Chondrocytic and pharmacokinetic properties of Phlpp inhibitors

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ABSTRACT

Objective: The pleckstrin homology domain leucine-rich repeat protein phosphatases (Phlpp1/2) were recently identified as potential therapeutic targets for cartilage regeneration in osteoarthritic joints. Phlpp inhibitors NSC 117079 and NSC 45586 increase chondrocyte proliferation and matrix production, but the pharmacodynamics and pharmacokinetics of these compounds are not known.

Design: Chondrocytic effects of Phlpp inhibitors, NSC 117079 and NSC 45586, were measured by western blotting of Phlpp substrates, glycosaminoglycan (GAG) assays, and transcriptomic assays. Liquid chromatography/mass spectroscopy assays were established to measure NSC 117079 and NSC 45586 in vitro and in vivo. The effects of NSC 117079 and NSC 45586 on articular cartilage structure in vivo after intra-articular injection were determined by histology.

Results: The Phlpp inhibitors NSC 117079 and NSC 45586 were highly stable in vitro and stimulated GAG, Sox9, proteoglycan 4 and collagen 2 production in maturing but not more differentiated chondrocytes in vitro. Both molecules reduced Phlpp1/2 levels and suppressed matrix degradation to functionally extend their inhibitory effect on these phosphatases. In vivo, NSC 117079 was eliminated from the bloodstream within 4 h after intra-venous injection, while NSC 45586 was eliminated in 8 h and had a higher volume distribution. Both molecules increased articular cartilage area on lateral and medial tibial plateaus and femoral condyles by 15% in C57Bl/6 mice between four and five weeks of age.

Conclusion: These data advance our understanding of how Phlpp inhibitors promote and preserve cartilage formation and provide a basis for understanding their safety and activity in vivo.

1. Introduction

Osteoarthritis (OA) is a degenerative and chronic musculoskeletal disease that causes significant disability [1,2]. It is predicted that 67 million people (one-third of the United States population) will have OA by year 2030 [3]. Less than 5% of these individuals will have joint replacement surgery and the majority will only receive palliative care for OA pain [4]. Palliative drugs like steroidal, non-steroidal anti-inflammatory drugs, and mu-opioids account for about one third of direct expenditures for OA treatment in the USA [5]. While these drugs can relieve pain, they do not prevent joint degeneration or promote tissue healing and can only be administered for a limited period of time because they cause toxicity, cardiovascular events and addiction [6]. Novel regenerative therapies and disease modifying OA drugs (DMOADs) that repair and preserve cartilage are needed to reduce morbidity and societal burdens of OA [7].

Pleckstrin homology domain leucine-rich protein phosphatases (Phlpp1/2) remove phosphate groups from serine and threonine residues on several intracellular substrates that control proliferation, protein synthesis and survival. We recently showed that a small molecule inhibitor of Phlpp1/2, NSC 117079 [8], promoted chondrocyte expansion and matrix production [9,10]. NSC 117079 also relieved allodynia and preserved locomotion as well as cartilage structure in mice following meniscal destabilization [9]. Chondrocytes and mice deficient in Phlpp1 showed similar phenotypes in vitro and in vivo [11]. Phlpp1 suppresses several anabolic pathways including Akt2 [12–18] and Phlpp inhibitors

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reverse this suppression to promote cell anabolism. Within chondrocytes, Phlpp inhibitors and genetic deletion of Phlp1 increase phosphorylation of Akt and other Phlp substrates [9,10]. Phlpp inhibitors stimulate production of chondrocytic growth factors and receptors, including Fgfl8 [10] and Pthlr [10,19]. In the present study, we compared the effectiveness of NSC 117079 to another Phlpp inhibitor, NSC 45586 [8], in promoting chondrocytic activity and developed LC/MS assays to define the pharmacokinetic properties of these molecules in vitro and in vivo.

2. Methods

2.1. Reagents

Phlpp inhibitors NSC 117079 and NSC 45586 (Supplementary Figure 1 A and D) were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis of the National Cancer Institute.

