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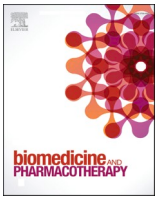

Unravelling the cardioprotective effects of calcitriol in Sunitinib-induced toxicity: A comprehensive *in silico* and *in vitro* study

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ABSTRACT

Sunitinib (SUN), a drug used to treat advanced renal cell carcinoma and other cancers, causes cardiotoxicity. This study aimed to identify a potential drug candidate to counteract SUN-induced cardiotoxicity. We analysed real-world data from adverse event report databases of existing clinically approved drugs to identify potential candidates. Through *in silico* analyses and *in vitro* experiments, the mechanisms of action were determined. The study identified calcitriol (CTL), an active form of vitamin D, as a promising candidate against SUN-induced cardiotoxicity. In H9c2 cells, SUN decreased cell viability significantly, whereas CTL mitigated this effect significantly. The SUN-treated group exhibited increased autophagy in H9c2 cells, which was reduced significantly in the CTL group. Bioinformatics analysis using Ingenuity Pathway Analysis revealed the mechanistic target of rapamycin (mTOR) as a common factor between autophagy and CTL. Notably, rapamycin, an mTOR inhibitor, nullified the effects of CTL on cell viability and autophagy. Furthermore, SUN treatment led to significant reductions in cardiomyocyte diameters and increases in their widths, changes that were inhibited by CTL. SUN also induced morphological changes in surviving H9c2 cells, causing them to adopt a rounded shape, whereas CTL improved their morphology to resemble the elongated shape of the control group. In conclusion, the findings of the present study suggest that CTL has the potential to prevent SUN-induced cardiomyocyte damage through autophagy, particularly via mTOR-mediated pathways. The findings indicate that CTL could serve as an effective prophylactic agent against SUN-induced cardiotoxicity, offering a promising avenue for further research and potential clinical applications.

1. Introduction

Sunitinib (SUN) is a tyrosine kinase inhibitor (TKI) used to treat adult patients with gastrointestinal stromal tumours, advanced renal cell carcinoma, and pancreatic neuroendocrine tumours. However, multiple studies have shown that SUN is associated with severe left ventricular dysfunction in these patients [1,2]. Common Terminology Criteria for Adverse Events ver. 3.0 grade 2 or 3 left ventricular dysfunction occurs in approximately 13 % of patients treated with SUN [2].

If grade 2 or 3 heart damage develops, SUN must be withdrawn or the dose reduced, and if grade 4 heart damage develops, the dose must be discontinued. Cardiotoxicity may occur in patients who can be treated effectively with or require SUN, forcing them to discontinue the drug. In addition, the development of cardiovascular diseases worsens the prognosis of cancer survivors [3]. There is currently no effective prophylactic drug for SUN-induced left ventricular dysfunction, and its

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development is an urgent issue.

Autophagy is a major intracellular degradative system that works for cell survival by breaking down non-essential cellular components and supplying nutrients [4,5]. However, excessive activation or inactivation of autophagy makes degradation of cellular components unbalanced, resulting in adverse effects on cell survival and functional maintenance [6,7]. Cells that die because their homoeostasis has been disrupted may also exhibit certain features of apoptosis or necroptosis. However, not all autophagy-related cell death is due to apoptosis, and autophagy is considered to be a phenomenon that occurs upstream of apoptosis and necroptosis [8]. Previous studies have shown that SUN causes cardiomyocyte damage owing to dysregulation of autophagy [9].

In this study, we aimed to identify drugs for clinical use that have the potential to prevent SUN-induced cardiotoxicity among various approved drugs using global real-world data from adverse event reports. As safety is important for prophylactic agents used against the adverse events of anticancer drugs, the drug repositioning method, which selects prophylactic agents from among existing approved drugs with abundant safety information, is useful for the selection of such candidates. We sought to clarify the preventive effects and mechanisms of action of a candidate drug against SUN-induced cardiotoxicity using *in silico* analyses and *in vitro* experiments.

2. Methods

2.1. Analysis of FAERS data

Data were downloaded from the Food and Drug Administration (FDA) Adverse Event Reporting System (FAERS) database for 13,037,860 adverse events (AEs) reported from Q1 2006 to Q4 2019 (https://www.fda.gov/drugs/surveillance/questions-and-answers-fdas-adverse-event-reporting-system-faers; data accessed on March 2020). Duplicate data were excluded according to FDA recommendations, and the remaining 11,014,290 reports were used for analysis. The data were processed using the SQLite database ver.3.33.0 (SQLite Consortium, Charlotte, NC, USA), and statistical analysis was performed using R ver.4.0.3 (The R Foundation, Vienna, Austria).

