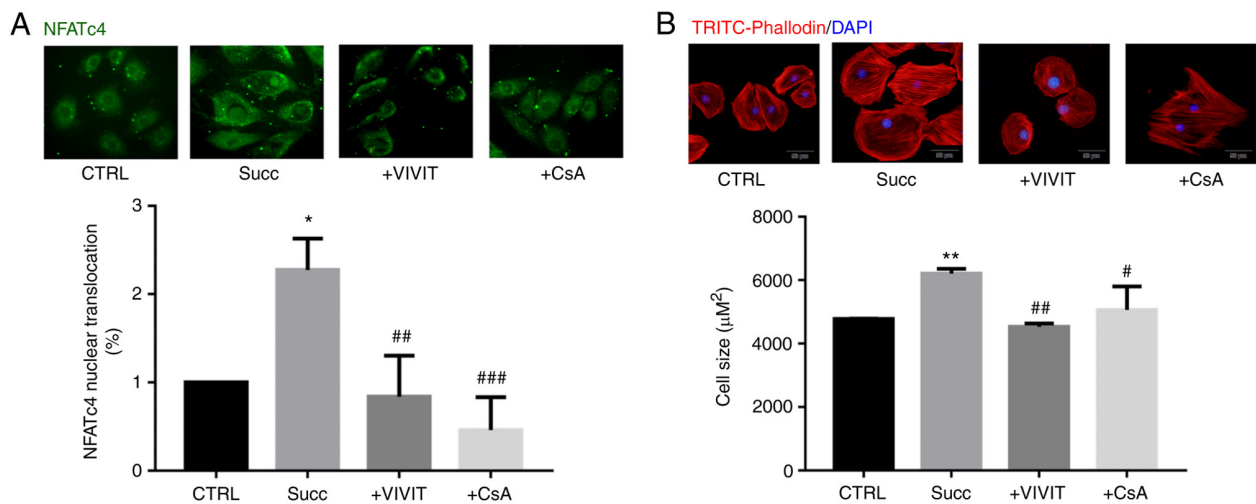


Figure 3. Succinate stimulated nuclear translocation of NFATc4 and its association with cardiomyocyte hypertrophy. (A) Immunofluorescence microscopy image of succinate-stimulated nuclear translocation of NFATc4 labeled with Alexa Fluor 488 (n=3 each; counting 50 cells). Magnification, x400. (B) Inhibitory effect of VIVIT and CsA on succinate-induced hypertrophy of H9c2 cardiomyocytes (n=3, counting 50 cells). Magnification, x200. Comparisons between groups were analyzed using Dunnett's multiple comparison test following one-way ANOVA. Data are expressed as mean \pm standard deviation. *P<0.05 and **P<0.01 vs. control group; #P<0.05, ##P<0.01 and ###P<0.001 vs. succinate-stimulated group. NFATc, nuclear factor activated T cell.

up to 20% compared with the model group (n=3; counting 50 cells; Fig. 4B). RT-qPCR revealed that MeBavaC restored

the gene expression of α -actinin induced by sodium succinate for 6 h, which is a hypertrophic marker (n=3; Fig. 4C).