2.2. Isolation and culture of mouse chondrocytes

Primary immature mouse articular chondrocytes (IMCs) were isolated from 5-day-old C57Bl/6J male mice and plated in micromasses at a seeding density of 2 × 10^5 cells per micromass in 35 mm petri plates. Three micromasses were cultured in DMEM supplemented with 2% FBS and 1% antibiotic/antimycotic as previously described [10,20]. After three days, the medium was switched to DMEM supplemented with 2% FBS, ITS (Insulin Transferrin Selenium) (Gibco), 0.05 mg/mL ascorbic acid and 10 μM β-glycerophosphate to promote chondrogenesis. Small-molecule Phlpp inhibitors (25 μM NSC 117079, 25 μM NSC 45586) or vehicle (0.05% DMSO) were added to the cultures for the indicated time periods with media changes every three days. Each experiment was performed in triplicate and repeated at least three times. Results from a representative experiment are shown.

2.3. GAG assays

Micromasses were digested overnight in a solution containing 0.05 M phosphate buffered saline (PBS), pH 6.5, 5 mM cysteine, 5 mM EDTA and 125 μg/mL papain. GAG concentrations were assessed using the Glycosaminoglycan Assay Kit (Chondrex #6022).

2.4. RNA isolation and semi-quantitative real-time PCR

Total RNA was extracted from primary chondrocyte cells using TRIzol (Invitrogen) and chloroform. RNA (2 μg) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Resulting cDNAs were template for semi-quantitative real-time PCRs containing the following gene-specific primers: Phlp1 (5′- AGCTGAAAGCCATCCAAACA-3′, 5′- GCTAGGTCCACA-CACTTG-3′), Phlp2 (5′ GGCGTACGCGCTTGTTGT-3′, 5′ AGCGTCGGTTGCGCATC-3′), Col2a1 (5′- ACTGTTAAGTGCCGAC-GAC-3′, 5′- CCACACCAATTCCTGTC-3′), Prg4 (5′- ACCACACCCATCCTAACA-3′, 5′- ATCCAGCTTACATGC-3′), Sox9 (5′- CTGGGGGAGATTTTTCCAG-3′, 5′- AGTCTCGAGCCGC- GACTC-3′), Ywzh (5′- GCCCTAAATGGTCTGTCACC-3′, 5′- GCCATTGTTGCTACTGCCG-3′), Mmp13 (5′-TGAAACCAGCAGCCT- CAGTCTC-3′, 5′-GACCTTCAGAGGTGTTGC-3′). Fold changes in gene expression for each sample were calculated using the 2^-ΔΔCt method [21] relative to control after normalization of gene-specific Ct values to Ywzh Ct values. Each experiment was performed in triplicate and repeated at least three times. Results from a representative experiment are shown.

2.5. Western blotting

Cell lysates were collected in a buffered SDS solution (0.1% glycerol, 0.01% SDS, 0.1 M Tris, pH 6.8) on ice. Total protein concentrations were obtained using the Bio-Rad DC Assay (Bio-Rad). Proteins (40 μg) were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Western blotting was performed with antibodies at 1 μg/mL for Actin (Invitrogen, A4700), pAkt2Ser474 (Cell Signaling Technology, 8599), pPKC Ser660 (Cell Signaling Technology, 9371), Akt (Cell Signaling Technology, 4685), PKC (Cell Signaling Technology, 46809), pErk1/2Thr202/Tyr204 (Cell Signaling Technology, 4370), Erk1/2 (Cell Signaling Technology, 9102), Mmp13 (Abcam, 219620), Prg4 (EMD Millipore, 2200), Phlp1 (Proteintech, 22789-1-AP), Phlp2 (Bethyl Laboratories, A300-661A), DIPEN (MyBioSource, MBS442010) and corresponding secondary antibodies conjugated to horseradish peroxidase (HRP) (Cell Signaling Technology). Antibody binding was detected with the Supersignal West Femto Chemiluminescent Substrate (Pierce Technology, Rockford, IL). Band intensities of proteins of interest were quantified on western blots using ImageJ and normalized to the intensity of the loading controls.

2.6. LC/MS assays for Phlpp inhibitors

The LC-MS/MS system (Waters, Milford, MA) consisted of an Acquity 1 class ultra-performance liquid chromatography (UPLC) system, containing a quaternary solvent manager and sample manager-FTN coupled to a Xevo G2-QS quantitative time-of-flight mass spectrometer (QTof) equipped with an electrospray ionization (ESI) source. Detection was accomplished by multiple reaction monitoring (MRM). Data were acquired and analyzed using Masslynx v4.1 software. The liquid chromatographic separations were accomplished using an Agilent Poroshell EC-C18 (particle size 2.7 μm) column.

