ORIGINAL RESEARCH

ALP Inhibitors Inhibit Inflammatory Responses and Osteoblast Differentiation in hVIC via AKT-ERK Pathways

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ABSTRACT

Objective • The aim of this study was to explore the calcification process of aortic valve interstitial cells and its potential association with osteogenic differentiation and alkaline phosphatase (ALP) activity.

Methods • The study patients were divided into 3 groups: the control group, the osteogenic induction medium (OM) group and the OM+ALP inhibitor group. Cell calcification was measured by alizarin red S staining and alizarin red S dye released by extracellular matrix (ECM) was quantified by spectrophotometry. Immunohistochemical staining was performed on valve tissues of patients harboring calcified and non-calcified aortic valve disease. Expression of bone morphogenetic protein (BMP), runt related transcription factor 2 (RUNX2), osteocalcin and osteopontin (OPN), was evaluated using immunohistochemistry, and expression of osteogenic specific markers (BMP, RUNX2 and OPN) was detected using Wesern blot analysis. RNA sequencing was analyzed to further study the exact mechanism of ALP inhibitors in terms of inhibiting the osteogenic differentiation of valvular interstitial cells (VIC). The mRNA levels of tumor necrosis factor alpha (TNF-α), Toll-like receptor 4 (TLR4) and NOD-like receptor thermal protein domain associated protein 3 (NLRP3), were detected using reverse-transcription quantitative polymerase chain reaction (RT-qPCR). In addition, Western blot analysis was performed to evaluate the expression of phosphorylated extracellular regulated protein kinases (ERK), nuclear factor κ B inhibitor α (IκBα) and protein kinase B (AKT) in protein.

Results • Alizarin red staining was positive in the OM and OM+ALP inhibitor groups, and calcified nodules were formed in VIC, which showed a significant difference compared with the control group ($P < .05$). The semi-quantitative level of calcification in the OM group was higher than in the control group ($P < .05$), and the semi-quantitative level of calcification in the OM+ALP inhibitor group was lower than in the OM group ($P < .05$). ALP staining intensity, ALP activity and messenger RNA (mRNA) levels of BMP, RUNX2, osteocalcin, OPN, ERK, IκBα, AKT, TNF-α, Toll-like receptor 4 (TLR4) and NLRP3 inflammasome (NLRP3) in the OM group were higher than in the control group ($P < .05$). ALP staining intensity, ALP activity and mRNA expressions of BMP, RUNX2, osteocalcin, OPN, phosphorylated ERK, IκBα, AKT, TNF-α and NLRP3 in the OM+ALP inhibitor group were lower than in the OM group ($P < .05$). Compared with the control group, 723 genes were upregulated and 248 genes were downregulated in the OM group. Compared with the OM group, 352 genes were upregulated and 586 genes were downregulated in the OM+ALP inhibitor group.

Conclusion • We suggest that ALP inhibitors have potential in terms of inhibiting the inflammatory response and osteoblast differentiation of human VIC (hVIC) via the TLR4, AKT, ERK and NLRP3 pathways. (*Altern Ther Health Med.* 2023;29(1):58-65).

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INTRODUCTION

Calcified aortic valve disease (CAVD) has become the most common heart valve disease, and is characterized by calcification, leading to aortic stenosis and subsequent heart failure.[1] CAVD is highly associated with patients harboring type 2 diabetes (approximately 45%), with increased risk for degenerative aortic valve disease and cardiovascular mortality. However, the risk factors responsible for this condition are largely unknown. There is no pharmacological

method available to slow CAVD progression, and surgical repair and valve replacement have been endorsed as the main treatment methods in CAVD.[2]

The human aortic valve (AV) is an avascular trilobal structure with a thickness of approximately 1 mm, which is composed of outer valve endothelial cells (VEC) and 3 inner layers: the fibrous, sponge and ventricular layer.[3] VECs maintain the steady state of the valve by regulating permeability, adhesion of inflammatory cells and interstitial cell paracrine signals. Differentiation of valvular interstitial cells (VICs) phenotype from fibroblasts to myofibroblasts is extremely important for wound-healing *in vivo*, as well as for the production of natural extracellular matrix during valve tissue engineering.[4] With further research, CAVD is currently regarded as an active cell-driven regulatory process, which mainly involves chronic inflammation, lipid deposition, angiogenesis and osteogenic phenotype VIC. Numerous clinical studies are available that show that the drugs currently on the market fail to prevent or slow the progression of CAVD during the treatment of arteriosclerosis.[5] Therefore, it is both extremely urgent and significant to identify drugs that inhibit the osteogenic phenotype transformation of aortic valve interstitial cells and delay valve calcification.

In vitro and clinical studies have shown that a series of active osteogenesis processes that contribute to CAVD, and the osteogenesis activity is caused by inflammation. Aortic valve interstitial cells (AVICs) are the main cell type found in aortic valve leaflets, which mainly participate in the CAVD process by inducing inflammation and osteoblast differentiation.[6] Other mechanisms include the deposition of atherosclerotic lipoprotein in a stenosed aortic valve and the activation of the renin-angiotensin system. Inflammation is the leading factor in CAVD.[7]

Alkaline phosphatase (ALP) is closely related to aortic calcification and is a reliable indicator for evaluating vascular calcification. High serum ALP levels are strongly associated with the incidence of cardiovascular events, and high serum ALP levels are involved in the cardiovascular damage process, leading to an increased risk for a poor prognosis. Hence, it is of great clinical value and significance to identify valid drugs that may regulate the inflammatory reaction and delay the occurrence of aortic valve calcification. In our study, we aimed to investigate the calcification process of AVICs and its association with osteogenic differentiation and ALP activity.

