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SNF472, a novel anti-crystallization agent, inhibits induced calcification in an in vitro model of human aortic valve calcification

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ABSTRACT

The purpose of the present study was to investigate whether SNF472, the hexasodium salt of myo-inositol hexaphosphate (IP6 or phytate): 1. Inhibits induced calcification in cultured aortic valve interstitial cells (VIC) as an in vitro model of aortic valve stenosis and 2. Whether inhibition is different in VIC obtained from healthy and calcified aortic valves.

VIC from healthy ($n = 5$) and calcified ($n = 7$) human aortic valves were seeded in basic growth medium, osteogenic differentiation medium alone, or in osteogenic medium with SNF472 (3, 10, and 30 μM) and cultivated for 3 weeks. Calcification was quantified spectrophotometrically after Alizarin Red staining. In VIC from calcified valves, a complete inhibition of calcification was observed with SNF472 concentrations of 10 and 30 μM ($p < .01$), significantly stronger than in VIC from healthy valves. When SNF472 was added to VIC after 1 week in osteogenic medium, 30 and 100 μM SNF472 inhibited the progression of ongoing calcification by 81 and 100% ($p < .01$), respectively. The same concentrations of SNF472 given after 2 weeks reduced calcification by 35 and 40% respectively (not significant).

SNF472 inhibited both the formation and the progression of calcification with the strongest effect in VIC from calcified valves.

1. Introduction

Aortic valve stenosis is the most common valve disease in the Western world and it is the third leading cause of cardiovascular disease [1]. The risk factors include age, diabetes, male gender, coronary artery disease, smoking, renal failure, obesity and metabolic syndrome [2]. The strongest risk factor, however, is the presence of a bicuspid aortic valve [3]. The exact mechanisms of calcification are not known, but shear stress and inflammation may be among the initiators (for reviews see [4–8]). Early events of the calcification process are disruption of the endothelial layer of the leaflets with reorganization of the valve matrix, immune cell infiltration, and leaflet thickening, ultimately causing calcification in the leaflet matrix [8,9]. Aortic stenosis can be divided into two distinct phases: “an early initiation phase dominated by lipid deposition, injury and inflammation with many similarities to atherosclerosis, and a later propagation phase where pro-calcific and pro-osteogenic factors take over and ultimately drive disease progression” [10]. Unfortunately, there is a lack of good animal models of aortic valve calcification [11,12]. Some mouse models develop aortic stenosis; however, mouse models have serious limitations. Both the animals and the aortic valves are very different from humans including a totally different morphology of the leaflets [11]. Large animal models are more relevant, but are much more expensive and time consuming [13]. In vitro models with human aortic valve interstitial cells (VIC) may be superior for initial screening studies. Because the VIC are believed to play a key role in the calcification process, induced calcification in cultured human VIC avoiding species differences may be the best in vitro model of valve calcification [5,8,14,15]. However, VIC change their phenotype when aortic valves calcify [16]. Consequently, VIC from healthy or calcified valves may behave differently to

Abbreviations: IP6, myo-inositol hexaphosphate; FBS, fetal bovine serum; VIC, valve interstitial cells; DMEM, Dulbecco's Modified Eagle Medium

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calcifying stimuli and inhibition.

The overall incidence of aortic stenosis is expected to increase over the next decades due to an aging population [17]. No pharmacological treatment is effective against valve calcification. The only available option for treatment is surgical or catheter-based aortic valve replacement [18,19]. Elucidating the cellular and molecular mechanisms of heart valve calcification may open up for new therapies. With an aging population, pharmacological inhibition of calcific aortic valve disease ought to be a priority of research.

Myo-inositol hexaphosphate (phytate or IP6) is an endogenous inhibitor of pathological calcification. It mainly acts by preventing precipitation of new calcium and phosphorus ions on forming or growing hydroxyapatite crystals [20,21]. Probably the direct inhibition on hydroxy-apatite formation is most important to inhibit calcification, however, phytate has other physiological and basic effects on cell functions which theoretically may play a role. These include antioxidant properties [22], anti-inflammatory effects via nuclear factor κB [23], decreasing cell proliferation and differentiation [24], anti-apoptotic effects and protection of the mitochondrial membrane potential [25].