For NSC 117079, the MRM transition m/z 302.01 > 238.05 under negative ESI was monitored following chromatographic separation using linear gradient elution with mobile phase A composed of water containing 0.1% ammonium hydroxide and mobile phase B composed of methanol containing 0.1% ammonium hydroxide. The elution was initiated at 69% A and 31% B, followed by linear increase of B to 35% over 4.25 min. B was held at 35% for 0.05 min and returned to initial conditions over 1.7 min. The flow rate was maintained at 0.3 mL/min (total run time of 6.5 min). The precursor ion mass of 474.04 m/z (positive ionization) or 472.03 m/z (negative ionization) for NSC 117079 was not detected on the LC-MS. However, its product ions (302.012 m/z, negative ionization and 304.028 m/z, positive ionization) were detected on the LC-MS (Supplementary Figure 1 B–C).

For NSC 45586, the MRM transition m/z 391.15 > 373.14 under negative ESI was monitored following chromatographic separation using linear gradient elution with mobile phase A composed of water containing 2 mM ammonium acetate and mobile phase B composed of methanol containing 2 mM ammonium acetate. The elution was initiated at 60% A and 40% B, followed by linear increase of B to 75% over 4.25 min. B was held at 35% for 0.05 min and returned to initial conditions over 1.7 min. The flow rate was maintained at 0.4 mL/min (total run time of 6.5 min) (Supplementary Figure 1 E–F). Calibration curves for NSC 117079 or NSC 45586 were produced in 1:1 MeOH:H2O and linearity was achieved from 1 to 1000 nM with an R2 value > 0.999.

2.7. In vitro stability of Phlpp Inhibitors

To test the stability of Phlpp inhibitors in vitro, 8 μM of NSC117079 or NSC45586 were added to culture medium (DMEM supplemented with 10% FBS) and mouse plasma (Innovative Research). Samples were collected in triplicate after incubation at 37 °C for 5, 15, and 30 min, and 1, 2, 4, 8, 12, 24, 48 and 72 h. Molecule concentrations were measured with LC/MS.

2.8. Intravenous injections and plasma collection

C57Bl/6J male mice (8 weeks old) were placed under anesthesia...
using isoflurane. Tails were placed in warm water for two to 3 min to dilate the intraventricular vein. Animals were administered Phlpp inhibitors diluted in saline to the concentration of 1.0 mg/kg, 2.5 mg/kg or 5.0 mg/kg (n = 8 per group). Volumes of 100 μL were injected into the

Fig. 1. Phlpp inhibitors stimulate rapid phosphorylation of substrates in chondrocytes. Proteins were isolated from chondrocyte micromass cultures after 30 min of exposure to Phlpp inhibitors NSC 117079 or NSC 45586. (A) Western blot analysis was performed to measure pSer660 PKC, pSer474 Akt2 and pT202/Y204 Erk42/44 and total levels of these enzymes. (B) Levels of phosphorylated proteins were quantified by ImageJ and determined in relation to total levels of AKT, PKC and ERK.

Fig. 2. Phlpp inhibitors promote chondrocyte maturation and glycosaminoglycan production during early stages of differentiation. (A) Primary mouse articular chondrocytes were isolated, placed into micromasses and cultured in chondrogenic medium for nine days. Blue shading indicates the presence of NSC 117079 or NSC 45586. (B) Micromasses were stained with Alcian blue after nine days in the presence of Phlpp inhibitors as indicated in A. (C) GAG production was measured using Chondrex Glycosaminoglycans Assay after nine days in the presence of Phlpp inhibitors as indicated in A. n = 3 per sample. The mean ± SD of triplicate samples is shown for each time point. *p < 0.05 of Phlpp inhibitors (blue squares = NSC 117079, red triangles = NSC 45586) versus the vehicle (Veh, black circles).
tail vein with a 27-gauge needle over 15–30 s. Whole blood was collected at 2–3 min, 30 min, 60 min, 4 h, 8 h and 48 h post injection from mice by cardiac puncture after euthanasia. Plasma was obtained by centrifugation for 10 min at 10,000 rpm. Pharmacokinetic parameters were calculated by standard noncompartmental methods.