METHODS AND MATERIALS

Study Materials

Isolation of VICs. Isolated VICs were collected from patients via acute aortic dissection under aseptic conditions. The mean patient age was 58.56 ± 9.96 years, the male/female ratio was 15:15 and the disease course was 10.52 ± 2.96 months. The average age of normal controls was 59.12±10.15 years, the male/female ratio was 16:14, and the disease course was 9.26 ± 2.29 months. After thoroughly washing with 0.1 mL phosphate buffered saline, the surface of the valve leaflet was gently scraped with a blade 2 to 3 times. Primary human VIC was obtained by resuspension and centrifugal precipitation in 5 mL high-glucose DMEM containing 10% fetal bovine serum (FBS). The 3rd generation cells were used in all experiments.[8]

VIC culture. VIC was inoculated in a 24-well plate at a density of 2×10^5 cells/well. The medium was supplemented every 3 days for 2 weeks.

Cell treatment and grouping. For the osteogenic differentiation (OD) model, hVIC was cultured in osteogenic induction medium (OM) (Huxma-90021; Cyagen Biosciences, Santa Clara, California, USA). The ALP inhibitor was dissolved in dimethyl sulfoxide (DMSO). The study patients were grouped as follows: the control group (no OM or ALP inhibitor treatment), OM group (OM treatment only) and the OM+ALP inhibitor group (OM combined with ALP inhibitor treatment).

Methods

Calcification analysis. Cells were cultured in OM with and without ALP inhibitor for 21 days. The degree of cell calcification was measured according to the manufacturer's instructions using alizarin red S (#0223; ScienCell Research Laboratories, Carlsbad, California USA) staining. After washing with distilled water twice, an image was taken to evaluate the degree of calcification. Calcium salt deposition in osteoblast-induced and uninduced cell matrix was detected via the alizarin red semi-quantitative method to study the changing trend of calcium salt deposition in the cell matrix. The calculation formula was: measured value of samples A = absorbance value of samples; A- absorbance value of single cell group A.

ALP staining. VIC was induced by OM for 21 days and stained for 15 minutes with an ALP staining kit (#1102-100; InnovativeCellularTherapeutics, Shanghai, China). At the end of the process, the staining of VIC after different treatments was observed under the microscope (CarlZeiss AG, Jena, Germany). ALP activity was measured with an ALP detection kit (Nanjing Jiancheng Institute of Bioengineering, Nanjing, Jiangsu, China).

Immunohistochemical staining. Patients with CAVD and normal controls were selected by the researchers. Pathology specimens from patients with rheumatic valvular heart disease, endocarditis and congenital aortic valve disease were excluded from the study. The primary antibodies used in this study were BMP, Runx2, osteocalcin and OPN, which were incubated overnight at 4°C. Biotin-labeled secondary antibodies (bioss, IHC001, IHC003) were incubated for 30 minutes, washed with PBS for 3 minutes, 3 times and horseradish peroxidase was added for 15 minutes. DAB was used to detect partial protein levels. The nucleus was stained with hematoxylin.

Western blot analysis. Total protein was isolated from tissues or cells 48 hours after transfection using a radioimmunoassay buffer. The protein was separated via SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane and sealed with 5% skimmed milk powder at room temperature for 1 hour. Subsequently, the PVDF membrane was incubated with the corresponding diluted

antibody at 4°C overnight. The following primary antibodies were used: BMP (1:1000; Abcam, Boston, Massachusetts, USA), Runx2 (1:1000; Abcam), osteocalcin (1:500; Abcam) and OPN (1: 500; ABCAM), AKT (1:2000; Abcam), p-AKT (1:1000; Abcam), IκBα (1:2000; Abcam), IκBα (1:2000;, Abcam), p-IκBα (1:1000; Abcam), ERK (1:2000; Abcam), p-ERK (1:1000; Abcam) was used as the standardized medium. The blot was incubated with horseradish peroxidase coupled goat anti-rabbit immunoglobulin G (IgG) secondary antibody (ab97051, 1:2000; Abcam) for 1 hour.

mRNA expression profile. RNA sequencing (RNA-seq) was applied to quantitatively study the changes in cell mRNA spectrum with different treatments. Isolated RNA was sequenced by BGICo., Ltd. (Shenzhen, China) using RNA-seq on a BGISEQ-500 instrument. Sequencing results were further analyzed to identify differentially expressed genes (DEG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using R language, version 3.5.1.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). The Quantitec reverse transcription (RT) kit from Takara was used for reverse transcription. Quantitative PCR (qPCR) was performed using SYBR® Green (B21202; bimake.com, Houston, Texas, USA) and Roche LightCycler® 96 system (Roche LifeScience, TNF-α, TLR4, NLRP3 and GAPDH were quantified using qPCR based on specific conditions. GAPDH was used as a reference control gene for other genes. The RT level of the target gene was calculated by relative quantification (2-delta-delta CT method). All experiments were repeated 3 times.