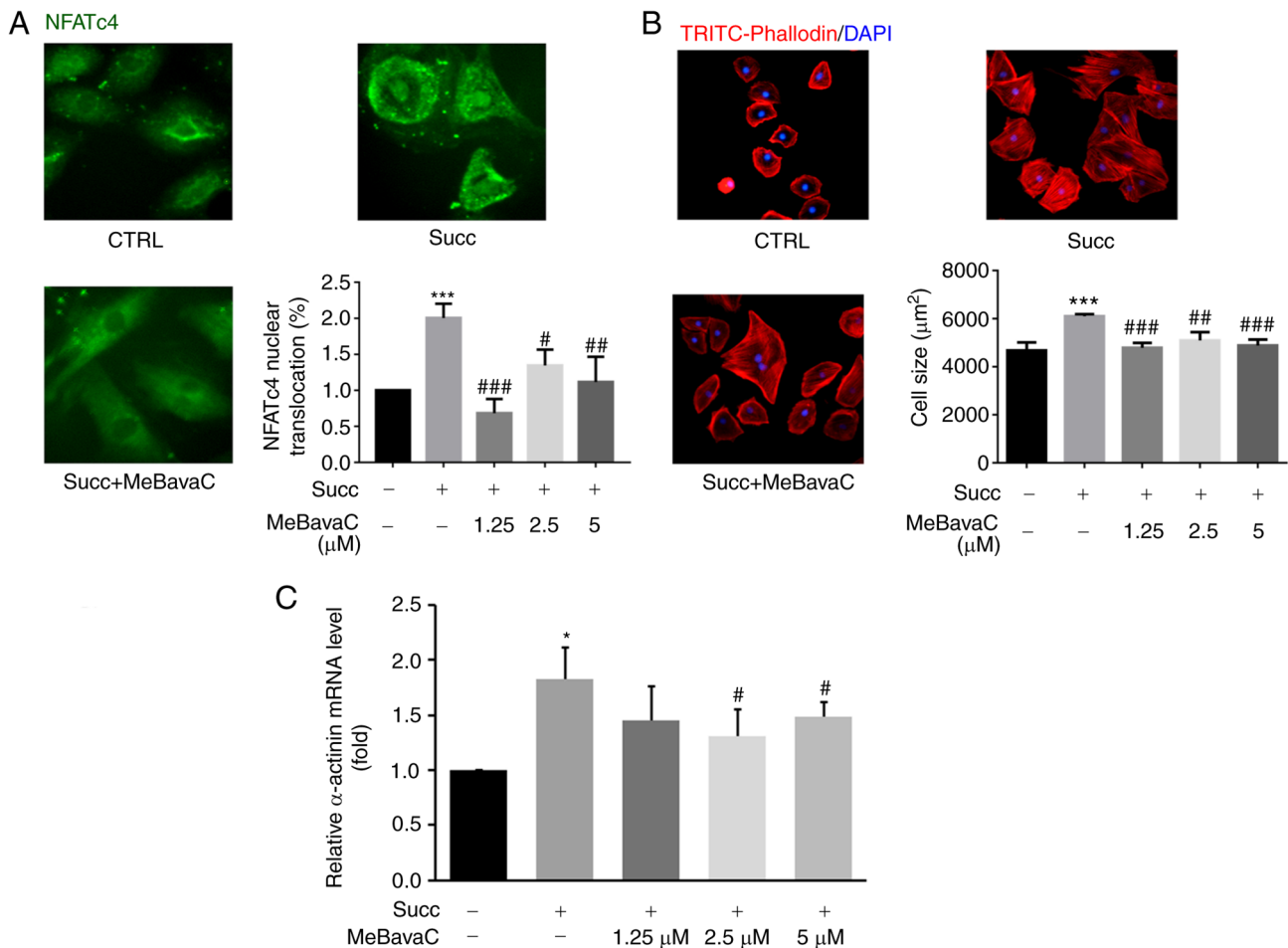


Figure 4. Inhibitory effect of MeBavaC on succinate-stimulated nuclear translocation of NFATc4 and cardiomyocyte hypertrophy. Effect of MeBavaC on (A) nuclear translocation of NFATc4 (magnification, x400) and (B) cell size (magnification, x200) imaged through immunofluorescence microscopy of succinate-induced hypertrophy of H9c2 cardiomyocytes (n=3; counting 50 cells). (C) Effect of MeBavaC on succinate-stimulated α -actinin mRNA expression in succinate-stimulated H9c2 cardiomyocytes (n=3). Comparisons between groups were analyzed using Dunnett's multiple comparison test following one-way ANOVA. Data are expressed as mean \pm standard deviation. *P<0.05, and ***P<0.001 vs. control group; #P<0.05, ##P<0.01 and ###P<0.001 vs. succinate-stimulated group. MeBavaC, 4'-O-methylbavachalcone; TRITC, tetramethylrhodamine.