AEs were defined according to the 31 extracted terms from the "Cardiac failure (SMQ 20000004)" group, which is based on the Medical Dictionary for Regulatory Activities (MedDRA)/J ver. 22.1, International Glossary of Pharmaceutical Terms of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (Supplementary Table 1). The risk of AEs was assessed using the reporting odds ratio (ROR) and 95 % confidence interval (CI). Heart failure RORs were calculated for patients with or without SUN treatment and for those with concomitant drug and SUN treatment, following the methods described in a previous study [10]. Drugs with ROR < 1 and 95 % CI < 1 were defined as those that decreased the frequency of reporting AEs; these drugs were selected as potential candidates.

2.2. Analysis of VigiBase data

We used 24,528,737 AEs reported from the start of VigiBase data collection to March 2021. VigiBase is a World Health Organisation (WHO) global individual case safety report database managed by the Uppsala Monitoring Centre. The data originate from $>$ 130 countries and from various sources. AEs were defined according to 70 extracted codes based on MedDRA/J ver. 25.1 J (Supplementary Table 2). Data processing, statistical analyses, and evaluation methods were the same as those used for FAERS analysis. The likelihood of a causal relationship was not the same in all reports. The information reported in this study does not represent the opinions of the WHO.

2.3. Cell culture

The embryonic rat cardiomyocyte-derived cell line, H9c2 (2–1, CRL-1446), was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). H9c2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) High Glucose (#08459–64; Nacalai Tesque, Kyoto, Japan) supplemented with 10 % foetal bovine serum (FBS; S1600–500; Biowest, Nuaillé, France), 1 % penicillin (100 U/mL)/streptomycin (100 U/mL; P/S; 168–23191; FUJIFILM Wako Pure Chemicals, Richmond, VA, USA), and 20 ng/mL human epidermal growth factor (E9644; Sigma-Aldrich, St. Louis, MO, USA) at 37 ℃ in a 5 % CO_2 atmosphere. For the assay, 10–15 passage cells were used.

The Japanese renal cell carcinoma cell line, OS-RC-2 (RCB0735), was obtained from the RIKEN BioResource Center (Kyoto, Japan). OS-RC-2 cells were cultured in RPMI 1640 medium (#30264–56; Nacalai Teque) supplemented with 10 % FBS and 1 % P/S at 37 ℃ in a 5 % CO_2 atmosphere. For the assay, 7–14 passage cells were used.

The human renal cell carcinoma cell line, 786-O (CRL-1932), was obtained from the ATCC. For the assay, four to six passage cells were used. The 786-O cells were cultured in the same manner as that of the OS-RC-2 cells.

2.4. Cell viability assay

Cell viability was assessed using Cell Counting Kit-8 (341–08001; Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions.

H9c2 cells were seeded into 96-well plates (4860–010; Iwaki, Japan) at 5.0×10^3 cells/well. After being left in culture for 48 h, the cells were treated with SUN (HY-10255A; MedChemExpress, Monmouth Junction, NJ, USA) in dimethyl sulfoxide (DMSO; at 2.5, 5, 10, and 20 μM), or pre-treated with CTL (034–24921; FUJIFILM Wako Pure Chemicals) in DMSO (at 10 and 100 nM) or Rap (Funakoshi, Tokyo, Japan) in EtOH (10 nM) and then treated with SUN 30 min later. After 24 h of SUN treatment, the cells were washed once with phosphate-buffered saline (PBS) and the absorbance of WST-8 formazan was measured at 450 nm using an iMark microplate reader (Bio-Rad, Hercules, CA, USA).

OS-RC-2 or 786-O cells were seeded into 96-well plates at 5.0×10^3 cells/well. After being left in culture for 24 h, the cells were pre-treated with CTL in DMSO (100 nM) and then treated with SUN in DMSO (5, 10, 20, and 30 μM) 30 min later. The absorbance of WST-8 formazan was measured at 450 nm 24 h after SUN treatment using a microplate reader.

2.5. Autophagic flux measurements in H9c2 cardiomyocytes

Autophagic flux was assessed using DAPGreen (D676; FUJIFILM Wako Pure Chemicals) or DALGreen (D675; FUJIFILM Wako Pure Chemicals), according to the manufacturer's instructions. To evaluate autophagy, DMEM High Glucose supplemented with 10 % FBS and 1 % P/S was used at all stages as the culture medium. Fluorescence intensity was quantified using the ImageJ (ver. 1.54) software (National Institutes of Health, Bethesda, MD, USA).