2.9. Urinary recovery/in vivo metabolism

Mice were administered Vehicle or 1.0 mg/kg Phlpp inhibitors by intravenous injections and housed in glass metabolism chambers. Urine was collected over two to 4-h intervals in vessels placed under the cage. Specimens were kept on dry ice during the collection period and stored at −20 °C until analysis. The walls of the chamber were rinsed with water after each interval. In each experiment urine was also collected from untreated animals to provide drug and metabolite-free material for comparison.

2.10. Intra-articular injections

C57Bl/6J mice were purchased from Jackson Labs at three weeks of age. Four-week-old C57Bl/6J mice were given either a single 3 μL intra-articular injection of saline (vehicle) or 8 μM NSC 117079 or NSC 45586 into the right knee joint (n = 3 to 5 mice). The estimated concentration of the inhibitors in the joint space is 4 μM, which is the IC50 for both molecules. Intra-articular injections were done with 50 μL syringes as previously described (Hamilton 7637-01) [9]. One-week post injection mice were sacrificed and whole knee joints were fixed in 10% neutral buffered formalin, decalcified in 15% EDTA for 14 days, embedded in paraffin, sectioned at 5 μm, and then stained with Safranin O/Fast Green. The medial and lateral femoral condyles and tibial plateaus were imaged.
using a Zeiss LSM 900 confocal, inverted, wide-field microscope using a 10x air, 0.8-NA objective equipped with an Axiocam 560 mono charged-coupled device camera. Brightfield microscopy was used to accurately capture the Safranin O staining. The area of articular cartilage was measured on every third section from each bone, resulting in 9–15 data points per test group.

2.11. Immunohistochemical staining

Immunohistochemical (IHC) staining was performed with antibodies directed to Mmp13 (Abcam, 219620), Prg4 (EMD Millipore, 2200), Phlpp1 (Proteintech, 22789-1-AP) Phlpp2 (Bethyl Laboratories, A300-661A), and Histone 3 (Abcam 176842) was accomplished using the Mouse and Rabbit Specific HRP (ABC) Detection IHC Kit (Abcam) using the substrate 3,3'-diaminobenzidine (Sigma Aldrich, St. Louis, MO).

3. Results

3.1. Phlpp inhibitors promote chondrocyte maturation in vitro

We previously reported that the Phlpp inhibitor, NSC 117079, promoted chondrocyte maturation and matrix production in vitro [9,10]. Here we tested a second Phlpp inhibitor, NSC 45586 [8] and defined stages of chondrocyte maturation that are most affected by Phlpp inhibitors. Both molecules increased phosphorylation of intracellular substrates PKC and Akt2 by two to six-fold within 30 min (Fig. 1A and B). Erk1/2 is not known to be a direct substrate of Phlpp1/2 and there was no change in its phosphorylation status after 30 min.

To compare the effects of the inhibitors on chondrocyte maturation, primary mouse chondrocytes were cultured in micromasses with NSC 117079 or NSC 45586 for 3, 6 or 9 days over a period of nine days (Fig. 2A). Chondrocytes that were exposed to either of the Phlpp inhibitors at the beginning of the experiment and for a minimum of six days, specifically from days 0–6 and days 0–9, had advanced matrix production as indicated by strong Alcian blue staining (Fig. 2B). Consistent with these results, GAG secretion was significantly higher in chondrocyte micromass cultures that contained Phlpp inhibitors from days 0–6 and days 0–9, but not in cultures containing Phlpp inhibitors for just three days (days 0–3, days 4–6, or days 7–9), or in cultures where Phlpp inhibitors were added after day 4 (Fig. 2C).

