PLCγ1 inhibition combined with inhibition of apoptosis and necroptosis increases cartilage matrix synthesis in IL-1β-treated rat chondrocytes

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Keywords
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Osteoarthritis (OA) is an age-related, chronic degenerative disease. With the increasing median age of the population, this disease has become an important public health problem. New, disease-modifying therapies are needed. A potential novel molecular target is phospholipase Cγ1 (PLCγ1), a critical enzyme with important functions including calcium signaling regulation and cell proliferation. In rat chondrocytes treated with IL-1β (20 ng·mL⁻¹ for 36 h), inhibition of PLCγ1 with U73122 (2 µM for 12 h) increased levels and expression of the cartilage matrix components Collagen2 and Aggrecan. This beneficial effect of PLCγ1 inhibition was counteracted by increased chondrocyte apoptosis and necroptosis, increased cell death, and increase levels of ROS, all potentially negative for OA. Combined treatment of IL-1β + U73122-treated chondrocytes with inhibitors of apoptosis (Z-VAD, 10 µM) and necroptosis (Nec-1, 30 µM) enhanced the increases in levels and expression of Collagen2 and Aggrecan, and prevented the increases in cell death and ROS levels. These results suggest that PLCγ1 inhibition may be a viable approach for an OA therapy, if combined with targeted inhibition of chondrocyte apoptosis and necroptosis.

Abbreviations
Nec-1, necrostatin-1; OA, osteoarthritis; PLCγ1, phospholipase Cγ1; ROS, reactive oxide species; RT-PCR/qPCR, real-time quantitative polymerase chain reaction; Z-VAD, Z-VAD-FMK.
totic body formation [23,24]. Necroptosis is another and is accompanied by pyknosis, karyolysis, and apoptosis [21,22]. Studies showing that U73122 increases cell apoptosis in neonatal rats reduced OA damage [20]. However, there are also systems, which can destroy organelles such as mitochondria and lead to cell death [25,26]. Previous studies have shown that both apoptosis and necroptosis in chondrocytes are OA risk factors [27]. Those inhibitors were added for the last 12 h of the 36 h of treatment of OA, we used IL-1β-activated chondrocytes to mimic the OA state and investigated the effects of inhibiting PLCγ1 (with U73122) [20], apoptosis (with caspase inhibitor Z-VAD-FMK or Z-VAD), and necroptosis (with RIP1 inhibitor necrostatin-1 or Nec-1), alone and in combination, on production of the cartilage matrix components Collagen2 and Aggrecan.

### Materials and methods

**Antibodies and reagents**

Antibodies used in this study were purchased from the following companies: antibodies against Collagen2 (1 : 1000), Bcl-2 (1 : 1000), Bax (S757) (1 : 1000), P53 (1 : 1000), RIP1 (1 : 1000), and RIP3 (1 : 1000) were purchased from Abcam Inc. (Cambridge, MA, USA); antibodies targeting PLCγ1 (1 : 1000), p-PLCγ1 (Y783) (1 : 1000), and caspase3 (1 : 1000) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA); and antibodies against Aggrecan (1 : 1000) and β-actin (1 : 40 000) were purchased from Sigma-Aldrich in China (Shanghai, China), respectively. Inhibitors used in this study (U73122, Z-VAD, and Nec-1) were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Cytokine used in this study such as recombinant rat IL-1β was purchased from PeproTech (Rocky Hill, NJ, USA). Other reagents were of the highest grade commercially available.

**Isolation, culture, and treatment of rat chondrocytes**

All the operations were approved by the committee on the Ethics of Animal Experiments of Xiamen University (ID no. 20170301).

Chondrocytes were isolated from knee cartilage of neonatal Sprague Dawley rats (within 1–2 days after birth) by mechanical and collagenase digestions [31,32]. Primary chondrocytes were cultured in DMEM/F12 with 10% fetal bovine serum and penicillin (100 U·mL⁻¹)/streptomycin (0.1 mg·mL⁻¹). At 80% confluence, the cells were then plated in 60-mm cell culture dishes at 1 : 3 or 1 : 4 at 37 °C, 95% humidity, and 5% CO2, and F2 generation cells were used for the experiments. IL-1β (10, 20, and 40 ng·mL⁻¹) when studied alone was added to the culture for 36 h to activate the cells [32]. The inhibitors U73122 (1, 2, 4, and 6 μM), Z-VAD (10 μM), and Nec-1 (30 μM) were added to the IL-1β-stimulated chondrocyte cell culture for 12 h. Those inhibitors were added for the last 12 h of the 36 h of IL-1β. When studied together, inhibitors were added at 10-min intervals.