Main sources of IP6 are fibrous plant food such as vegetables, grains, and nuts; but it has limited gastrointestinal absorption [26]. IP6 has been shown to counteract cardiovascular calcification in animal models [27,28], but has never been tested in relation to valve calcification. However, in an elderly population, an inverse correlation was found between urinary phytate content and mitral annulus calcification [21]. SNF472 is a formulation of IP6 [29] which inhibits cardiovascular calcification in non-uremic and uremic rat models [20]. Currently, the possibility of SNF472 to inhibit vascular calcification in patients on hemodialysis is being investigated in a Phase 2 clinical trial (clinicaltrials.gov Identifier: NCT02966028) in which all patients have been recruited and last patient out is expected for August 2019. A Phase 2 clinical trial (clinicaltrialsregister.eu NCT02790073) in uremic calciphylaxis patients (the most aggressive manifestation of vascular calcification) showed improvements in wound healing and pain in patients treated with SNF472 on top of standard of care. Finally, IP6 regulates mineralization in osteoblast cultures [30] and valve calcification has similarities to bone formation [8].

We hypothesize that SNF472 inhibits calcification in cultured VIC. The purpose of the present study was 1. To study if SNF472 inhibits experimentally induced in vitro calcification of the aortic valve in a model using VIC; 2. To study if this effect is different whether VIC from healthy and calcified valves are used; and 3. To investigate if SNF472 stops or limits an already ongoing calcification process in the same model.

2. Materials and methods

2.1. Valve interstitial cells isolation and cultivation

Aortic valves were harvested at the Department of Cardiothoracic Surgery, Oslo University Hospital, Oslo, Norway with the approval of the local Ethical Committee and performed in accordance with principles of the Declaration of Helsinki. All patients gave informed consent. Healthy valves were collected from explanted hearts of heart transplant recipients without a history of heart valve disease and with macroscopically normal valves. Calcified aortic valve leaflets were obtained during aortic valve replacement.

After the removal of the valve endothelial cells, VIC were isolated. Briefly, the leaflets were placed in the 30 ml of basic growth medium (DMEM (Life technologies, 41966-052), 10% FBS (HyClone, SH30070.03), 50 μg/ml gentamycin (Life technologies, 15750-060)) with the addition of 1 mg/ml collagenase II (Worthington, LS004177) and incubated overnight at 37 °C/5% CO_2. Next day, the digested tissue was homogenized by pipetting with serological pipet and centrifuged at 300 × *g* for 5 min and the supernatant carefully removed by aspiration. The pellet was washed in 10 ml of fresh DMEM and centrifuged as described previously. The supernatant was removed by aspiration, the cell pellet was resuspended in basic growth media and seeded at 75 cm^2 flask (Nunc, 734-2066). VIC were cultivated at 37 °C/5% CO2 with growth media changed twice a week until they reached ~90% confluency. Then, cells were passaged at ratio of 1:2, propagated and frozen.

The purity of the VIC population was regularly assessed by the expression of smooth muscle actin (αSMA), and sorting against CD31 as an endothelial marker. In no case did we observe contaminating endothelial or inflammatory cells in VIC cultures.

2.2. VIC osteogenic differentiation and inhibition

For osteogenic differentiation VIC from healthy (n = 5) and calcified (n = 7) human aortic valves were seeded in 24-well tissue culture plates at 30 × 10^4 cells/well (Nunc, 734-0992) in basic growth medium and cultured overnight at 37 °C, 5% CO_2. The next day, osteogenic differentiation was induced by supplementing the medium with 50 μM ascorbic acid (Sigma-Aldrich, A4544), 0.1 μM dexamethasone (Sigma-Aldrich, D4902) and 10 mM beta-glycerophosphate (Sigma-Aldrich, G9422). Osteogenic medium was changed twice a week for 3 weeks. The control group was cultivated in basic growth media for the same period of time and media was changed following the same protocol. Treatment groups were incubated with variable concentrations of SNF472 (Almac Sciences, Q000003708).

2.3. Alizarin Red calcium staining and quantification

Alizarin Red staining was performed to assess calcification. The cell medium was removed, the cells were washed with PBS and fixed with 70% ethanol for 1 h at room temperature. Then cells were washed with Milli-Q water and stained with Alizarin Red (Sigma-Aldrich, A5533) according to the manufacturer's instructions. In order to quantify calcium accumulation, Alizarin Red staining was extracted and measured spectrophotometrically. Briefly: for 24-well plate 200 μl 10% acetic acid was added to each well and incubated for 30 min at room temperature with gentle agitation. After that, cells were detached using a cell scraper and the resulting suspension was transferred to a 1.5 ml microcentrifuge tube and vortexed vigorously for 30 s. The cells were then heated to 85 °C for 10 min before the tubes were transferred to ice for 5 min and chilled. Then, the cells were centrifuged at 15000 × *g* for 15 min. The supernatant was transferred to a new 1.5 ml microcentrifuge tube, 75 μl of 1 M NaOH were added to each tube to achieve pH between 4.1 and 4.5. Finally, 50 μl from the tube were transferred to a clear bottom 96-well plate and the absorbance at 405 nm was measured on a plate reader (Molecular Devices, E11191).