Role of MAPK on 4'-O-methylbavachalcone in improving cardiomyocyte hypertrophy. The phosphorylation activities of ERK1/2 and JNK kinases, two essential members of MAPK signaling, were activated by sodium succinate for 15 min as detected using western blotting, but not by p38 kinase (n=3; Fig. 5A-C). Pretreatment with MeBavaC for 30 min significantly inhibited ERK1/2 and JNK phosphorylation with a maximum inhibition of ~15 and 40%, respectively (n=3; Fig. 5A-C). However, sodium succinate-induced cardiomyocyte hypertrophy was blocked by 30 min of pretreatment of the ERK1/2 pathway inhibitor PD98059 (20 μ M), but not the JNK pathway inhibitor SP600125 (20 μ M), as indicated by a decreased cardiomyocytes size (~30%; n=3; counting 50 cells; Fig. 5D and E). These results indicated that ERK1/2 signaling partly serves a role in mediating succinate-induced cardiomyocyte hypertrophy.

Molecular docking analysis of 4'-O-methylbavachalcone and the SUCNR1 receptor. To elucidate the binding mechanism of MeBavaC on SUCNR1 in detail, a docking simulation analysis was performed. As presented in Fig. 6A, MeBavaC and succinate bound SUCNR1 at extremely close position. Binding to

both the Cys168 and Val169 groups of the SUCNR1 receptor, succinate was bound via hydrogen bonds, whereas MeBavaC was bound via the alkyl and Pi-alkyl groups (Fig. 6B and C). Markedly, MeBavaC was predicted to be more compatible with the orthosteric site of SUCNR1 than was succinate, with the two having a docking score of -8.1 and -4.7 kcal/mol, respectively. The carbonyl of succinate is integrally docked to a pseudooxanion whole (Fig. 6C and D), which is generally a proteinaceous substructure preferring multiform oxygen atoms and consists of Tyr26, Tyr29 and Arg276. Nevertheless, MeBavaC formed stable Pi-cation with Lys18 as well as Pi-Alkyl with Leu75. Taken together, these results indicate that MeBavaC inhibited SUCNR1 activity.

Discussion

Succinate is an intermediate product of mitochondrial metabolism and mitochondrial damage results in succinate leakage. A number of cardiovascular events, such as myocardial infarction, atrial fibrillation and heart failure, promote succinate leakage from cardiomyocytes (20,37-39). Succinate is the only metabolite whose levels are significantly