**Western blotting analysis**

Protein extracts were subjected to SDS-PAGE (8–15%) and transferred to a PVDF membrane (GE Healthcare, Hertfordshire, UK) as described before [33,34]. Briefly, the PVDF membrane was clipped and incubated with the above-mentioned primary antibodies at 4 °C overnight, followed by complete elution of the primary antibodies and the addition of the corresponding secondary antibodies at room temperature for 1 h. An enhanced chemiluminescence

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(ECL) detection kit was used to detect antibody reactivity (Pierce, Rockford, IL, USA).

Cell viability assay
For the cell viability assay, $1 \times 10^4$ cells were cultured in 96-well plates and were treated with IL-1β and the inhibitors as described. At the end of the treatment period, 3-(4-dimethylthiazol-2-y)-2, 5-diphenyl-tetrazolium bromide (MTT reagent) was added to the cultures as per the manufacturer’s instruction and as described in a previous study [35]. After 4 h, DMSO was added to stop the reaction and solubilize the formazan. The optical density was measured at 490 nm with GloMax 20/20 luminometer (Promega, Madison, WI, USA).

Real-time quantitative polymerase chain reaction (RT-PCR/qPCR)
Total RNA was extracted from the chondrocytes using TRIzol (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized with 1 µg of total RNA at 37 °C for 15 min using a PrimeScript RT Master Mix Kit (Takara, Dalian, China). Real-time PCR was then performed using a Roche LightCycler 96 (Roche, Basel, Switzerland) with a SYBR Premix Ex Taq II Kit (Takara). The results were normalized to GAPDH and analyzed using SDS software v2.1 as previously described [33, 34]. The following primers were used in quantitative PCR for measuring gene expression relative to GAPDH.

Primer sequences used in this study were as follows:

Collagen2:
Forward 5-TCTAAGGCTGCAATGGTA-3,
Reverse 5-GGACCAACTTTGCTTGGAGGAC-3;
Aggrecan:
Forward 5-TCCGCTGGTCTGATGGACAC-3,
Reverse 5-CCAGATCATCACTACGCAGTCCTC-3;
GAPDH:
Forward 5-CAAGCTACGCAAGCCTGAC-3,
Reverse 5-ACATACTCAGCACCAGCATC-3.

Apoptosis and necroptosis analysis
The detection of apoptotic cells was performed using a Beckman CytoFluor (Beckman, Brea, CA, USA) with the Annexin V-FITC/PI detection kit (Beyotime Biotechnology, Shanghai, China), as per the manufacturer’s instructions. The results were analyzed using CYTEXPERT 1.2.11.0 and FLOWJO 10 as previously described. The percentage of cell death (including dying and dead cells) was calculated from the total number of viable apoptotic cells and nonviable apoptotic cells (from Annexin V-FITC with and without PI staining) [36]. Protein levels of apoptosis and necroptosis indices Bcl-2, Bax, P53, pro/cleaved-caspase3, RIP1, and RIP3 were analyzed by Western blotting.

Reactive oxide species (ROS) analysis
ROS were analyzed with the ROS detection kit (Beyotime Biotechnology), as per the manufacturer’s instructions. The results were analyzed using CYTEXPERT 1.2.11.0 and FLOWJO 10 as previously described [37].

Statistical analysis
Data are expressed as the mean ± 95% confidence interval (CI) of three independent experiments for each experiment. One-way analysis of variance (ANOVA) with the Dunnett test was used to compare the control group with treatment groups by GRAPHPAD PRISM 5 software (GraphPad Software, San Diego, CA, USA). Differences at a value of $P < 0.05$ were regarded as statistically significant.

Results
Effects of PLCγ1 inhibitor U73122 on Collagen2 and Aggrecan levels in IL-1β-treated rat chondrocytes
To mimic the OA state, rat chondrocytes were treated with different concentrations of IL-1β (10, 20, and 40 ng·mL$^{-1}$) for 36 h. Collagen2 and Aggrecan levels decreased significantly at IL-1β 20 and 40 ng·mL$^{-1}$ (Fig. 1A). To determine effects of PLCγ1 inhibition, chondrocytes treated with IL-1β (20 ng·mL$^{-1}$) were treated with the PLCγ1 inhibitor U73122 at 1, 2, 4, and 6 µM for 12 h. Compared with the IL-1β-treated control group, U73122 at 2 µM significantly decreased the phosphorylation of PLCγ1 (Figs. 1B and S1). This was accompanied by significantly higher Collagen2 and Aggrecan protein levels (Figs.1B and S1) and mRNA levels (Fig. 1C).