The average absorbance of Alizarin Red staining of VIC cultured in osteogenic medium without inhibitor supplementation was set as 100%. The background absorbance was set as 0%.

2.4. Experimental groups

Series 1: A dose-response study with the same VIC donors in all groups.

Group 1.1: VIC cultures kept in the basic growth medium. After 3 weeks they were stained with Alizarin Red (n = 12, five VIC cultures from healthy valves, seven VIC cultures from calcified valves).

Group 1.2: VIC cultures kept in osteogenic medium. After 3 weeks stained with Alizarin Red (n = 12, five VIC cultures from healthy valves, seven VIC cultures from calcified valves).

Group 1.3: same as group 1.2 + 3 μM SNF472 (n = 12).

Group 1.4.: same as group 1.2 + 10 μM SNF472 (n = 12).

Group 1.5.: same as group 1.2 + 30 μM SNF472 (n = 12).

Series 2: Inhibition of ongoing calcification with the same VIC donors from calcified valves in all groups.

Group 2.1: VIC cultures were kept in the basic growth medium. After 1 week stained with Alizarin Red ($n = 8$).

Group 2.2: same as group 2.1 but stained after 3 weeks ($n = 8$).

Group 2.3: VIC cultures were kept in osteogenic medium. After 1 week stained with Alizarin Red ($n = 8$).

Group 2.4: same as group 2.3 but stained after 2 weeks ($n = 8$).

Group 2.5: same as group 2.3 but stained after 3 weeks ($n = 8$).

Group 2.6: VIC cultures were kept in osteogenic medium for 3 weeks before being stained and quantified. After 1 week with osteogenic medium 30 μM SNF472 was added ($n = 8$).

Group 2.7: same as group 2.6 but 100 μM SNF472 ($n = 8$).

Group 2.8: same as group 2.6, but 30 μM SNF472 was added after 2 weeks with osteogenic medium ($n = 8$).

Group 2.9: same as group 2.8 but 100 μM SNF472 ($n = 8$).

For series 2 the inhibition of calcification progression was calculated for each SNF472 concentration and time point when added. Progression of calcification in the non-treated group between week 2 and week 3, and how much the calcification progressed in the treated group between week 2 and week 3 were calculated. Based on these data percentage inhibition in the treated group was calculated. The same was done between weeks 1 and 3 when SNF472 treatment started at week 1.

2.5. Statistics

Statistics was analyzed by GraphPad Prism (version 8, USA). Outliers were identified and removed using ROUT approach [31]. The VIC cultures which failed to differentiate and calcify under uninhibited induction of osteodifferentiation were excluded from inhibition studies. Normality of the data was assessed using a Shapiro-Wilk test. For the results that were normally distributed a one-way ANOVA for parametric distributions with DMS post-test was applied. For the results that were not normally distributed a nonparametric one-way ANOVA (Kruskal–Wallis test) with Dunn's post-test was applied. Healthy/Calcified VIC inhibition trends shown as mean ± standard error of the mean (SEM) other data are shown as dotplot or mean ± standard deviation (SD). Grouped two-way ANOVA and Mixed-effects model with Bonferroni's multiple comparison test were performed comparing healthy and calcified VIC behavior under the influence of the inhibitor. A value of $p < .05$ was considered statistically significant.

3. Results

Microscopy images of the calcium deposits stained by Alizarin Red in the different treatment groups of VIC from calcified valves are shown in Fig. 1. Calcium content was visually detected in the differentiated cultures without inhibitor and with 3 μM SNF472, while cultures treated with 10 and 30 μM SNF472 did not show macroscopic signs of calcification.

When the amount of calcium staining was quantified, we found that SNF472 inhibited calcification of VIC in a dose-dependent manner. When all donors were considered, a concentration of 3 μM SNF472 added at the start of osteodifferentiation inhibited calcification by 57% (Fig. 2A). Concentrations of 10 and 30 μM SNF472 inhibited calcification by 74 and 75% respectively (Fig. 2A). Calculated half maximal effective concentration (EC_{50}) inhibition was 2.0 μM (Fig. 2B).