H9c2 cells were seeded onto 8-well μ-slides (ib80829; NIPPON Genetics, Tokyo, Japan) at 1.5×10^4 cells/well. After being left in culture for 72 h, the cells were pre-treated with 100 nM CTL with 0.1 μM DAPGreen for 30 min. Cells were treated with 5 μM SUN for 24 h and fixed with 4 % paraformaldehyde solution (09154–85; Nacalai Tesque) for 10 min, whereafter 25 μM DAPI solution (19178–91; Nacalai Tesque) was added and the cells observed under a confocal laser scanning microscope (LSM 700; ZEISS, Germany).

Similarly, H9c2 cells were seeded onto 8-well μ-slides at 1.5×10^4 cells/well. After being left in culture for 72 h, the cells were pre-treated with 100 nM CTL or 10 nM Rap with 0.1 μM DALGreen for 30 min. Cells were treated with 5 μM SUN for 24 h, exposed to 5 mg/mL Hoechst 33342 solution (346–07961; FUJIFILM Wako Pure Chemicals), and incubated for 5 min. Subsequently, the cells were washed three times with PBS and observed under a confocal laser scanning microscope.

2.6. Analysis of ingenuity pathway analysis data

The Ingenuity Pathway Analysis (IPA) application (Qiagen, Hilden, Germany) is a database of approximately 7.6 million biological functions and interactions of genes, proteins, tissues, drugs, and diseases. In this study, we initially extracted autophagy- and CTL-related molecules from the Ingenuity Pathway Knowledge Database. We then searched for biomolecules involved in the association between autophagy and CTL by analysing the direct and indirect actions of each molecule.

2.7. Fluorescence Immunostaining

H9c2 cells were seeded onto 8-well μ-slides at 1.5×10^4 cells/well. After being left in culture for 72 h, the cells were pre-treated with 100 nM CTL, incubated for 30 min, and then treated with 5 μM SUN. After 24 h of SUN treatment, the cells were fixed with a 4 % PFA solution for 10 min. The cells were then treated with 0.1 % polyoxyethylene (10) octylphenyl ether (160–24751; FUJIFILM Wako Pure Chemicals) and left on ice for 10 min. The cells were incubated with the following primary antibodies: myosin 4 monoclonal antibody (14–6503–82; 1:1000; Thermo Fisher Scientific, Waltham, MA, USA) and smooth muscle actin rabbit polyclonal antibody (23081–1-AD; 1:2500; Proteintech) at 4 ℃ overnight. The cells were then incubated with the following secondary antibodies: Alexa Fluor 488 goat anti-mouse IgG (H+L; A11001; 1:1000; Thermo Fisher Scientific) and Alexa Fluor 568 goat anti-rabbit IgG (H+L; A11036; 1:1000; Thermo Fisher Scientific) with slight agitation (50 rpm) for 1 h at room temperature (approximately 25 ℃). A 25 μM solution of DAPI was added to the cells that were thereafter observed under a confocal laser scanning microscope. Images were taken, and the cell length and width were measured for three individual cells using the ImageJ 1.54 g; java 1.8.0_345 [64-bit] software. The mean value of each group was calculated.

2.8. Statistical analysis

All data were analysed using R ver. 4.0.3 and are shown as the mean ± standard error of the mean. Differences between two groups were compared using Fisher's exact test. One-way analysis of variance (ANOVA), followed by Dunnett's or Tukey's post hoc tests, was performed to compare three or more groups. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Extraction of candidate drugs via database analysis

Of 11,014,290 FAERS reports, 156,656 reported cardiac failure, excluding duplicates; 125,240 of the 24,528,737 VigiBase reports were also of cardiac failure reported during the study period. A comparison of the frequency of cardiac failure reports between SUN users and non-users showed that this frequency in FAERS was 1.42 % in non-users compared with 2.87 % in users; in VigiBase, the frequency was 0.51 % in non-users and 1.40 % in users, indicating a high frequency of cardiac failure reports. The ROR was also significantly higher in FAERS (2.05 [CI_{min}: 1.93, CI_{max}: 2.19; $P < 0.01$]) and in VigiBase (2.77 [CI_{min}: 2.54, CI_{max}: 3.02; $P < 0.01$]) [Table S3].

Among the drugs concomitantly used with SUN, those with significantly lower RORs for cardiac failure were identified as denosumab (ROR: 0.30, CI_{min}: 0.1, CI_{max}: 0.95; $P = 0.02$) and vitamin D (ROR: 0.50, CI_{min}: 0.26, CI_{max}: 0.96; $P = 0.03$) from FAERS and vitamin D (ROR: 0.37, CI_{min}: 0.1, CI_{max}: 0.96; $P = 0.04$) from VigiBase (Table 1). Therefore, we considered vitamin D as a candidate drug because it significantly reduced the ROR for cardiac failure in the cases reported in both the FAERS and VigiBase databases.