Higher concentrations of U73122 (4 and 6 µM) also significantly reduced PLCγ1 phosphorylation but did not increase Collagen2 and Aggrecan levels compared with the IL-1β-treated control group (the Aggrecan level at 6 µM was significantly decreased). Interestingly, compared with the 1 µM group, U73122 at 2 µM had a significantly greater decrease in PLCγ1 phosphorylation but a smaller increase in Collagen2 and Aggrecan levels.

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U73122 with Z-VAD and Nec-1 promotes ECM synthesis

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Effects of U73122 alone and combined with Z-VAD and Nec-1 on chondrocyte apoptosis and necroptosis

The MTT assay showed that IL-1β (20 ng·mL⁻¹) did not affect chondrocyte proliferation with or without PLCγ1 inhibition with U73122 (2 μM) (Fig. 2A). However, compared with the IL-1β-treated group the percent dead cells increased significantly in the IL-1β + U73122-treated group (Annexin V-FITC/PI assay; Fig. 2B). ROS levels significantly increased in the IL-1β-treated group vs untreated control group (Fig. 2C), and levels were further increased significantly in the IL-1β + U73122 group.

Changes in markers of apoptosis and necroptosis were consistent with IL-1β-induced chondrocyte apoptosis and necroptosis that were further increased by PLCγ1 inhibition with U73122 (Fig. 2D). Bcl-2 level decreased in the IL-1β group (vs untreated group) and further decreased significantly in the IL-1β + U73122 group (vs IL-1β-treated group). Bax and p53 levels increased in the IL-1β group (vs untreated group) and further increased significantly in the IL-1β + U73122 group (vs IL-1β-treated group). In the IL-1β group, there was a small but not significant decrease in the pro-caspase3 level and a significant increase in the cleaved-caspase3 level (vs untreated
U73122 combined with apoptosis and necroptosis inhibitors increased Collagen2 and Aggrecan levels in IL-1β-treated rat chondrocytes

Treatment with Z-VAD or Nec-1 alone had no significant effect on Collagen2 or Aggrecan levels compared with the IL-1β + U73122-treated control group (Fig. 3A). Both Z-VAD and Nec-1 produced small decreases in Collagen2 mRNA level but had no effect on Aggrecan mRNA level. (Fig. 3B). Combined treatment with Z-VAD and Nec-1 significantly increased protein and mRNA levels of Collagen2 and Aggrecan compared with the IL-1β + U73122-treated control group.

Discussion

As one of the most important members of the phospholipase family, the role of PLCγ1 in osteoarthritis, especially in chondrocytes, has become a research focus [38,39]. In a previous study in a Sprague Dawley rat model of OA, we showed that intra-articular injection of the PLCγ1 inhibitor U73122 reduced cartilage damage [20]. This suggested that targeted inhibition of PLCγ1 might be a potential therapy for OA. In this current study in IL-1β-treated rat chondrocytes, we found that a low concentration (2 μM) of the PLCγ1 inhibitor U73122 decreased PLCγ1 phosphorylation and improved cartilage matrix synthesis, with increased levels of Collagen2 and Aggrecan. However, higher concentrations of U73122 reduced levels of Collagen2 and Aggrecan. These observations are consistent with our previous results [40] and with those of Gao [41], who found U73122 10 μM reduced Collagen2 and Aggrecan mRNA levels in rat nucleus pulposus cells. Our subsequent experiments showed this decreased matrix synthesis to result from increased apoptosis and necroptosis of chondrocytes at the higher concentrations of U73122 and higher level of PLCγ1 inhibition.

The conclusion from these observations is that PLCγ1 inhibition and cartilage matrix synthesis is not...
a simple linear relationship, and there might be a
crossover point between them such that a successful
therapy for OA would provide some but not complete
PLC\textsubscript{c}1 inhibition.

Increased chondrocyte death and ROS levels are risk
factors for OA\cite{42}, and the results of our experiments
are consistent with these factors being associated with
reduced cartilage matrix synthesis. Increases in cell
death and ROS levels are closely related to pro-
grammed cell death (PCD), especially apoptosis and
necroptosis, and apoptosis and necrosis inhibitors can
reduce both factors\cite{43}. Our current results are also
consistent with this. A study by Yuan\textit{et al.}\cite{21}
showed that inhibition of PLC\textsubscript{c}1 phosphorylation with
U73122 10 lM increased apoptosis in pheochromocy-
toma 12 (PC12) cells induced by hydrogen peroxide. A
study by Jiang\textit{et al.}\cite{22} also showed that knockdown
of PLC\textsubscript{c}1 can increase apoptosis of vascular smooth
muscle cells. The increased level of chondrocyte apop-
tosis after PLC\textsubscript{c}1 inhibition with U73122 in our stud-
ies was similar to that observed by Xiao\textit{et al.}\cite{39}. In
addition, chondrocyte apoptosis is a risk factor for the
initiation and development of OA, and inhibition of
chondrocyte apoptosis can relieve OA\cite{27,28}. Our cur-
rent study results are consistent with these data.