Statistical analysis

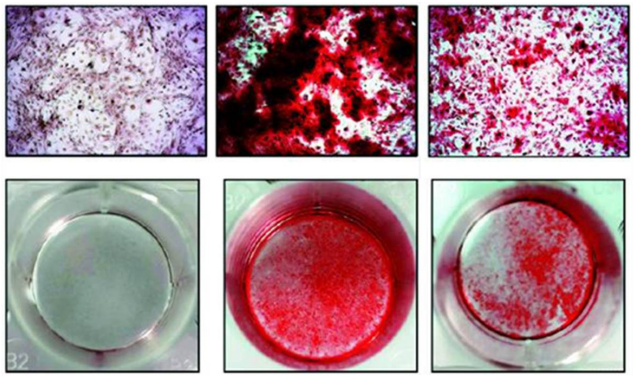
Statistical analysis was performed using IBM® SPSS version 21.0 statistical software (IBM Corp., Armonk, New York, USA). R language (version 3.5.1) was then applied to further analyze the RNA sequencing results. The measured data and other data were analyzed and expressed as mean ± standard deviation (SD). All semi-quantitative measurements were captured by ImageJ (National Institutes of Health, USA) software. Statistical comparison was performed via ANOVA to evaluate the differences between groups. $P < .05$ reflected statistical significance.

RESULTS

Comparison of VIC Calcification in the Study Groups

Alizarin red staining was positive in the OM and OM+ALP inhibitor group, and calcified nodules of VIC were formed, showing a significant difference compared with the control group ($P < .05$). The semi-quantitative level of calcification in the OM group was significantly higher than in the control group ($P < .05$), while markedly lower results were found in the OM+ALP inhibitor group compared with the OM group, and in the OM+ALP inhibitor group were higher than in the control group ($P < .05$). Based on these results, we found that ALP inhibitor was able to inhibit the calcification of VIC induced by OM (see Figure 1; Table 1).

Figure 1. Alizarin red staining



Note: The semi quantitative calcification level in the OM group was higher than in the control group ($P < .05$), and lower in the OM+ALP inhibitor group than in the OM group ($P < .05$).

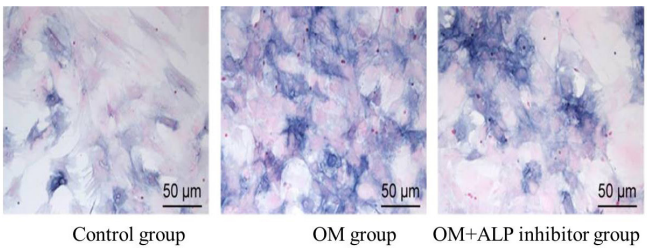
Abbreviations: ALP, alkaline phosphatase; OM, osteogenic induction medium.

Table 1. Semi-Quantitative Level of Calcification (n = 15)

Groups	Dyeing intensity	Semi-quantitative calcification level
Control	1.02 ± 0.13	1.00 ± 0.16
OM	5.78 ± 0.47	6.18 ± 0.46
OM+ALP inhibitor	2.26 ± 0.18	3.13 ± 0.12
Variance ratio	16.032	12.325
P value	<.001	<.001

Abbreviations: ALP, alkaline phosphatase; OM, osteogenic induction medium.

Figure 2. ALP staining.



Note: The ALP staining intensity and activity in the OM group were higher than in the control group ($P < .05$), and lower in the OM+ALP inhibitor group than in the OM group ($P < .05$).

Abbreviations: ALP, alkaline phosphatase; OM, osteogenic induction medium.

Table 2. ALP Staining Intensity and Activity (n = 15)

Groups	Dyeing intensity	Activity of ALP (μmol/min/mg)
Control	1.00 ± 0.11	2.37±0.25
OM	4.23 ± 0.25	36.48±2.34
OM+ALP inhibitor	1.89 ± 0.14	13.56±1.27
Variance ratio	13.632	15.230
P value	<.001	<.001

Abbreviations: ALP, alkaline phosphatase; OM, osteogenic induction medium.

Table 3. Expression Level of BMP, RUNX2 and OPN Protein (n = 15)

Groups	BMP	RUNX2	Osteocalcin	OPN
Control	0.58 ± 0.09	0.96 ± 0.14	1.12 ± 0.13	1.03 ± 0.12
OM	1.89 ± 0.12	3.25 ± 0.24	4.37 ± 0.45	3.58 ± 0.64
OM+ALP inhibitor	0.97 ± 0.12	1.15 ± 0.18	2.06 ± 0.17	1.89 ± 0.23
Variance ratio	15.231	12.306	14.237	18.529
P value	<.001	<.001	<.001	<.001

Abbreviations: ALP, alkaline phosphatase; OM, osteogenic induction medium.

Table 4. Expression Level of BMP, RUNX2 and OPN Protein (n = 15)

Groups	BMP	RUNX2	OPN
Control	1.02 ± 0.15	0.85 ± 0.14	0.93 ± 0.17
OM	6.33 ± 0.47	5.78 ± 0.25	5.88 ± 0.37
OM+ALP inhibitor	2.85 ± 0.23	1.96 ± 0.18	2.14 ± 0.26
Variance ratio	15.238	18.267	16.329
P value	<.001	<.001	<.001

Abbreviations: ALP, alkaline phosphatase; OM, osteogenic induction medium

Table 5. Database of Essential Genes (DEG) Analysis (n = 15)