Table 1
Effect of vitamin D on sunitinib-related heart failure based on reports in the FAERS and VigiBase databases.

	Drug name	Cardiac failure reporting rate (number of reports)		ROR	*P*-value
		With drug of interest	Without drug of interest	(95 % CI)	
FAERS	Denosumab	2.87 % (981/34,177)	2.80 % (978/34,898)	0.30 (0.1–0.95)	0.02
	Vitamin D	1.41 % (9/637)	2.81 % (972/34,607)	0.50 (0.26–0.96)	0.03
VigiBase	Vitamin D	0.52 % (4/765)	1.42 % (521/36,796)	0.37 (0.1–0.95)	0.04

Statistical analysis was performed using Fisher's exact test. FAERS, FDA Adverse Event Reporting System; ROR, reporting odds ratio; CI, confidence interval.

3.2. Protective effects of candidate drugs in cardiomyocytes

The efficacy of vitamin D, as determined through database analysis, was analysed *in vitro*. Although there are various types of vitamin D preparations available as pharmaceuticals, we used CTL because it occurs naturally in the body and is considered to have high bioactivity.

Initially, we evaluated cell viability in H9c2 cells, which significantly decreased after SUN treatment in a concentration-dependent manner (Fig. 1A; $P < 0.01$), whereas CTL significantly increased cell viability (Fig. 1B; $P < 0.05$).

3.3. Effects of candidate drugs on sunitinib-induced autophagy

Next, we investigated the mechanism by which CTL prevents SUN-induced cardiomyocyte damage. We evaluated autophagy using H9c2 cells. DAPGreen fluorescence was observed in the SUN-treated group ($P < 0.01$) but was significantly weaker in the CTL co-treated group (Fig. 2A, B; $P < 0.05$).

3.4. Effect of calcitriol on autophagy-related factors

We used IPA analysis to comprehensively investigate the mechanisms of action involved and explore the interactions between autophagy-related molecules and CTL. We found that molecules such as protein kinase B (Akt) and the mechanistic target of rapamycin (Rap), mTOR, were shared between the autophagy-related molecules and CTL (Supplementary Fig. 1), and that these are involved in pathways that regulate autophagy [11,12].

3.5. Impact of mTOR inhibition on the effects of calcitriol

To investigate the involvement of mTOR identified through IPA analysis, we examined whether the mTOR inhibitor, Rap, could influence the protective effect that CTL exerts on cardiomyocytes.

WST-8 assay results showed that H9c2 cell viability, which increased after CTL treatment, was significantly decreased by Rap (Fig. 3A; $P < 0.01$). In the autophagy evaluation, fluorescence intensity that had been attenuated by CTL treatment was enhanced by Rap (Fig. 3B, C; $P < 0.05$).

3.6. Effects of candidate drugs on sunitinib-induced changes in cardiomyocyte morphology

Although autophagy may be involved, SUN-treated cells exhibited morphological changes that were observed microscopically. We evaluated the changes in cell morphology using immunofluorescence staining of the cytoskeleton and found that the diameter and width of

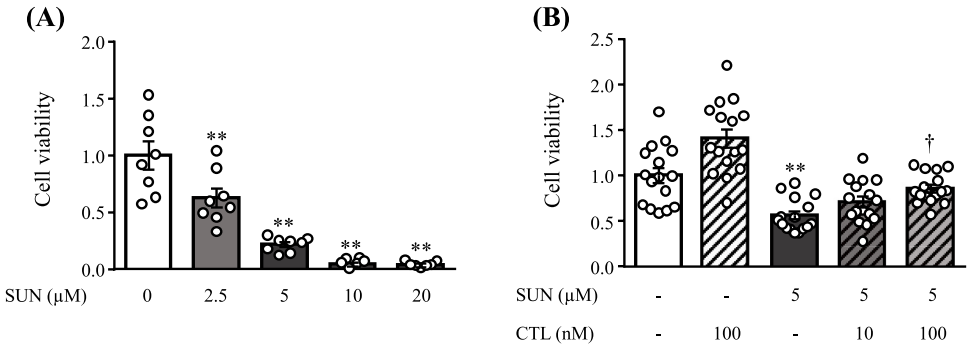

Fig. 1. Effect of sunitinib and calcitriol treatment on H9c2 cell viability. (**A**) Cell viability after treatment with each concentration of sunitinib (SUN) for 24 h determined using the WST-8 assay. n = 8; * *P < 0.01 vs. vehicle; Dunnett's test (one-way analysis of variance [ANOVA]). (**B**) Cell viability after pre-treatment with each concentration of calcitriol (CTL) for 30 min, followed by co-treatment with CTL and SUN for 24 h, as determined using the WST-8 assay. n = 16; * *P < 0.01 vs. vehicle; †P < 0.05 vs. SUN; Tukey's test (one-way ANOVA). Data are expressed as the mean ± standard error of the mean.