Although necroptosis is currently a focus for
research in programmed cell death, the direct relation-
ship between PLC\textsubscript{c}1 and necroptosis is still not clear.
However, it is clear that PLC\textsubscript{c}1 is an important regu-
lator of Ca\textsuperscript{2+}, and Chang\textit{et al.} have shown that Ca\textsuperscript{2+}
regulates RIP3 through CaMKII, thus affecting the
necroptosis level in rat ventricular cardiomyocytes
\cite{44,45}. We therefore had reason to believe there was a
relationship between PLC\textsubscript{c}1 and necroptosis in chon-
drocytes. This was confirmed, and our results showed
that chondrocytes necroptosis increased with PLC\textsubscript{c}1
inhibition with U73122 treatment.

It is worth noting that in this study, Z-VAD and
Nec-1 treatment alone did reduce chondrocyte apopto-
sis and necroptosis, respectively, but neither improved
cartilage matrix synthesis. Previous reports show that
apoptosis and necroptosis share the same signal path-
way in the early stage, and it is difficult to completely
inhibit programmed cell death by inhibiting just one

**Fig. 3.** U73122 combined with apoptosis
and necroptosis inhibitors increased
Collagen2 and Aggrecan levels in IL-1b-
treated rat chondrocytes. Chondrocytes
pretreated by IL-1b (20 ng\textcdot mL\textsuperscript{-1} for 36 h)
and U73122 (2 \textmu g for 12 h) were treated
with Z-VAD (10 \textmu g for 12 h) or and Nec-1
(30 \textmu g for 12 h). Protein (A) and mRNA (B)
levels of Collagen2 and Aggrecan were
analyzed by Western blotting and RT-PCR,
respectively. \beta-Actin was used as the
control for Western blotting, and GAPDH
was used as the control for RT-PCR.
Values are means and standard deviations,
the error bars represent SD. One-way
ANOVA with the Dunnett test was used
to calculate P values. These results are
representative of at least three
independent experiments in each
experiment. *P < 0.05, **P < 0.01,
***P < 0.001, ****P < 0.0001.
In our study, combined inhibition of apoptosis and necroptosis with Z-VAD and Nec-1 combined produced greater inhibition of cell death and increased cartilage matrix synthesis. The observation of enhanced cartilage matrix synthesis at a low concentration of U73122 but reduced cartilage matrix synthesis at a higher concentration is not rare in pharmaceutical research and clinical drug usage [47–49]. For example, except for leukemia [50], tretinoin is also used in photodamaged skin and cosmetology [51]. However, because of its strong inhibition of keratinization, a large overdose can damage the skin, causing skin erythema and ulceration [52,53]. Although U73122 at 2 μM improved cartilage matrix synthesis, this concentration did increase chondrocyte apoptosis and necroptosis levels, which would partially counteract the effect on cartilage matrix synthesis. Inhibition of the U73122-induced programmed cell death by simultaneous treatment with the apoptosis inhibitor Z-VAD and the necroptosis inhibitor Nec-1 increased chondrocyte proliferation and further enhanced cartilage matrix synthesis. Therefore, we propose PLCγ1 as a new molecular target as a potential disease-modifying therapy for OA (Fig. 4).

**Fig. 4.** Molecular mechanism of PLCγ1 inhibition combined with inhibition of apoptosis and necroptosis increases cartilage matrix synthesis in IL-1β-treated rat chondrocytes. Inhibition of PLCγ1 phosphorylation by U73122 improved cartilage matrix synthesis in IL-1β-treated rat chondrocytes. However, it also increased cell apoptosis and necroptosis, which reduced the effect on cartilage matrix synthesis. Inhibition of programmed cell death by simultaneous treatment with the apoptosis and the necroptosis inhibitor increased chondrocyte proliferation and further enhanced cartilage matrix synthesis.

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**Conflicts of interest**

The authors declare no conflict of interest.

**Data accessibility**

The data will be available from the corresponding author upon reasonable request.

**Author contributions**

XC and CX conceived the study and designed the experiments. XC, RC, and YX contributed to the data collection. XC and RC performed the data analysis and interpreted the results. XC wrote the manuscript. CX contributed to the critical revision of the article. All authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** U73343 can’t inhibit PLCγ1 and also can’t increase Collagen2 and Aggrecan levels in IL-1β-treated rat chondrocytes. Rat chondrocytes pretreated with IL-1β (20 ng/ml for 36 hours) were treated with U73122 or U73343 (2 μM for 12 hours).