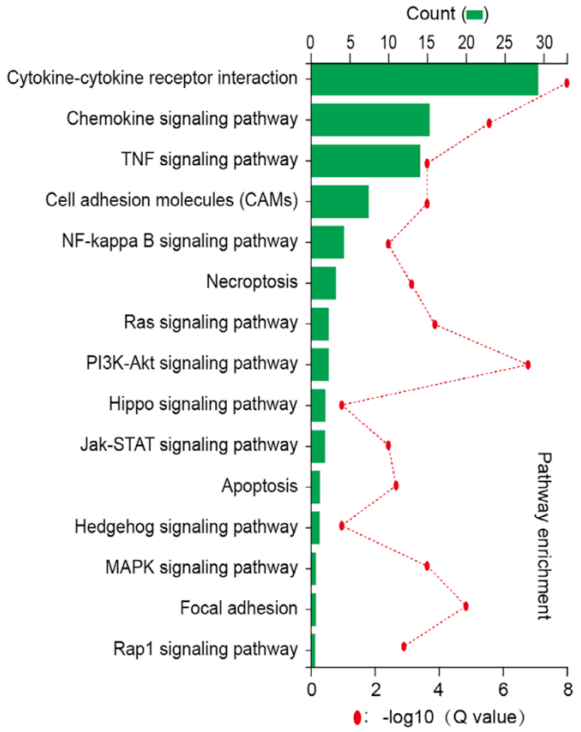
Groups	Upregulated genes	Downregulated genes
Control vs OM	723	248
OM vs OM+ALP inhibitor	352	586

Abbreviations: ALP, alkaline phosphatase; OM, osteogenic induction medium.

Comparison of ALP Levels in the Study Groups

Regarding ALP staining intensity and activity, the OM group was associated with higher staining than the control group ($P < .05$), while in the OM+ALP inhibitor group were lower than in the OM group, and higher in the OM+ALP inhibitor group than in the control group ($P < .05$), indicating the role of the ALP inhibitor in inhibiting the activation of ALP induced by OM (see Figure 2; Table 2).

Figure 3. KEGG enrichment analysis.



Note: Similar gene expression profiles were observed between the OM group and the OM+ALP inhibitor group, in which 352 genes were upregulated and 586 genes were downregulated.

Abbreviations: ALP, alkaline phosphatase; KEGG, Kyoto Encyclopedia of Genes and Genomes; OM, osteogenic induction medium.

Comparison of Bone-Specific Markers in the Study Groups Using Immunohistochemical Staining

The expression levels of BMP, RUNX2, osteocalcin and OPN were higher in the OM group than in the control group and the OM+ALP inhibitor group, and were higher in the OM+ALP inhibitor group than in the control group ($P < .05$) (see Table 3).

Comparison of Bone-Specific Markers in the Study Groups Using Western Blot Analysis

Higher expression levels of BMP, RUNX2 and OPN protein were found in the OM group than in the control group and OM+ALP inhibitor group, and these levels were higher in the OM+ALP inhibitor group than in the control group ($P < .05$). This result indicates the role of an ALP inhibitor in inhibiting the osteogenic differentiation induced by OM (see Table 4).

Identification of DEG and KEGG Enrichment Analysis

Compared with the control group, 723 genes were upregulated and 248 genes were downregulated in the OM group. In addition, similar gene expression profiles were observed between the OM group and the OM+ALP inhibitor group, in which 352 genes were upregulated and 586 genes were

downregulated. Based on DEG analysis, KEGG signal pathway enrichment analysis was carried out. Our results showed that these DEGs were highly enriched in PI3K-AKT, TNF, Jak-STAT and MAPK signaling pathways (see Figure 3; Table 5).

Levels of Inflammatory Cytokine mRNA

The mRNA expression levels of TNF-α, TLR4 and NLRP3 mRNA in the OM group were higher than in the control group ($P < .05$), while there were lower levels in the OM+ALP inhibitor group compared with the OM group, and higher levels in the OM+ALP inhibitor group than in the control group ($P < .05$). This suggests that the ALP inhibitor prevented an inflammatory reaction (see Table 6)

Comparison of ERK, IκBα and AKT Signal Pathways in the Study Groups

The expression levels of phosphorylated ERK, IκBα and AKT protein in the OM group were significantly higher than in the control group ($P < .05$), while lower levels were found in the OM+ALP inhibitor group than in the OM group, and there were higher levels in the OM+ALP inhibitor group than in the control group ($P < .05$). The results showed that osteogenic differentiation of hVIC were negatively influenced by the ALP inhibitor (see Figure 4; Table 7).

DISCUSSION

VEC forms a continuous monolayer that covers both sides of the valve, and is connected with the endothelial cell layer in the endocardium or adjacent great vessel area. In addition, VEC has been proven to inhibit and regulate the pathological proliferation and differentiation of VIC, and maintain the valve steady state through paracrine signals.[9] VIC plays an important role in maintaining the steady state and function of the aortic valves through proliferation, matrix metalloproteinase secretion and new ECM molecules. According to histological analysis and non-invasive imaging technology findings, the progress of CAVD can be divided into 3 stages: plaque-like lesions, late lesions showing microcalcification and end-stage lesions dominated by significant calcium deposition.[10] Recent studies have shown that normal human aortic valves contain relatively few macrophages, while a large number of leukocytes and macrophages had infiltrated resected calcified human aortic valves.[11-13]