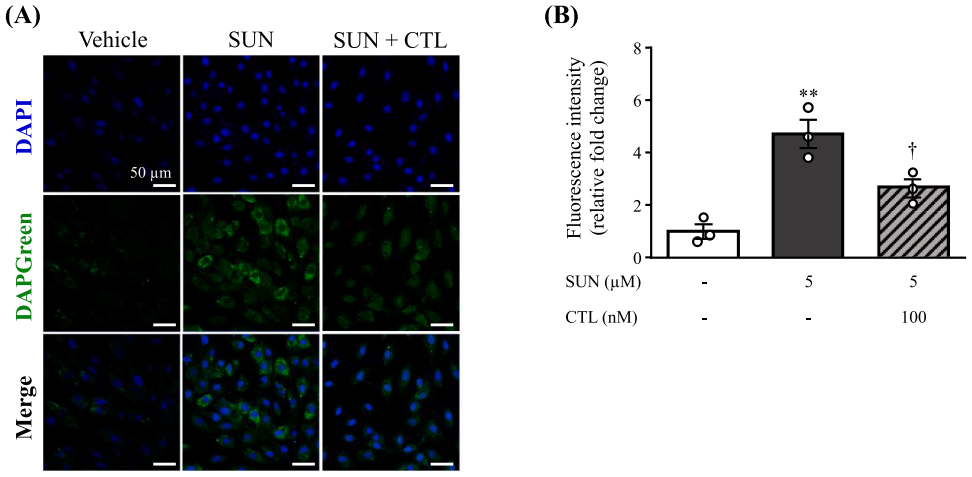

Fig. 2. Autophagy changes in H9c2 cells. (A) Fluorescence observed using DAPGreen. (**B**) Quantification of fluorescence intensity. n = 3;* *P < 0.01 vs. vehicle; †P < 0.05 vs· sunitinib (SUN); Tukey's test (one-way analysis of variance [ANOVA]). Data are expressed as the mean ± standard error of the mean. CTL, calcitriol.

cardiomyocytes were significantly reduced and increased, respectively, after SUN treatment, whereas CTL inhibited these changes (Fig. 4A–C; $P < 0.01$).

3.7. *Effects of candidate drugs on sunitinib anticancer activity*

To confirm whether CTL inhibited the anticancer effect of SUN, cell viability was evaluated in OS-RC-2 and 786-O human renal cancer cells. SUN treatment decreased cell viability in a concentration-dependent manner, whereas CTL did not increase this outcome (Fig. 5A, B).

4. Discussion

SUN is a TKI that is known to cause cardiotoxicity. In this study, we identified vitamin D as a new candidate drug against SUN-induced cardiotoxicity by analysing real-world data. We used CTL as a candidate drug and found that it significantly improved decreases in cell viability that had been induced by SUN, and inhibited SUN-induced autophagy. IPA analysis identified mTOR as a common factor between autophagy-related molecules and CTL. The mTOR inhibitor, Rap, cancelled the effects that CTL had on both cell viability and autophagy. We also evaluated the morphological changes in H9c2 cells and found that SUN induced changes in the surviving H9c2 cells, causing a rounded shape, whereas CTL improved their morphology to a shape similar to that of the elongated control group. Finally, we confirmed that CTL did not improve the decreased viability of renal cancer cells caused by SUN treatment when using OS-RC-2 and 786-O cells, nor did they affect the antitumor effects of SUN.

Based on several studies, SUN is known to cause severe left ventricular dysfunction in patients [1,2]. There are no effective countermeasures against SUN-induced cardiotoxicity, which remains a challenge in clinical practice. By analysing the FAERS and VigiBase databases, we identified vitamin D as a prophylactic drug candidate with high clinical potential for mitigating SUN-induced cardiotoxicity. We selected CTL, an active form of vitamin D, as the candidate drug because it occurs naturally in the body and is considered highly bioactive. CTL significantly improved the decrease observed in SUN-induced H9c2 cell viability in the WST-8 assay, suggesting that it has a protective effect on cardiomyocytes.