Transcripts of pro-chondrogenic genes Sox9 (Fig. 3A), Prg4 (Fig. 3B), and Col2a1 (Fig. 3C) were significantly increased in cultures containing either Phlpp inhibitor from days 0–6 and days 0–9. Mmp13 mRNA transcripts were decreased under these conditions (Fig. 3D). Phlpp1 and Phlpp2 transcript levels were also decreased in chondrocyte cultures with either inhibitor (Fig. 3E and F). Consistent with these transcriptomic results, protein levels of Phlpp1 and Phlpp2 were approximately 50–70% lower in cells exposed to NSC 117079 or NSC 45586 from days 0–6 and days 0–9 (Fig. 2G and Supplemental Figure 2). Phlpp2 protein levels were also reduced when Phlpp inhibitors were added during days 4–6 and 4 to 9 of the culture period. Prg4 protein levels increased in the presence of either Phlpp inhibitor from days 0–6 or 0–9. Mmp13 protein levels were reduced in some cultures but showed variability. DIPEN, an N-terminal neoepitope of aggrecan created by Mmp cleavage, was not changed when Phlpp inhibitors were present during just the first three or six days of cultures but declined in cells exposed to NSC 117079 or NSC 45586 during the last three or six days, respectively, of the nine-day culture period.

Fig. 4. Phlpp inhibitors are stable in vitro. (A, B) NSC 117079 and NSC 45586 were incubated in 50 mL DMEM and 10% FBS in triplicate. Each replicate is represented by a different line color. Medium (1 mL) was collected for analysis from each replicate at the indicated times. (C, D) NSC 117079 and NSC 45586 were incubated in mouse plasma for indicated times. Concentrations were measured by LC/MS.

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3.2. Phlpp Inhibitors are highly stable in vitro

To determine if culture conditions used for in vitro studies affect the stability of the Phlpp inhibitors, LC-MS/MS assays were developed for each compound (Supplementary Fig 1). NSC 117079 and NSC 45586 were highly stable, with no hydrolysis, oxidation or other degradation observed during 72 h at 37°C in culture medium containing 10% FBS (Fig. 4A and B). Both molecules were also stable for 72 h at 37°C in mouse plasma (Fig. 4C and D).

3.3. NSC 117079 and NSC 45586 have different pharmacokinetic properties in vivo

We next determined the pharmacokinetic properties of Phlpp inhibitors NSC 117079 and NSC 45586 in male C57BL/6J mice. Three concentrations (1.0, 2.5 or 5.0 mg/kg) of NSC 117079 or NSC 45586 were injected intravenously, and blood was collected over the next 48 h. NSC 117079 was detected in the bloodstream during the first 4 h (Fig. 5A) and NSC 45586 was detected during the first eight to 10 h (Fig. 5B). The half-life for NSC 117079 was approximately 1 h regardless of the dose (Table 1), while the half-life for NSC 45586 ranged from four to 6 h (Table 2). The volumes of distribution (Vd) of NSC 117079 and NSC 45586 were 8–14 L kg⁻¹ and 261–376 L kg⁻¹, respectively (Tables 1 and 2).

Compounds with low Vd tend to remain within the plasma and be excreted, while higher Vd means the drug or compound has lipophilic properties and can be distributed in various tissues [22]. To measure excretion, mice were injected intravenously with Phlpp inhibitors and placed into metabolic chambers for 8 h. Urine was collected in two to 4-h intervals and analyzed by LC-MS. High concentrations of NSC 117079 were present in the urine collected within first 2 h of injection and lower concentrations were excreted over the next 6 h (Fig. 5C). In contrast, NSC 45586 was not detected in the urine over 8 h (Fig. 5D). Thus, NSC 117079 and NSC 45586 have different pharmacokinetic profiles.

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Table 1

Pharmacokinetic calculations for NSC 117079 in vivo.

|           | 1.0 mg/kg | 2.5 mg/kg | 5.0 mg/kg |
|-----------|-----------|-----------|-----------|
| AUC, mg*h/L | 874.9     | 2124      | 4362      |
| Clearance, mL·kg⁻¹·min⁻¹ | 9.14      | 7.53      | 7.34      |
| Cmax, mM   | 570       | 1197      | 3893      |
| Tmax, hr   | 0.5       | 0.5       | 0.5       |
| Volume of Distribution (Vd), L | 14.04     | 13.37     | 8.220     |
| Half life, hr | 1.06       | 1.23      | 0.776     |

Table 2

Pharmacokinetic calculations for NSC 45586 in vivo.