Analysis of the results showed a different pattern of response between those cells isolated from healthy donors and those isolated from calcified valves. There was a significantly stronger inhibition in cultured VIC from calcified valves compared to VIC from healthy valves found in two-way ANOVA analysis of both cell source and treatment effect ($p < .01$, Fig. 3A–C). In VIC from calcified valves some inhibition (but not significant) of calcification was observed at 3 μM SNF472 (88%). Concentrations of 10 and 30 μM SNF472 completely inhibited calcification (100% inhibition, $p < .01$), and the calculated EC_{50} for these calcified donors was 2.3 μM. Calcification was even less with the two highest concentrations of SNF472 than in the control group without osteodifferentiation. When non-calcified donors alone were analyzed, an EC_{50} of 4.8 μM was calculated.

Grouped two-way ANOVA and Mixed-effects model with Bonferroni's multiple comparison test were performed comparing healthy and calcified VIC behavior under the influence of the inhibitor for Fig. 3A. One-way ANOVA for parametric distributions with DMS post-test statistical analysis was applied for Fig. 3B. Nonparametric one-way ANOVA (Kruskal–Wallis test) with Dunn's post-test was applied for Fig. 3C.

In order to make more clinically relevant findings, we investigated whether addition of SNF472 could inhibit ongoing calcification. When compared to uninhibited calcification after 3 weeks, the addition of 30 and 100 μM SNF472 after 1 week of osteodifferentiation significantly decreased the calcification at 30 μM SNF472 and completely inhibited the progression of calcification from first to third week of differentiation at 100 μM SNF472 (Fig. 4A) by 81 and 100% respectively as shown in Fig. 4B. When SNF472 was added after 2 weeks, the progression of calcification during the last week was non-significantly attenuated by 35 and 40% for 30 and 100 μM respectively (Fig. 4B).

4. Discussion

SNF472 inhibited in vitro calcification in VIC cultures in a dose-dependent manner when added at the start of osteodifferentiation. When SNF472 was added during ongoing osteodifferentiation, it reduced progression of calcification and the effect was larger the earlier it was added. The strongest inhibition was observed in VIC isolated from calcified valves. The highest concentrations used ($\leq$ 100 μM) is considerably less than concentrations known to cause adverse effects ($>$ 400 μM) in animal experiments (unpublished results). In hemodialysis patients, a maximum peak concentration of 100 μM in blood has been achieved without toxic effects in physical status, body weight, cardiorespiratory function, body temperature, calcium ion levels, heart rate, ECG parameters or blood pressure (manuscripts under review). Consequently, clinical use of SNF472 for the treatment of valve calcification is expected to be safe with the presently used concentrations ($\leq$ 100 μM). However, further studies for long-term administration are needed.

So far there is no clinically available pharmacological treatment of aortic valve calcification. A major advantage of SNF472 is that it is already being developed for use in patients with end-stage renal disease on hemodialysis and calciphylaxis patients. Other drugs that have been discussed as therapy for aortic stenosis, such as statins, angiotensin II inhibitors, aldosterone blockade, bisphosphonates, inhibitors of nitric oxide uncoupling, as well as serotonin receptor blockers and inhibitors of ectonucleotidases, are at present not alternatives for clinical therapy [32–34].

Although the VIC are key players in the valve calcification process, a limitation of this study is that inhibition of calcification was investigated in a cell culture model. Unfortunately, there is a lack of good animal models of heart valve calcification, although some genetically engineered mice will develop aortic calcifications [35]. Other models include aortic valve calcification caused by warfarin treatment in rats [36] (for reviews see [11,13,37]). Unfortunately, none of these models are representative of the clinical picture of aortic valve stenosis, being more offsprings from atherosclerosis models. An interesting model is aortic stenosis in mice caused by mechanical injury of the leaflets [38]. All taken together, there is a need for improved animal models. [8]. Using human VIC in culture is a good and easily available in vitro model to study the molecular and cellular mechanisms of calcification. In particular, it is an easy and quick model to assess potential inhibitors. Furthermore species differences are avoided when using human cells. However, cells in culture have distinct limitations and in the development of clinical therapy animal studies will be necessary.