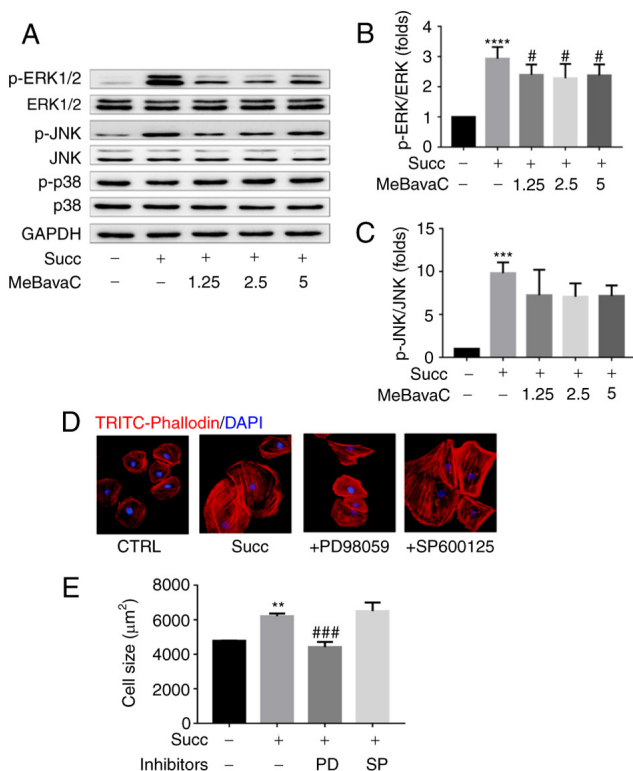


Figure 5. Effect of MeBavaC on succinate-stimulated MAPK activity and association with cardiomyocyte hypertrophy. (A, B and C) Effects of MeBavaC on succinate-stimulated MAPK phosphorylation levels (n=3). (D and E) Effects of ERK1/2 and JNK pathways inhibitors (PD98059 and SP600125) on succinate-induced hypertrophy of H9c2 cardiomyocytes (n=3; counting 50 cells). Magnification, x200. Comparisons between groups were completed using Dunnett's multiple comparison test after one-way ANOVA. Data are analyzed as mean \pm standard deviation. **P<0.01, ***P<0.001 and ****P<0.0001 vs. control group; #P<0.05 and ###P<0.001 vs. the succinate-stimulated group. MeBavaC: 4'-O-methylbavachalcone; p-, phosphorylated; TRITC, tetramethylrhodamine.

increased in the coronary sinus compared with in the venous blood in patients with STEMI, suggesting that it is released in the heart. Succinate concentrations in arterial, coronary sinus and peripheral venous blood are higher in patients with STEMI than in patients without STEMI or with stable angina pectoris (20). The mean succinate concentration in the coronary sinus blood of patients with STEMI is reported to be 2.73 μ M, which is \sim twice that of patients with angina pectoris and without STEMI (20). In addition, enhanced levels of succinate are closely associated with an increased risk of atrial fibrillation due to the extreme quartiles of TAC metabolites in patients with atrial fibrillation (37). Elevated myocardial energy expenditure has been associated with reduced left ventricular ejection fraction and an independent predictor of cardiovascular mortality. In patients with heart failure, serum succinate concentrations also significantly increased with increasing myocardial energy expenditure elevation (38). Succinate accumulates during ischemia and two-thirds of ischemic succinate accumulation is extracellular (40). In addition, succinate produced by gut microbiota is also directly related to the development of cardiovascular disease (41,42). Compared with healthy animals, the blood succinate concentration of spontaneously hypertensive rats, ob/ob mice, fa/fa rats and db/db mice was significantly increased. Therefore, ultra-high

succinate concentrations in the blood may be involved in the occurrence of numerous cardiovascular events. In the present study, 1 mM of sodium succinate incubated with H9C2 cardiomyocytes for 48 h promoted cardiomyocyte hypertrophy and increased the expression of cardiomyopathy-associated gene α -actinin (Fig. 2), which is partly consistent with the finding of Aguiar *et al* (24). Their findings indicate that intravenous injection of 0.066 mg/kg of sodium succinate salt into 8-week-old Wistar rats with cardiac ischemia once daily for 5 days can lead to cardiac hypertrophy. They further stimulated cardiomyocytes from neonatal (3-5 days old) Wistar rats with 1 mM of sodium succinate, which triggered hypertrophy of cardiac cardiomyocytes by activating the SUCNR1 receptor and through phosphorylation of ERK1/2 and expression of CaMKII δ (24). *In vivo* experiments in rats have also indicated that succinate enhanced pressure overload-induced right ventricular hypertrophy. Yang *et al* (43) selected three succinate concentrations of 30, 50 and 100 mg/kg/day in the pre-experiment. As rats given a dose of 100 mg/kg/day succumbed before the end of the experiment, 50 mg/kg/day was chosen as the follow-up experimental dose. Stimulation with 50 mg/kg/day succinate resulted in right ventricular hypertrophy in rats and enhanced ANP gene expression and Akt signaling (43). High sodium succinate concentrations (1 mM) also promote platelet aggregation and cross-cellular biosynthesis of leukotriene C₄ by activating SUCNR1 (36).