BMP is a group of ligand proteins with multiple functions that are far beyond the scope of promoting bone formation.[14] The transforming growth factor beta (TGF-β) ligand family is composed of subfamilies, including BMP, growth differentiation factor (GDF), activin and inhibin, nodes, left and right determinants, etc. Some of these are named for their structural and functional similarities and environments. Like other TGFβ-β family ligands, BMP ligand dimer participates in signal transduction by promoting the assembly of heterogeneous complexes of BMPI and II receptors on the cell surface.[15] TGF-β1 in most cells signals via ALK5 and TGFBR2, while activin and GDF bind ACVR2A/B and ALK4, 5 or 7. BMP receptors can also activate non-SMAD pathways.[16] BMP signals are also regulated by antagonists, such as Gremlin and Noggin, which bind to and inhibit BMP2 and BMP4. In a similar fashion, matrix Gla protein is a secreted carboxyglutamic acid-modified protein that can bind to and inhibit BMP2 and BMP4 signal transduction.[17]

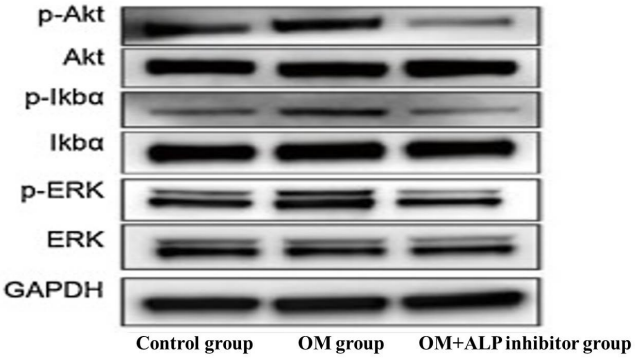
BMP affects the formation of bone and cartilage. In the classic signaling pathway, BMP initiates signaling by binding to the heterogeneous complex of type I and type II receptors.

Table 6. The mRNA Level of Inflammatory Cytokines (n = 15)

Groups	TNF-α	TLR4	NLRP3
Control	1.06 ± 0.13	1.08 ± 0.17	1.23 ± 0.12
OM	5.32 ± 0.39	3.24 ± 0.16	4.35 ± 0.28
OM+ALP inhibitor	2.18 ± 0.24	1.95 ± 0.17	2.24 ± 0.23
Variance ratio	13.024	15.097	16.879
P value	<.001	<.001	<.001

Abbreviations: ALP, alkaline phosphatase; OM, osteogenic induction medium.

Figure 4. Protein expression of phosphorylated ERK, IκBα and AKT were detected by Western blot analysis.



Note: Phosphorylated ERK and IκBα and Akt protein expression levels were higher in the OM group than in the control group ($P < .05$), and the OM+ALP inhibitor group, phosphorylated ERK, IκBα and Akt protein expression levels in the OM group were lower than in the OM group ($P < .05$).

Abbreviations: ALP, alkaline phosphatase; OM, osteogenic induction medium.

Table 7. Expression Level of Phosphorylated ERK, IκBα and AKT Protein (n = 15)

Groups	p-AKT	p-IκBα	p-ERK
Control	0.48 ± 0.05	0.36 ± 0.07	0.75 ± 0.11
OM	1.28 ± 0.21	1.23 ± 0.06	2.64 ± 0.21
OM+ALP inhibitor	0.56 ± 0.09	0.45 ± 0.05	1.03 ± 0.16
Variance ratio	12.329	13.264	14.264
P value	<.001	<.001	<.001

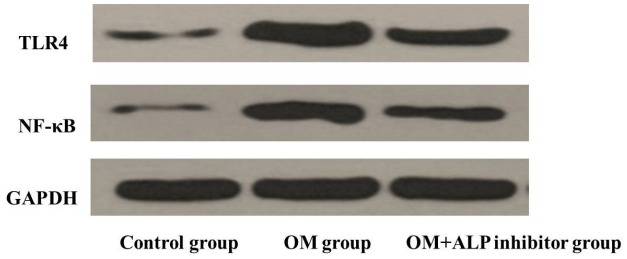
Abbreviations: ALP, alkaline phosphatase; OM, osteogenic induction medium

Table 8. Expression Level of TLR4 and NF-κB Protein (n = 15)

Groups	TLR4	NF-κB
Control	0.41 ± 0.03	0.32 ± 0.09
OM	1.37 ± 0.17	1.29 ± 0.15
OM+ALP inhibitor	0.61 ± 0.11	0.48 ± 0.07
Variance ratio	13.469	12.792
P value	<.001	<.001

Abbreviations: ALP, alkaline phosphatase; OM, osteogenic induction medium.

Figure 5. Protein expression of TLR4 and NF-κB was detected by Western blot analysis.



Type II receptor kinase phosphorylates type I receptor, and then exerts intracellular signal transduction by activating receptor-regulated SMADs (R-SMADs). After being released from the receptor complex, R-SMADs interact with SMAD, a common mediator, and transfer to the nucleus to regulate the transcription of the target gene. Among these factors, BMP-2 appears to play a central role in the differentiation of plastic cell population into osteogenic phenotype by inducing MSX-2, Runx2 and ALP.[18] Functional toll-like receptors (TLR-2) and TLR-4 are important mediators of inflammation. VIC is stimulated by BMP-2 signaling to obtain osteoblast-like phenotype.