The mechanism of action of SNF472 is based on its ability to

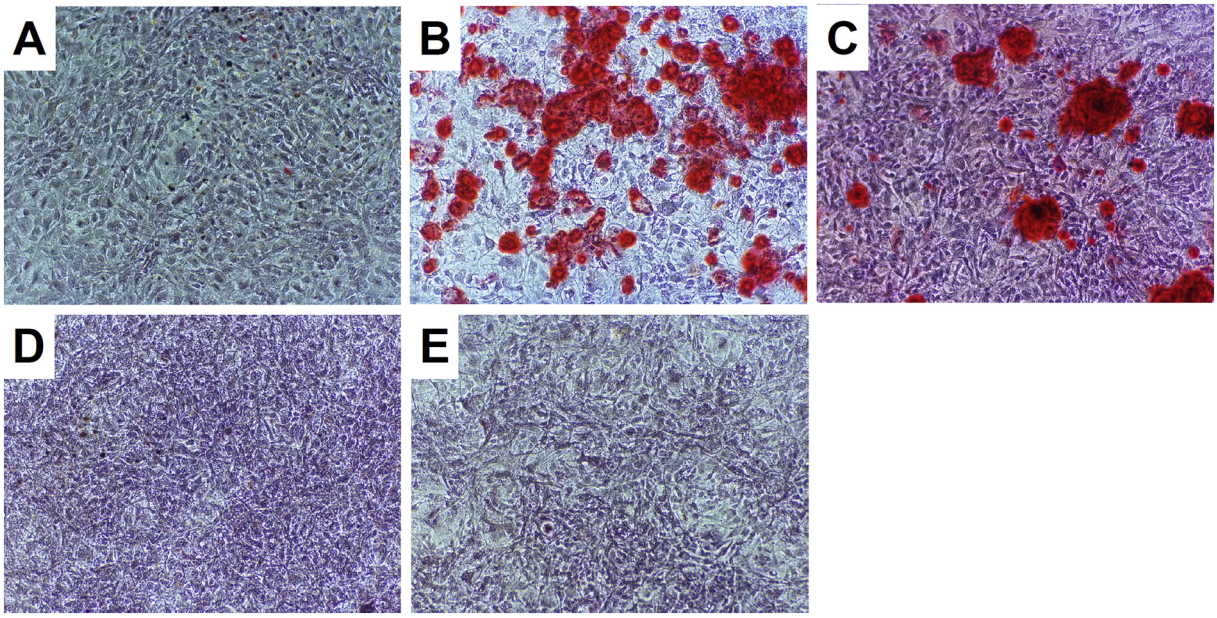


Fig. 1. Microphotography of the cultured VIC calcification inhibition.
Microphotography of Alizarin Red staining of valvular interstitial cells (VIC) from calcified valves cultured for 3 weeks in basic growth media (A), osteogenic medium (B), and osteogenic medium with the presence of increasing concentrations of SNF472: 3 μM (C), 10 μM (D) and 30 μM (E). Magnification 100×.

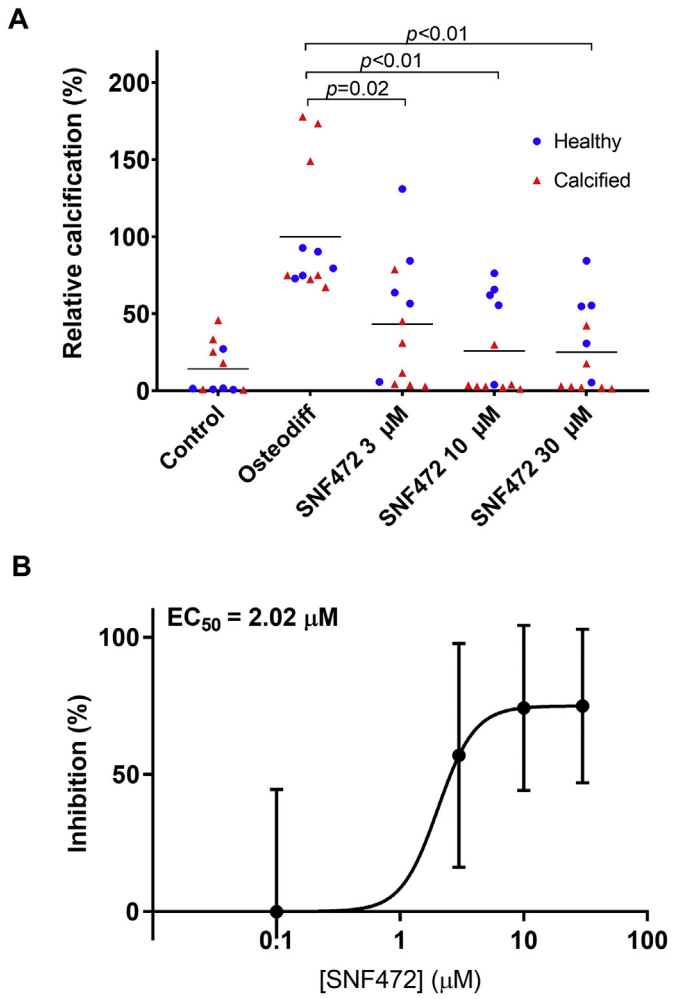
selectively bind to hydroxyapatite crystals surface [20]. SNF472 blocks binding of additional ions and crystal growth is inhibited [39,40]. Although at high concentrations SNF472 can chelate divalent anions such as calcium, the doses required to inhibit cardiovascular or experimental aortic valve calcification in VIC (EC50 of 2.0 μM as measured in the current study) are too low to provoke measurable calcium chelation [20]. These results are in line with previous results obtained in vitro and in vivo. In a novel in vitro model of calcification induction in human plasma, the IC50 of SNF472 for the inhibition of hydroxyapatite crystallization was 2.1 μM [40]. In vivo, the subcutaneous administration of 10 mg/kg SNF472 to rats (leading to circulating SNF472 levels in the range of 10 μM) completely stopped the progression of heart calcification in a vitamin D-induced rat model of calcification [20]. This distinguishes SNF472 from other known natural calcium inhibitors such as Fetuin-A, which also has lower potency in inhibiting formation of calcium-phosphate crystals [40]. Possible additional effects of phytate include antioxidant properties [22], anti-inflammatory effects via nuclear factor κB [23], decreasing cell proliferation and differentiation [24], anti-apoptotic effects and protection of the mitochondrial membrane potential [25].