Succinate is primarily sensed by the G-protein-coupled receptor SUCNR1, which serves a role in extracellular metabolic stress signaling. SUCNR1 is widely expressed in various tissues, including the kidney, liver, heart and retinal, resulting in a wide range of physiological and pathological effects. The increase in blood succinate concentration in hypertension, obesity and diabetes model animals is consistent with SUCNR1 activation (19). SUCNR1 serves specific proinflammatory and antiinflammation roles in macrophages. When SUCNR1-expressing macrophages are activated by inflammatory signals, the accumulated succinate is released into the extracellular milieu. SUCNR1 activation binding to autocrine or paracrine succinate can perpetuate inflammation by enhancing IL-1 β production in macrophages (44). Several cardiac hypertrophy-related signals, including phosphorylation of ERK1/2, expression of CaMKII δ and translocation of histone deacetylase 5 into cytoplasm, are triggered by SUCNR1 activation and serve critical roles in the hypertrophic remodeling of the myocardium (24), indicating that cardiac hypertrophy is induced in a SUCNR1-dependent manner. SUCNR1 stimulation activates G α i and initiates G α q activity (45,46), which is required to trigger intracellular Ca²⁺ flux. SUCNR1 requires PLC β activation to increase intracellular Ca²⁺ through an inositol phosphate-dependent mechanism (47). The canonical Gi-G β γ -Ca²⁺ signaling also requires the participation of the G α q subunit in living cells (48). Ca²⁺ flux is the upstream signal of the CaMKII δ and calcineurin-NFAT pathways and abnormal Ca²⁺ flux is one of the molecular bases of cardiac hypertrophy (12,49,50). The results of the present study also revealed that succinate treatment promoted cardiomyocyte size enlargement and increased α -actinin expression (Figs. 2 and 4). The gene α -actinin is associated with dilated cardiomyopathy, impaired ventricular diastolic function and cardiac hypertrophy (51,52) and