Autophagy is the intracellular process of self-digestion. Cells use autophagy to dispose of waste products and remove dangerous substances from the cell, recycling nutrients and regulating metabolites during this process [13,14]. However, excessive autophagy is known to cause cell death because it degrades cellular components more than necessary [7]; inadequate autophagy adversely affects the normal cell cycle because unnecessary components that need to be removed are not degraded and accumulate in the cell [6]. Several studies have reported that autophagy plays an important role in SUN-induced cardiotoxicity, which is thought to be induced by a dramatic increase in autophagy [14,15]. Previous studies have shown that SUN administration increases the expression levels of HMGB1 protein in myocardial cell nuclei [9]; the increased expression of HMGB1 promotes the progression of autophagy

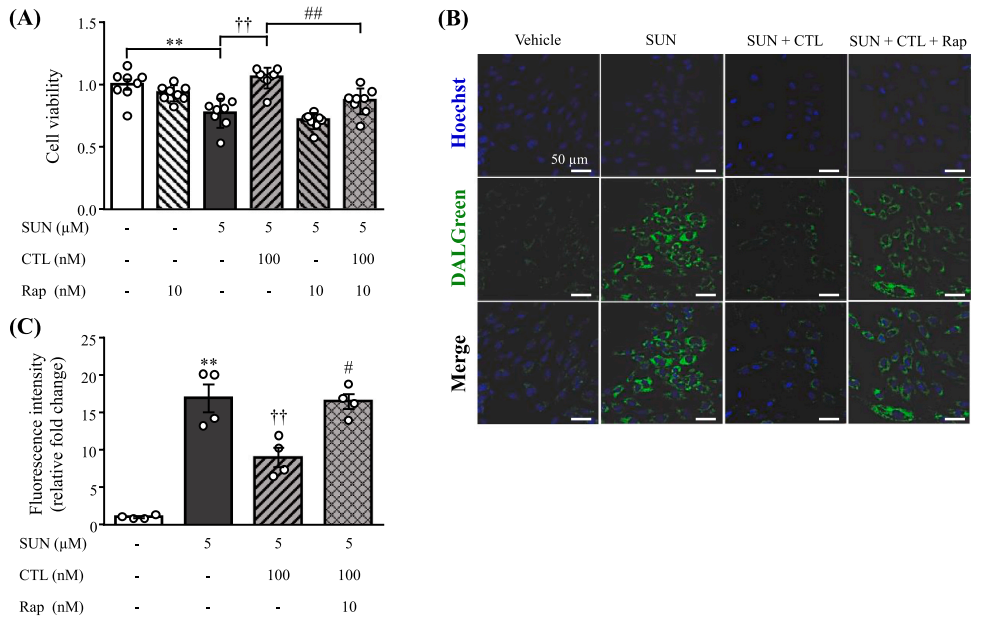

Fig. 3. Cell viability and autophagy changes in H9c2 cells co-treated with calcitriol. (A) Cell viability after pre-treatment with calcitriol (CTL) or rapamycin (Rap) for 30 min, followed by co-treatment with CTL, Rap, and sunitinib (SUN) for 24 h, as determined via the WST-8 assay. n = 8; * *P < 0.01 vs. vehicle; ††P < 0.01 vs. SUN; ##P < 0.01 vs. SUN + CT; Tukey's test (one-way analysis of variance [ANOVA]). (**B**) Fluorescence observed using DALGreen. (**C**) Quantification of fluorescence intensity. n = 4; * *P < 0.01 vs. vehicle; ††P < 0.01 vs. SUN; #P < 0.05 vs. SUN + CTL; Tukey's test (one-way ANOVA). Data are expressed as the mean ± standard error of the mean.