A surprising finding was that inhibition of calcification by SNF472 was significantly stronger in VIC from calcified valves. This finding is very relevant for possible clinical use strengthening the possible therapeutic benefits of SNF472, because pharmacological therapy will not be initiated unless some calcification and slight to moderate aortic stenosis have been observed.

SNF472 blocked calcification even when calcification was ongoing. However the present study showed that the effect was more pronounced the earlier the agent was given, suggesting it is important to initiate treatment with SNF472 as early as possible in order to inhibit or limit the calcification process. At concentrations levels ≥10 μM, the administration of SNF472 totally inhibited the calcification process when given ≤1 weeks after the process started in the VIC cultures. The routine of administration of SNF472 used in this study might have not been optimal because degradation studies in the cell culture medium showed that there was no free SNF472 after 24 h (unpublished observations), when the medium was changed twice a week. However, when bound to hydroxyapatite crystal surfaces, SNF472 may still maintain its anti-crystallization properties. This has already been suggested by in vitro studies showing that binding of SNF472 to hydroxyapatite is stable for at least 3 days and in vivo animal studies in which every other day intravenous administration of SNF472 to rats inhibited vascular calcification to the same extent than daily administration [20]. For possible clinical use, the drug should be given in the initiation phase or as early as possible in the propagation phase. Even when given at a later stage, the drug may slow down the calcification process and delay or even make intervention for aortic stenosis unnecessary. SNF472 is currently in clinical development for use in patients with end-stage renal disease on hemodialysis and calciphylaxis patients. A Phase 2 clinical trial (clinicaltrials.gov Identifier: NCT02966028) is ongoing to assess the effect of SNF472 in cardiovascular calcification progression in hemodialysis patients. All patients in this study have already been recruited and last patient out is expected for August 2019. Another Phase 2 clinical trial (clinicaltrialsregister.eu NCT02790073) in uremic calciphylaxis patients (the most aggressive manifestation of vascular calcification) showed improvements in wound healing and pain in patients treated with SNF472 on top of standard of care [41]. The mean SNF472 circulating levels in this clinical trial were around 30 μM which is also consistent with the results presented in this manuscript. The positive results of this Phase 2 study allowed to design a randomized, placebo-controlled Phase 3 trial in calciphylaxis patients, which is under preparation.

A critical issue in clinical treatment of developing calcification of the aortic valve will be to develop strategies and technology to detect early changes in the leaflets. More sensitive methods than traditional echocardiography may be used, although the quality and sensitivity of echocardiography is constantly developing. More sensitive technologies can be employed, including PET-CT imaging modalities for use in patients to detect inflammation and mineralization [42]. Even more sophisticated methods are being developed such as molecular imaging [43]. This technique however is at present only available for preclinical studies.