|           | 1.0 mg/kg | 2.5 mg/kg | 5.0 mg/kg |
|-----------|-----------|-----------|-----------|
| AUC, mg*h/L | 179.7     | 379.9     | 800       |
| Clearance, mL·kg⁻¹·min⁻¹ | 44.5      | 42.1      | 40.1      |
| Cmax, mM   | 30.6      | 42.5      | 129.5     |
| Tmax, hr   | 1         | 1         | 4         |
| Volume of Distribution (Vd), L | 261       | 376       | 347       |
| Half life, hr | 4.07       | 6.19      | 6.01      |

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Fig. 5. Pharmacokinetic analysis of Phlpp inhibitors in male mice. (A, B) C57BL6/J male mice were intravenously injected with 1, 2.5 or 5 mg/kg NSC 117079 (A) or NSC 45586 (B) (n = 2 per concentration). Blood was collected at regular time points beginning within 1 min of the injection through 48 h. Plasma concentrations were determined using LC/MS. Each line represents values from the same mouse. (C, D) Mice were placed in metabolic chamber and urine was collected in time frames 0–2 h, 2–4 h and 4–8 h. Urine samples were analyzed by LC/MS. Each line represents a different mouse (n = 4).
3.4. Phlpp inhibitors increase articular cartilage thickness in vivo

To test the effects of NSC 117079 and NSC 45586 on articular cartilage in vivo, we injected inhibitors or vehicle intra-articularly into four-week-old male mice and examined cartilage structure one week later. Safranin O staining showed that femorotibial cartilage was thicker on both the medial and lateral surfaces in mice injected with NSC 117079 (Fig. 6A) or NSC 45586 (Fig. 6D). The overall articular cartilage area on the medial and lateral tibial plateaus and femoral condyles were increased 15–25% in joints injected with NSC 117079 and 10–15% in joints injected with NSC 45586 (Fig. 6B and E). The distributions of NSC 117079 on NSC 45586 to plasma were measured by LC/MS following intra-articular injection. Low (nanomolar) levels of NSC 117079 (Fig. 6C) and NSC 45586 (Fig. 6F) were detected in blood plasma between one and 48 h after the intra-articular injection.

Finally, histological sections from these animals were incubated were antibodies to proteins whose expression was changed by Phlpp inhibitors in vitro (Fig. 4: Phlpp1, Phlpp2, Prg4, Mmp13). Antibodies to Histone 3 were included to identify all cell nuclei. As observed in vitro, NSC 117079 and NSC 45586 increased Prg4 expression and reduced Phlpp1, Phlpp2 and Mmp13 levels in articular cartilage in vivo, particularly on the tibial plateau (Fig. 7).
4. Discussion

Osteoarthritis is the most common degenerative joint disease and causes significant disability worldwide [23]. No disease modifying drugs are available to effectively prevent or treat OA. The ideal DMOAD would promote chondrocyte survival and proliferation and hyaline cartilage matrix thickness. Phlpp1 and Phlpp2 are promising targets for DMOAD candidates as they are present in articular cartilage from osteoarthritic joints, but not in articular cartilage of non-OA joints ([11] and unpublished data). Genome-wide association studies indicate that genetic variants in or near PHLPP1 are associated with human OA [24]. In previous experiments, we showed Phlpp1 inactivation by pharmacologic inhibition and/or genetic deletion prevented injury-induced cartilage damage in mice [11]. Phlpp1 inhibition also protected intervertebral discs, neurons, and intestinal and heart tissues from degeneration after ischemic injuries or inflammation-induced damage [25–29]. In this study we sought to improve understanding of how Phlpp inactivation promotes chondrocyte maturation and to define the pharmacokinetics of available Phlpp inhibitors, NSC 117079 and NSC 45586, in mice.

Knee articular cartilage has limited but measurable repair capacity [30,31]. We previously showed that NSC 117079 increases glycosaminoglycans (GAG) production by chondrocyte micromasses [10]. Here we demonstrated that NSC 45586 (IC_{50} = 4 μM) also promotes chondrocyte maturation and matrix synthesis in vitro and in vivo. GAG production was highest when Phlpp inhibitors were added at the beginning of chondrocyte cultures and remained in the culture for at least six days. Prg4 transcript and protein levels were also higher in vitro during these periods. Moreover, Prg4 protein levels were elevated within articular cartilage one week after injection of each Phlpp inhibitor. In addition to their positive effects on matrix production, both NSC 117079 and NSC 45586 suppressed Mmp13 and DIPEN expression by chondrocytes. While Mmp13 suppression was modest and variable, DIPEN reductions were most evident at later stages of chondrocyte maturation. Thus, NSC 117079 and NSC 45586 increase cartilage thickness by stimulating matrix synthesis and suppressing matrix degradation.