The fiber and calcification reconstruction of the ECM layer is the pathological sign of calcified valve disease, which is a common pathological cardiovascular disease.[19] TGF-β is a known regulator of vascular smooth muscle cell calcification and osteoblast differentiation, and it has been proven that TGF-β is present in calcified aortic valve (CAV). In endothelial cells of calcified human AV fibers, the expression of phosphorylated SMAD1/5/9 was significantly higher than in cells without calcified valve fibers, while the expression of phosphorylated SMAD2/3 remained relatively unchanged, which was consistent with activation of the BMP signal in calcified AV fibers (see Figure 1). In mouse studies, endothelial AV cells showed increased SMAD1/5/9 phosphorylation and increased expression of osteochondral genes ALP, Runx2 and osteopontin. In our study, it was proven that OM induced increased expression of BMP, Runx2, osteocalcin and OPN, which was inhibited by the ALP inhibitor.

The serum ALP level in patients with CAVD is higher than in patients without CAVD, and studies have shown that serum ALP is a risk factor for cardiac valve calcification, further confirming the close relationship between high serum ALP levels and cardiac valve calcification. ALP is a plasma membrane-bound glycoprotein. These enzymes are widely distributed in nature, including prokaryotes and higher eukaryotes, except in some higher plants.[20] ALP is produced in the early growth stage and is commonly found on the cell surface and in the matrix vesicles of all tissues, bones and calcified cartilage. Subsequently, although some genes are upregulated (such as osteocalcin), ALP expression is decreased.[21] BMP/RUNX2 and the Wnt signal cascade communicate with each other; this is the main pathway that regulates osteoblast differentiation, chondrogenesis and ALP expression. In addition, since various model gene products, such as HOX10, were detected in the BMP/RUNX2 system, it is relatively reasonable to think that they have the ability to promote the differentiation of mesenchymal stem cells into osteoblasts and directly control the expression of TNAP (and other genes) via chromosome remodeling.[22]

Recently, a large number of non-coding RNAs, such as microRNA and long non-coding RNA, have been reported that regulate the expression of ALP during mineralization.[23] Wnts and its co-receptor LRP5/6 bind to curly family receptors. Wnt/Frizzled/LRP5 activation begins with a typical cascade reaction defined by β-catenin aggregation and nuclear transfer. Activation of BMP-BMPR-SMADs signal or FGF-FGFR-MAPK signal can increase ALP expression and osteoblast differentiation. IGF is a powerful ligand that can activate the PI3K/Akt pathway to promote ALP expression and osteoblast differentiation. Various studies have reported ALP expression in osteoblasts.

Based on our results, ALP inhibitor was able to inhibit OM-induced ALP activation. As the exact mechanism of ALP inhibitor inhibiting OM-induced calcification of AVIC is unknown, we performed high-throughput gene expression analysis to quickly and accurately identify the related molecular signaling pathways. DEG selected by transcriptome sequencing was highly enriched in TNF, PI3K-AKT, mTOR, toll-like receptor and NOD-like receptor signaling pathways. Among these, NOD-like receptor signaling pathways were the most common signaling pathways mediated by inflammatory reaction.

Inflammation is recognized as the main response of innate immunity. The pathogenesis of CAVD is multifactorial, including inflammation, hemodynamic factors, fibrosis and active calcification. In the development of CAVD, increasing evidence is available that shows that inflammation plays a pivotal role in the initiation and transmission of diseases, especially the function of TLR.[24] TLR mediates inflammation and the outside immune system, and is significant in controlling infection and maintaining tissue homeostasis. Other mechanisms include the deposition of atherosclerotic low-density lipoprotein (LDL) and lipoprotein (a) in the stenosed aortic valve and the activation of renin-angiotensin system. Typical calcification during CAVD is considered to be an inflammation-dependent process. The progression of

CAVD can be divided into 3 stages. Inflammation involves the initial stage and the transmission stage, while in the final stage, calcification rather than inflammation is dominant.

Congenital immune receptors, or toll-like receptors (TLR), are particularly used to identify conserved motifs in pathogens; that is, so-called pathogen-related molecular patterns (PAMP) and endogenous molecules released when tissues are damaged. The TLR family is composed of transmembrane receptors that recognize a wide range of ligands, and each member senses a specific set of molecular patterns. In recent studies, the new role in the cardiovascular system of TLR in inflammatory diseases such as atherosclerosis and ischemia/reperfusion injury and CAVD have been elucidated. The discovery of pathogen cargo in the narrow aortic apex prompted researchers to investigate the hypothetical role of TLR in CAVD. IL-37 negatively regulates the osteogenic reaction of AVIC to PAMP (such as TLR4/2 agonist) and DAMP (such as oxLDL) by inhibiting the activity of ERK1/2 and NF-κ B. It is worth noting that IL-37 transgenic mice can prevent early aortic valve disease caused by long-term exposure to LPS and other proinflammatory agents. Of note, the anti-inflammatory effect of IL-37 is specific to the TLR4/2 ligand.

The adaptive immune system plays a key role in cardiovascular disease. Several studies have demonstrated the correlation between inflammation and aortic valve calcification. AVIC expresses functional toll-like receptors TLR2 and TLR4, which are important mediators of innate immune response and inflammation. TLR4 and TLR2 ligands induce the early activation of p38MAPK in aortic valve interstitial cells. Stimulation of TLR2 and TLR4 signaling pathways leads to pro-inflammation and differential expression of bone factors in AVIC and PVIC. The expression of TLR2, TLR4 and BMP-2 was different in AVIC separated from normal valves and stenotic valves. Compared with VIC in the aortic valves of adults and children, adult AVIC showed greater inflammatory and osteogenic response to TLR4 stimulation than pediatric AVIC.