In conclusion, SNF472 was shown being a promising agent which ought to be investigated for future pharmacological treatment and prevention of calcific aortic valve disease. Treatment should be started as early as possible when fibrosis and inflammation of the leaflets have started. However, even at a later stage with considerable ongoing calcification, the substance may slow down and delay surgery, or even stop



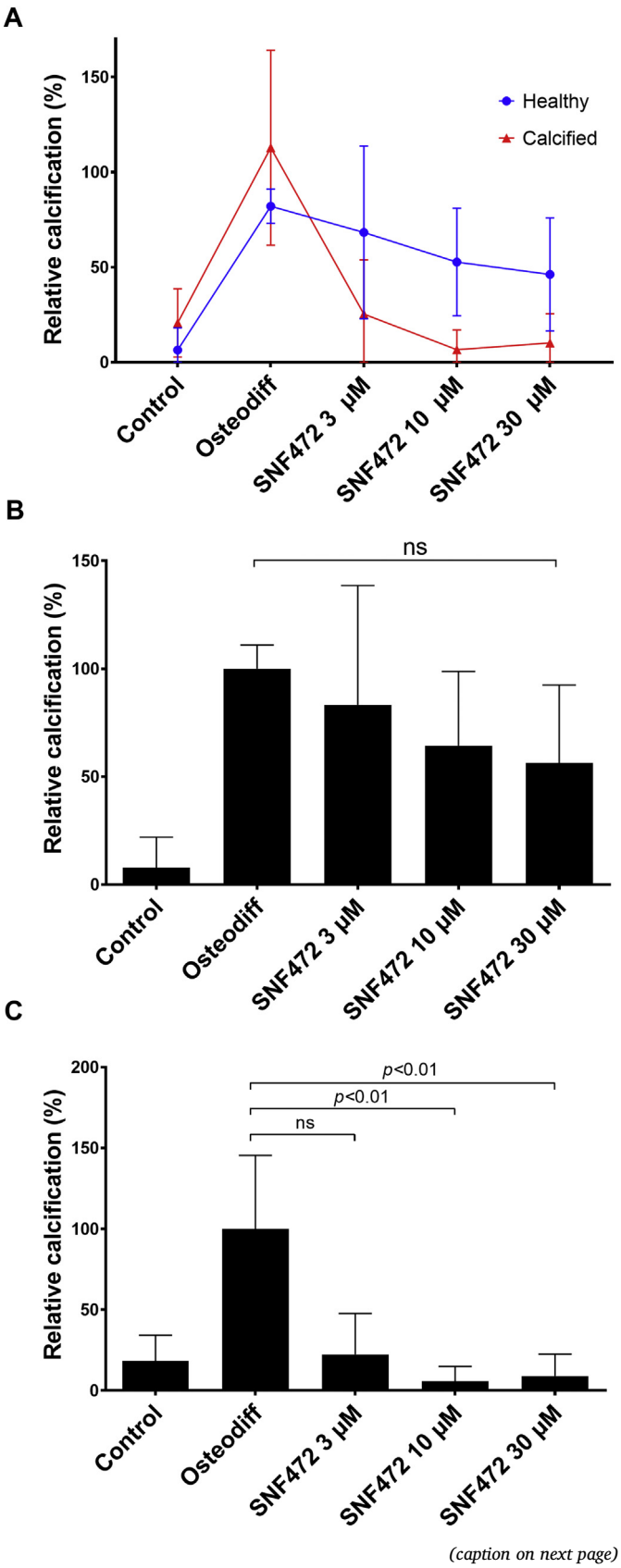


Fig. 2. Dose-dependent inhibition of calcification.
Cultures of aortic valve interstitial cells (VIC) (n = 12 in each, five from healthy valves, seven from calcified valves) were kept in basic growth media (Control), osteodifferentiation media (Osteodiff) and osteodifferentiation media + different concentrations of SNF472 (3, 10, and 30 μM) for 3 weeks. Calcification was measured by Alizarin red staining and quantified spectrophotometrically. (A) Relative calcification, values are dot plots + mean. (B) Calcification inhibition curve and half maximal effective concentration (EC_{50}) for SNF472. A one-way ANOVA for parametric distributions with DMS post-test statistical analysis was performed.

the calcification process.

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Declaration of competing interests

A. Zabirnyk, Sanifit Therapeutics independent contractor (including contracted research).

M. Bogdanova, none.

M. Ferrer, Sanifit Therapeutics independent contractor (including contracted research), stock option holder at Sanifit Therapeutics.

M.M. Perez, Sanifit Therapeutics Employment, stock option holder at Sanifit Therapeutics.