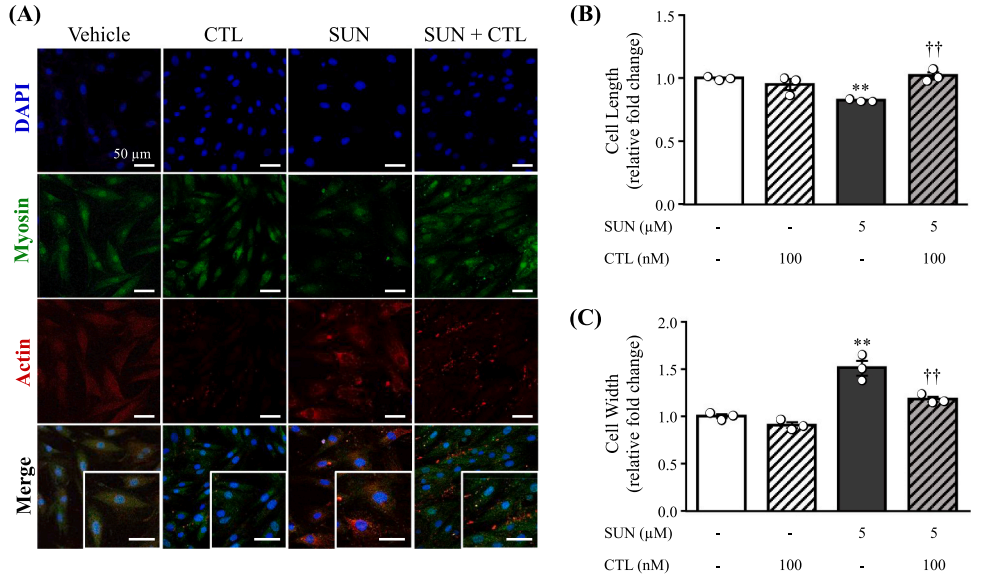

Fig. 4. Morphological changes in H9c2 cells. (**A**) Cytoskeleton, with actin and myosin observed using immunofluorescence staining. (**B, C**) Changes in cell (**B**) length and (**C**) width. n = 3; * *P < 0.01 vs. vehicle; ††P < 0.01 vs. sunitinib (SUN); Tukey's test (one-way analysis of variance [ANOVA]). Data are expressed as the mean ± standard error of the mean. CTL, calcitriol.

[16,17] and is one of the important factors in the pathogenesis of autoimmune myocarditis, myocardial infarction, and myocardial fibrosis [18,19]. Therefore, the induction of autophagy in cardiomyocytes may lead to a decrease in cardiac function. In this study, we observed an increase in autophagolysosomes after SUN treatment (Fig. 2A, B), suggesting that SUN is toxic to cardiomyocytes.

There are various reports on the relationship between vitamin D and autophagy. These studies showed that vitamin D may induce autophagy, thereby reducing apoptosis and preventing cell death, or it may exhibit a cytoprotective effect by suppressing overactive autophagy [20,21]. In the latter case, vitamin D receptor agonists restore autophagy to normal by decreasing the LC3-II/LC3-I ratio and p62 protein expression levels, which are commonly used as markers to evaluate autophagy and have cardioprotective effects [21]. In the present study, CTL decreased the number of autophagolysosomes that had been increased by SUN (Fig. 2A, B), suggesting that CTL exerts a cardiomyocyte-protective effect by suppressing excessive autophagy.

We suggest that mTOR is involved in the autophagy-regulating action of CTL. As a serine/threonine kinase, mTOR is activated by the phosphorylation of Akt, which is activated by phosphatidylinositol 3-kinase. Activation of mTOR leads to the inhibition of autophagy through the phosphorylation of several autophagy-related proteins, such as

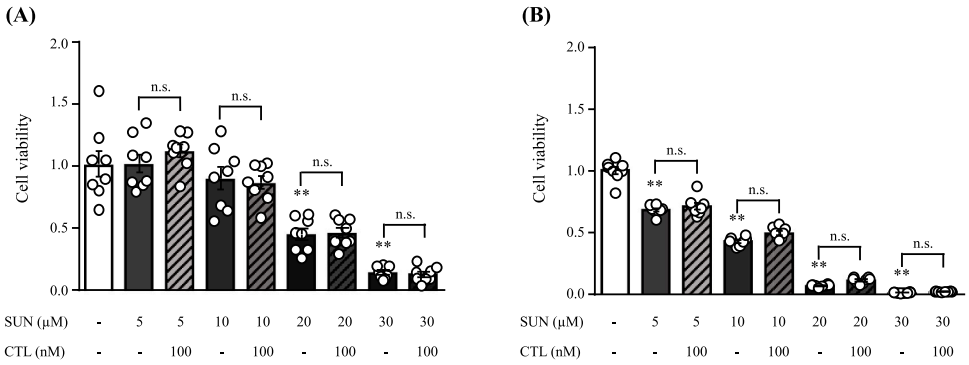

Fig. 5. Effect of calcitriol on the anticancer activity of sunitinib in human renal cancer cells. (**A, B**) Cell viability of (**A**) OS-RC-2 and (**B**) 786-O cells pre-treated with calcitriol (CTL) for 30 min, followed by co-treatment with each concentration of CTL and sunitinib (SUN) for 24 h, as determined using the WST-8 assay. n = 8; * *P < 0.01 vs. vehicle; Tukey's test (one-way analysis of variance [ANOVA]). Data are expressed as the mean ± standard error of the mean.