Our data confirm that these important inflammatory markers always exist in human tissues and isolated heart valves. On the other hand, AVIC is stimulated by TLR agonist upregulated BMP-2 and short stature-related transcription factor 2 (Runx2). The changes in Runx2 and osteopontin levels are preceded by phosphorylation of SMAD1 and extracellular signal-regulated kinase 1/2, but not p38 mitogen-activated protein kinase. The levels of Runx2 and osteopontin are decreased by silencing SMAD1, while the expression of osteopontin is decreased by the inhibition of extracellular signal-regulated kinase 1/2, but without affecting the expression of Runx2. In addition, AVIC stimulated by BMP-2 upregulates ALP, and the formation of calcified nodules in VIC depends on ALP activity. TNF-α is one of the important inflammatory factors that is expressed in aortic stenosis and positively correlated with the clinical severity of aortic stenosis. Treatment with TNF-α also promoted the expression of osteopontin and Runx2mRNA. Clinical evidence has shown that coronary artery calcification is related to inflammation caused by rheumatoid arthritis. In this study, we provide the first evidence of ALP inhibitor as an effective inflammatory inhibitor of OM-induced calcification of human AVIC. We confirmed the potential of anti-inflammatory intervention on CAVD.

NLRP3 inflammatory corpuscle is the core factor of the NOD-like receptor signaling pathway, and it is a cytoplasmic complex involved in the early inflammatory reaction.[25] It has been proven that NLRP3-3 inflammatory corpuscles contribute to phenotype transformation, proliferation and vascular remodeling of vascular smooth muscle cells in patients with hypertension. Furthermore, ALP inhibitor treatment significantly damaged the phosphorylation of AKT and ERK needed to promote cell proliferation. Therefore, our results confirmed that ALP inhibitor inhibited the growth of AVIC by inhibiting the phosphorylation of AKT and ERK.

RNA sequencing was performed to investigate the exact mechanism of ALP inhibitor in terms of inhibiting VIC osteogenic differentiation. Based on our results, 723 genes were upregulated and 248 genes were downregulated in the OM group compared with the control group. In addition, similar gene expression profiles were observed between the OM group and the OM+ALP inhibitor group, in which 352 genes were upregulated and 586 genes were downregulated. Based on DEG analysis, KEGG signal pathway enrichment analysis was performed and these DEGs were highly enriched in the medium.

Transcriptome sequencing analysis was used to study the global gene expression of hVIC after various treatments, to further study the mechanism of ALP inhibitors in reducing OM-induced calcification of hVIC. DEG selected by transcriptome sequencing was highly enriched in PI3K-AKT, TNF, Jak-STAT and MAPK signaling pathways. Compared with the OM group, 586 genes were downregulated in the OM + ALP inhibitor group, which confirmed that ALP inhibitors could effectively reverse the abnormal expression of hVIC induced by OM. In our study, the JAK-STAT pathway in DEGs was affected by ALP inhibitor in the OM group and the control group, as well as in the OM+ALP inhibitor group.

Study Limitations

This study had several limitations that should be acknowledged. On one hand, only *in vitro* experiments were conducted to identify the role of ALP inhibitors in the osteogenic differentiation of hVIC. The role of ALP inhibitors during CAVD progression *in vivo* requires further study. In addition, the targets of ALP inhibitor are understudied, and their potential mechanism should be established by scientific investigations to treat valvular disease.

CONCLUSIONS

Our study offers some important conclusions. ALP inhibitors exerted ability in inhibiting inflammatory reaction and osteoblast differentiation of hVIC via the AKT, ERK and

NLRP3 pathways. However, further studies are needed to confirm the potential medical value of ALP inhibitors in the prevention of CAVD.

CONFLICT OF INTEREST

None.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study protocol was reviewed and approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, China. All patients and their families voluntarily participated in the study and signed an informed consent form.