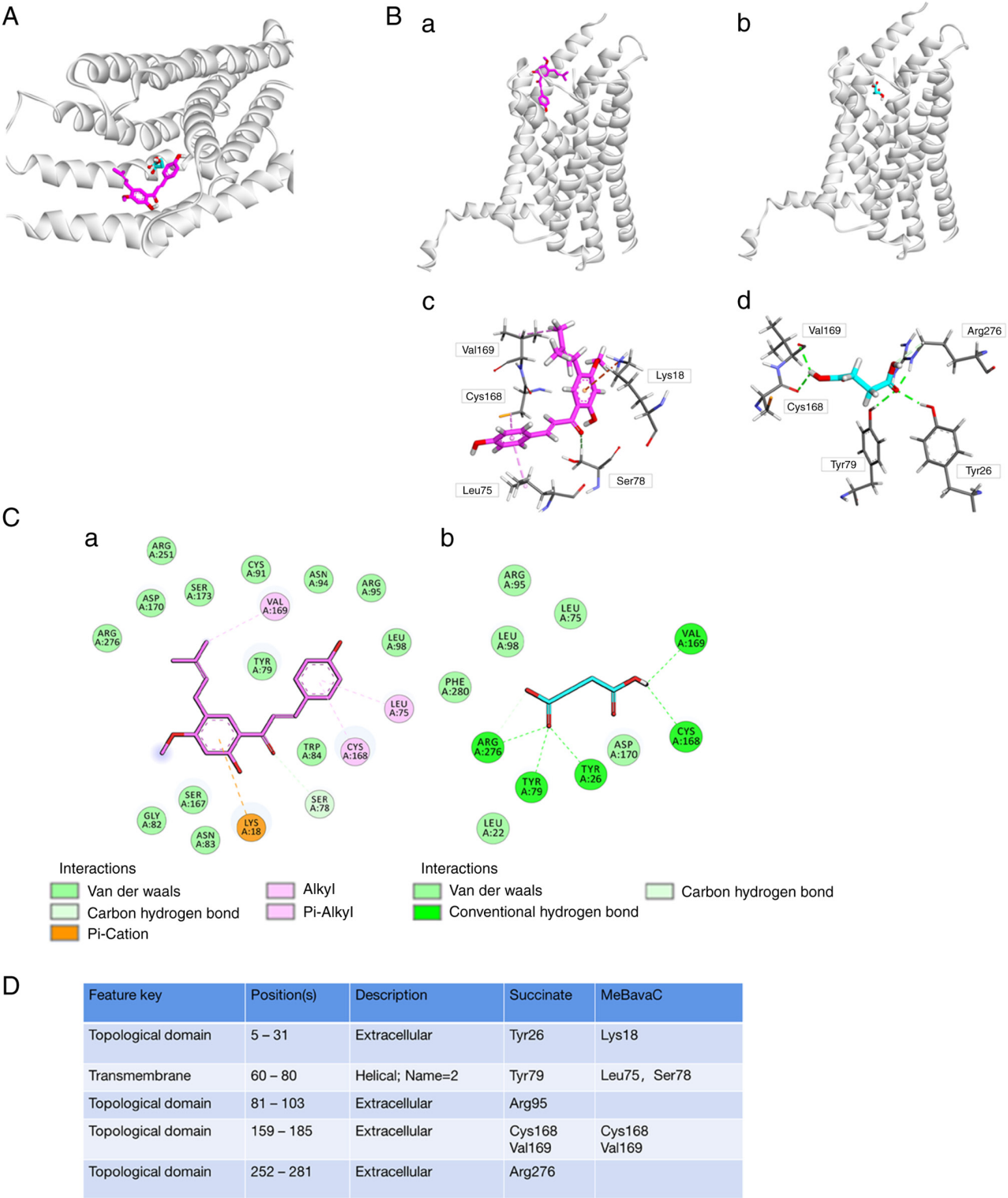


Figure 6. Molecular docking analysis of SUCNR1 with MeBavaC and succinate. (A) The orthosteric site of SUCNR1 (white) binding MeBavaC (magenta) and succinate (cyan). The docking modes were predicted by AutoDock Vina. (Ba and Bb) Overall stereoscopic docking modes of MeBavaC (magenta) and succinate (cyan) upon SUCNR1 (white); (Bc and Bd) Receptor-ligand interaction analysis of MeBavaC (magenta) or succinate (cyan) on the orthosteric site of SUCNR1; (Ca and Cb) 2D receptor-ligand interaction analysis of MeBavaC (magenta) or succinate (cyan) on the orthosteric site of SUCNR1; (D) Functional bond site for UCNR1 binding to MeBavaC or succinate. SUCNR1, succinate receptor 1; MeBavaC, 4'-O-methylbavachalcone.

α -actinin-1 promotes the activity of the L-type Ca^{2+} channel Cav 1.2 (53,54), which regulates cardiac hypertrophy (55,56). Together, these findings indicate the presence of at least two pathways by which stimulation of SUCNR1 increase intracellular Ca^{2+} flux to contribute to cardiac hypertrophy; by

triggering $\text{G}_{\alpha q}$ activity and $\text{PLC}\beta$ and activating the L-type Ca^{2+} channel Cav 1.2 opening.

Calcineurin-NFAT signaling is an important regulatory pathway of cardiac hypertrophy. However, whether succinate can activate calcineurin-NFAT signaling has not been

determined. To this end, the nuclear translocation of NFATc4 was observed in cardiomyocytes after they were treated with succinate. The results suggested that succinate-induced cardiomyocyte hypertrophy is mediated by the calcineurin-NFATc4 signaling (Fig. 3). Indeed, four NFAT members (NFATc1-c4) are expressed in cardiomyocytes; they trigger nuclear translocation upon calcineurin activation and yet promote cardiomyocyte hypertrophy (14,57). The data from the present study also indicated that succinate activated ERK1/2 and JNK signaling and that inhibition of ERK1/2 signaling, but not of JNK signaling, attenuated cardiomyocyte hypertrophy (Fig. 5). ERK1/2 stimulation induces cardiac hypertrophy, whereas JNK activation in cardiomyocytes directly antagonizes activated NFAT signaling (15,58). In cultured cardiomyocytes, ERK1/2 signaling inhibition attenuates hypertrophy induced by activated calcineurin. Nevertheless, the targeted inhibition of ERK1/2 signaling does not directly affect calcineurin-NFAT activation (58).