M. Kaljusto, none.

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Fig. 3. Comparison of calcification inhibition in VIC from healthy and calcified valves.
Cultures of aortic valve interstitial cells (VIC) (n = 12, five from healthy valves, seven VIC from calcified valves) were kept in basic growth media (Control), osteodifferentiation media (Osteodiff) and osteodifferentiation media + different concentrations of SNF472 (3, 10, and 30 μM) for 3 weeks. Calcification was measured by Alizarin Red staining and quantified spectrophotometrically. Panel A: Calcification inhibition trends for VIC from healthy and calcified valves. Presented as relative calcification, (mean ± SEM). (B) Inhibition of calcification in VIC from healthy valves (mean ± SD). (C) Inhibition of calcification in VIC from calcified valves (mean ± SD).

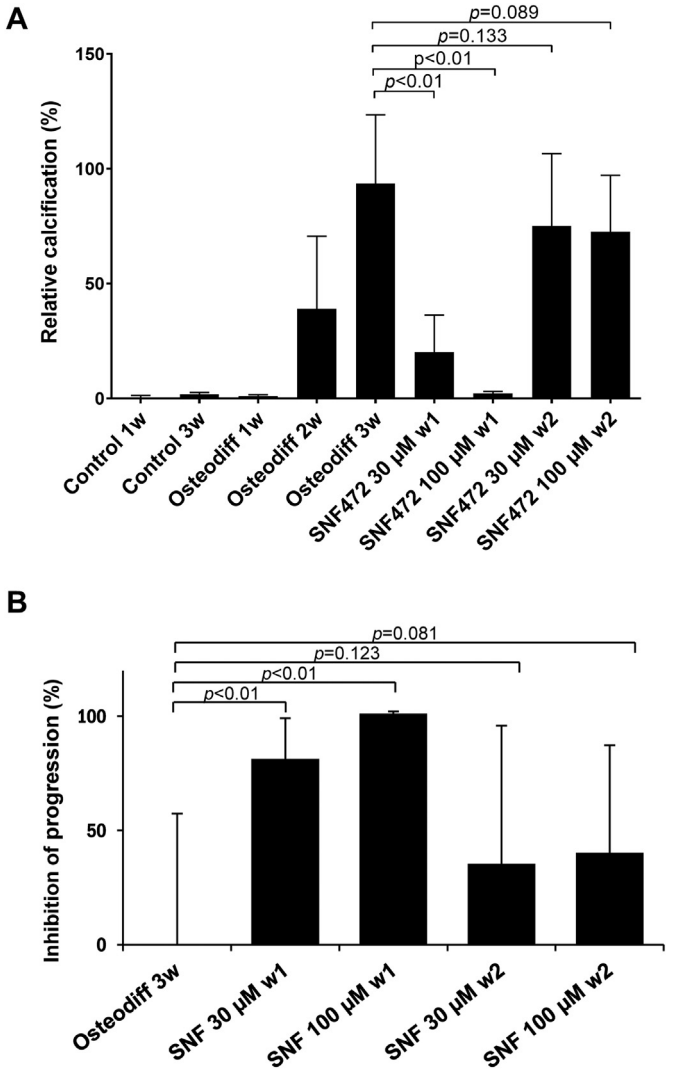

Fig. 4. Inhibition on ongoing calcification.
VIC cultures (n = 8 in each) undergoing cultivation in basic growth media for 1, 2 and 3 weeks (Control 1w, 2w, 3w subsequently); osteodifferentiation for 1, 2 and 3 weeks without treatment (Osteodiff 1w, 2w, 3w subsequently); or treated with SNF472 (30 or 100 μM after 1 (w1) or 2 weeks (w2)) in osteogenic medium (A). Inhibition of calcification, determined as the relation between increased calcification of the non-treated and treated groups between week 1 and week 3 (w1 groups) or between week 2 and week 3 (w2 groups) (B). All values are mean ± SD. A one-way ANOVA for parametric distributions with DMS post-test statistical analysis was performed.

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J. Vaage, Sanifit Therapeutics independent contractor (including contracted research).

The investigation was initiated and conducted in the labs in Oslo. The design of the study was done in Oslo with additional comments from Sanifit. Sanifit provided expertise on SNF472 and measured degradation in cell culture medium. The data were processed in Oslo. Statistics and figures were done during discussion and collaboration with Sanifit. The article and revisions were done in Oslo. The final revisions were done in collaboration with Sanifit.

Author contributions

AZ acquired the data and drafted the manuscript; AZ, MF performed statistical analysis; AZ, MB, MK, JK obtained the research material; AZ, MF, MB, MMP, KOS, JP, made critical revision on research design and manuscript for key intellectual content. JV conceived and designed the research and made critical revision of manuscript.

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