REFERENCES

1. Chen Y, Zhao X, Wu H. Arterial stiffness: A focus on vascular calcification and its link to bone mineralization. *Arterioscler Thromb Vasc Biol*. 2020;40(5):1078-1093. doi:10.1161/ATVBAHA.120.313131
2. Wang B, Lin HQ, Li F, Mao ZF, Dong NG. Aβ40 promotes the osteoblastic differentiation of aortic valve interstitial cells through the RAGE pathway. *Curr Med Sci*. 2020;40(5):931-936. doi:10.1007/s11596-020-2264-3
3. Jinnouchi H, Sato Y, Sakamoto A, et al. Calcium deposition within coronary atherosclerotic lesion: implications for plaque stability. *Atherosclerosis*. 2020;306:85-95. doi:10.1016/j.atherosclerosis.2020.05.017
4. Yu C, Wu D, Zhao C, Wu C. CircRNA TGFBR2/MiR-25-3p/TWIST1 axis regulates osteoblast differentiation of human aortic valve interstitial cells. *J Bone Miner Metab*. 2021;39(3):360-371. doi:10.1007/s00774-020-01164-4
5. Schroeder ME, Gonzalez Rodriguez A, Speckl KF, et al. Collagen networks within 3D PEG hydrogels support valvular interstitial cell matrix mineralization. *Acta Biomater*. 2021;119:197-210. doi:10.1016/j.actbio.2020.11.012
6. Wang X, Deb N, Lacerda CMR. Comparison of serotonin-regulated calcific processes in aortic and mitral valvular interstitial cells. *ACS Omega*. 2021;6(30):19494-19505. doi:10.1021/acsomega.1c01723
7. Torzewski M. The initial human atherosclerotic lesion and lipoprotein modification – A deep connection. *Int J Mol Sci*. 2021;22(21):11488. doi:10.3390/ijms222111488
8. van Engeland NCA, Bertazzo S, Sarathchandra P, et al. Aortic calcified particles modulate valvular endothelial and interstitial cells. *Cardiovasc Pathol*. 2017;28:36-45. doi:10.1016/j.carpath.2017.02.006
9. Li SJ, Kao YH, Chung CC, Cheng WL, Chen YJ. HDAC I inhibitor regulates RUNX2 transactivation through canonical and non-canonical Wnt signaling in aortic valvular interstitial cells. *Am J Transl Res*. 2019;11(2):744-754.
10. Gendron N, Rosa M, Blandinieres A, et al. Human aortic valve interstitial cells display proangiogenic properties during calcific aortic valve disease. *Arterioscler Thromb Vasc Biol*. 2021;41(1):415-429.
11. Liu Z, Wang Y, Shi J, et al. IL-21 promotes osteoblastic differentiation of human valvular interstitial cells through the JAK3/STAT3 pathway. *Int J Med Sci*. 2020;17(18):3065-3072. doi:10.7150/ijms.49533
12. Rattazzi M, Donato M, Bertacco E, et al. l-Arginine prevents inflammatory and pro-calcific differentiation of interstitial aortic valve cells. *Atherosclerosis*. 2020;298:27-35. doi:10.1016/j.atherosclerosis.2020.02.024
13. He W, Che H, Jin C, Li Y, Li F, Zhou R. LncRNA AFAP1-AS1 promotes M1 polarization of macrophages and osteogenic differentiation of valve interstitial cells. *J Physiol Biochem*. 2021;77(3):461-468. doi:10.1007/s13105-021-00821-0
14. Chang Z, Yan G, Zheng J, Liu Z. The lncRNA GAS5 inhibits the osteogenic differentiation and calcification of human vascular smooth muscle cells. *Calcif Tissue Int*. 2020;107(1):86-95. doi:10.1007/s00223-020-00696-1
15. Zheng D, Wang B, Zhu X, et al. LncRNA OIP5-AS1 inhibits osteoblast differentiation of valve interstitial cells via miR-137/TWIST11 axis. *Biochem Biophys Res Commun*. 2019;511(4):826-832. doi:10.1016/j.bbrc.2019.02.109
16. Yang W, Yu Z, Chiyoya M, et al. Menaquinone-4 accelerates calcification of human aortic valve interstitial cells in high-phosphate medium through PXR. *J Pharmacol Exp Ther*. 2020;372(3):277-284. doi:10.1124/jpet.119.263160
17. Zhou Y, Li J, Zhou K, Liao X, Zhou X, Shen K. The methylation of Notch1 promoter mediates the osteogenesis differentiation in human aortic valve interstitial cells through Wnt/β-catenin signaling. *J Cell Physiol*. 2019;234(11):20366-20376. doi:10.1002/jcp.28638
18. Lu P, Yin B, Liu L. MicroRNA-138 suppresses osteoblastic differentiation of valvular interstitial cells in degenerative calcific aortic Valve disease. *Int Heart J*. 2019;60(1):136-144. doi:10.1536/ihj.18-086
19. Li N, Bai Y, Zhou G, et al. miR-214 attenuates aortic valve calcification by regulating osteogenic differentiation of valvular interstitial cells. *Mol Ther Nucleic Acids*. 2020;22:971-980. doi:10.1016/j.omtn.2020.10.016
20. Vadana M, Cecoltan S, Ciortan L, et al. Molecular mechanisms involved in high glucose-induced valve calcification in a 3D valve model with human valvular cells. *J Cell Mol Med*. 2020;24(11):6350-6361. doi:10.1111/jcmm.15277
21. Gee T, Farrar E, Wang Y, et al. NFκB (Nuclear factor κ-light-chain enhancer of activated B cells) activity regulates cell-type-specific and context-specific susceptibility to calcification in the aortic valve. *Arterioscler Thromb Vasc Biol*. 2020;40(3):638-655. doi:10.1161/ATVBAHA.119.313248
22. Huang G, An L, Fan M, et al. Potential role of full-length and nonfull-length progranulin in affecting aortic valve calcification. *J Mol Cell Cardiol*. 2020;141:93-104. doi:10.1016/j.yjmcc.2020.03.012
23. Rogers MA, Chen J, Nallamshetty S, et al. Retinoids repress human cardiovascular cell calcification with evidence for distinct selective retinoid modulator effects. *Arterioscler Thromb Vasc Biol*. 2020;40(3):656-669. doi:10.1161/ATVBAHA.119.313366
24. Yang L, Wu D, Li M, et al. Upregulation of microRNA-195 ameliorates calcific aortic valve disease by inhibiting VWF via suppression of the p38-MAPK signaling pathway. *Int J Cardiol*. 2020;309:101-107. doi:10.1016/j.ijcard.2020.01.001
25. Sakaue T, Hamaguchi M, Aono J, et al. Valve interstitial cell-specific cyclooxygenase-1 associated with calcification of aortic valves. *Ann Thorac Surg*. 2020;110(1):40-49. doi:10.1016/j.athoracsur.2019.09.085