Fructus Psoraleae mainly contains coumarins, terpene phenols and prenylflavonoids, which are the material basis of drug therapy. MeBavaC is an active component of prenylflavonoids. The authors previously reviewed the mechanisms of five major psoralea prenylflavonoids and their multiple effects including anti-inflammation, cardiovascular protection, neuroprotection, osteoporosis improvement and intervention on diabetes and obesity (26). No cardiovascular pharmacological study of MeBavaC has been conducted and the present study revealed its mechanism in preventing cardiac hypertrophy. The results demonstrated that MeBavaC blocks both calcineurin-NFAT and ERK1/2 signaling and inhibits succinate-induced cardiomyocyte hypertrophy (Figs. 4 and 5). A number of prenylflavonoids, such as xanthohumol and icariin, have multiple cardiovascular protection functions. Xanthohumol inhibits abnormal ryanodine receptor Ca^{2+} release, possesses antiarrhythmic properties, attenuates isoproterenol-induced cardiac hypertrophy and fibrosis and protects rat myocardia from ischemia/reperfusion injury-induced ferroptosis (59-61). Icariin inhibits isoproterenol-induced cardiomyocyte hypertrophic injury and severe heart failure, protects cardiomyocytes from ischemia/reperfusion injury and attenuates Ang II-induced H9c2 cardiomyocyte hypertrophy and apoptosis (62-65). In Fructus Psoraleae, only bakuchiol, a terpene phenol, was reported to block aortic banding-induced cardiac hypertrophy by blocking the NF- κ B signaling pathway and to attenuate pressure overload-induced fibrosis and inflammation (66).

In 2011 year, compounds numbered 2C, 4C and 5G were identified as potent and selective antagonists for human SUCNR1 (67). Another group recently discovered an optimized SUCNR1 antagonist scaffold to improve oral exposure by designing Zwitterionic Derivatives with salt bridges (68). Using a humanized rat construct, the X-ray structure of optimized compound 20 binding to SUCNR1 was determined (67). On this published humanized rat SUCNR1 construct, the team also confirmed the structure of another antagonist, NF-56-EJ40, in complex with its receptor, SUCNR1 (34). In the present study, molecular docking analysis indicated that MeBavaC binds to the orthosteric site of succinate receptor SUCNR1 through alkyl and Pi-alkyl, which are more compatible than succinate with a higher docking score (Fig. 6). When

binding to the pseudo-oxyanionic protein subunit group of SUCNR1, MeBavaC formed stable Pi-cation complexes with Lys18, as well as Pi-alkyl bonds with Leu75 (Fig. 6). The results indicated that MeBavaC inhibited SUCNR1 activity.

The results of the present study revealed that succinate stimulated cardiomyocyte hypertrophy through the calcineurin-NFATc4 and ERK1/2 pathways. MeBavaC ameliorated succinate-induced cardiomyocyte hypertrophy mediated by calcineurin-NFATc4 and ERK1/2 pathways. Molecular docking analysis indicated that MeBavaC had a certain affinity for the succinate receptor SUCNR1 and the ability to inhibit SUCNR1 binding with succinate. Therefore, MeBavaC has fully demonstrated the potential ability to improve myocardial hypertrophy and heart failure, associated with elevated myocardial energy expenditure and an increased extra serum succinate concentration.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HS implemented most of the experiments, data collation and statistics and wrote the first draft of the manuscript. GZ implemented the molecular docking research, data collation and statistical analyses and wrote the first draft of the molecular docking section of the manuscript. SL participated in part of the experimental design, gave technical guidance on cardiomyocyte culture, conducted financial management and assisted in the revision of the manuscript. JL provided experimental design for the Western blot, validated some of the real-time PCR experiments, provided technical guidance on microscopy and monitored the progress of the experiments, and assisted in the revision of the manuscript. JWX proposed the research idea, obtained financial support and revised and finalized the manuscript. HS, GZ and JL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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