ULK1, ATG13, AMBRA1, and ATG14L, which promote autophagy initiation and autophagosome nucleation [11] and play important roles in the completion of autophagy [12]. Thus, mTOR plays a major role in the pathways that regulate autophagy. In this study, the combined use of Rap as an mTOR inhibitor decreased cell viability, which was improved after CTL exposure (Fig. 3A), and enhanced the fluorescence intensity of DALGreen, which was attenuated by CTL (Fig. 3B, C), suggesting that Rap inhibited the positive effects of CTL. As such, SUN induces cardiomyocyte damage by inducing autophagy, whereas CTL prevents cardiomyocyte damage by suppressing autophagy induction; the mTOR pathway may be involved in this molecular mechanism.

In addition to autophagy, cell morphology influences cardiac function. Cardiomyocytes generate various types of stress to maintain the sustained contractile function of the heart, and these hemodynamic stresses have a profound effect on cell structure. Hemodynamic changes lead to geometrical changes in cell shape. Increased preload and enlargement of the ventricle cause eccentric hypertrophy, with geometric changes in cellular shape, changing the aspect ratio for length and width from 7:1 to approximately 11:1. An increased afterload (namely, the pressure overload) causes concentric hypertrophy, which changes the cellular aspect ratio from 7:1–1:1 [22]. In the current study, SUN shortened the long diameter and lengthened the width of cardiomyocytes, whereas pre-treatment with CTL maintained a cell shape similar to that of the control group (Fig. 4A–C). It was reported that a deviation from the normal cell shape is associated with the inactivation of specific pathways, such as oxidative phosphorylation, protein kinase A, and β-adrenergic signalling pathways, in the heart [22]. SUN-induced changes in cardiomyocyte morphology may be linked to impaired function of the cardiac conduction system.

Finally, because SUN is used for the treatment of cancer, a drug that prevents cardiotoxicity must not interfere with its antitumor effect. CTL does not impact the SUN-induced decrease in renal cancer cell viability (Fig. 5A, B), suggesting that it can prevent cardiotoxicity without interfering with the antitumor effect of SUN. Moreover, in a recent review, vitamin D was reported to have antitumor effects against various cancers, both directly by regulating tumour cell differentiation, proliferation, and apoptosis, and indirectly by regulating immune cells in the malignant tumour microenvironment [23], suggesting that vitamin D does not affect the progression of renal cancer cells.

This study had some limitations. First, FAERS data may have biases, such as under-reporting or over-reporting, because it is a database of spontaneous reports. Vitamin D is used to treat osteoporosis, and many patients with osteoporosis are older women. Therefore, a detailed analysis of these background factors is required. In addition, because most FAERS reports are from the U.S., there is a possibility that the data collected may be affected by the unique U.S. system. Although the accuracy of our analysis was enhanced using VigiBase in combination with FAERS, a detailed analysis of patients' backgrounds is necessary to obtain more reliable results. Second, although we focused on mTOR, we could not fully evaluate its impact on other molecules and pathways. However, based on the results of experiments using mTOR inhibitors, the mTOR pathway is considered a major pathway. This is because the mTOR pathway is regulated by phosphorylation, which was not evaluated in this study. Third, the efficacy of vitamin D was only analysed *in vitro*; no *in vivo* or human studies have been conducted. As the analysis of the adverse event database suggests its efficacy, it is possible that vitamin D may be effective within the range of doses used in actual clinical practice; however, further studies are needed regarding finer doses and durations.

In conclusion, we identified vitamin D as a novel prophylactic candidate drug against SUN-induced cardiotoxicity by analysing global real-world databases and investigating its efficacy *in vitro*. CTL has the potential to prevent SUN-induced cardiomyocyte damage through autophagy, particularly via mTOR-mediated pathways. Therefore, CTL may be an effective prophylactic agent against SUN-induced cardiotoxicity.

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CRediT authorship contribution statement

Niimura Takahiro: Writing – original draft, Formal analysis. **Sakamoto Yoshika:** Writing – original draft, Visualization, Investigation, Formal analysis. **Tomochika Nanami:** Investigation. **Goda Mitsuhiro:** Writing – review & editing. **Izawa-Ishizawa Yuki:** Writing – review & editing. **Hamano Hirofumi:** Writing – review & editing. **Ishizawa Keisuke:** Writing – review & editing. **Zamami Yoshito:** Writing – review & editing. **Murakawa Wakana:** Investigation. **Yagi Kenta:** Writing – review & editing. **Aizawa Fuka:** Writing – review & editing.

Declaration of Competing Interest

All the authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118137.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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