The ability of NSC 117079 and NSC 45586 to affect the phosphorylation status of intracellular substrates in chondrocytes has been documented [10]. Here we show that phosphorylation of PKC and Akt2 is increased within 30 min of NSC 117079 and NSC 45586 addition to the culture. We also observed that NSC 117079 and NSC 45586 reduced Phlpp1 and Phlpp2 transcript and protein levels in chondrocytes in vitro and in vivo. These results indicate that NSC 117079 and NSC 45586 can inhibit Phlpp1/2 activity in at least two ways: by occupying and blocking their substrate binding site and by reducing their levels. Phlpp1/2 have nuclear functions and NSC 117079 and NSC 45586 may directly or indirectly affect Phlpp1/2 transcription [32]. While reductions in transcript levels could explain the decline of protein levels, Phlpp1 and Phlpp2 levels may also be reduced by proteasome-mediated degradation [33,34]. Additional studies are needed to determine if interactions with NSC 117079 and NSC 45586 make Phlpp1 and Phlpp2 more susceptible to degradation in chondrocytes.

Here we describe LC-MS/MS assays for quantifying NSC 117079 and NSC 45586 levels. NSC 117079 was undetectable in its precursor ion mass of 474.04 m/z (positive ionization) or 472.03 m/z (negative ionization); however, we were able to detect the product ion 302.012 m/z (negative ionization) and 304.028 m/z (positive ionization) on the LC-MS. Since the Xevo G2-XS QToF is an exact mass spectrometer, we are confident that we are detecting a product ion of NSC 117079 and suspect that source fragmentation causes the precursor ion to fragment before it reaches the quadrupole occurred.

NSC 117079 and NSC 45586 were very stable in vitro but had different in vivo pharmacokinetic profiles. NSC 117079 had a shorter half-life than NSC 45586 in plasma (1 h versus 3.5–6 h) and a lower volume distribution (V_d). Drugs with low V_d typically remain within the plasma, while drugs with higher V_d tend to have lipophilic properties, are distributed via the bloodstream to various tissues and have a high tendency to cross cell membranes. Our studies indicate that NSC 45586 is not an ideal drug candidate because it is lipophilic and has a high V_d in vivo. The tissue distribution of NSC 45586 was not determined but none was detected in the urine after 8 h. In contrast, the majority NSC 117079 injected intravenously was excreted in the urine within 4 h. Thus, NSC 117079 has a more favorable pharmacokinetic profile than NSC 45586.

In vivo, NSC 117079 and NSC45586 increased the area of articular cartilage one week after intra-articular injection in 4-week-old male C57Bl/6J mice. These results suggest that Phlpp inhibitors could restore thinning or damaged articular cartilage. Small molecules typically have
low retention times in joints and are eliminated from intraarticular spaces via capillaries [7]. NSC 117079 and NSC 45585 were detected in the plasma within one to 4 h of injection but at comparatively low (nanomolar) levels to what was injected (32 μM) into the joints. No NSC 117079 or NSC 45586 was detected in the blood plasma between 4- and 48-h post-injection. Thus, some but not all NSC 117079 and NSC 45586 was eliminated from the joints following injection. The amount of NSC 117079 or NSC 45586 retained in cartilage and other joint tissues was not tested in these studies.

In conclusion, the Phlpp inhibitor NSC 117079 shows more favorable pharmacokinetics and pharmacodynamics in mice than NSC 45586, but both compounds promote chondrocyte maturation in vitro and are useful for probing molecular mechanisms of Phlpp function in primary chondrocytes and other cells. Our results indicate that NSC 117079 and NSC 45586 functionally inhibit Phlpp enzymes by at least two mechanisms: rapid inhibition of phosphatase activity as indicated by increased phosphorylation of intracellular substrates within 30 min, and sustained suppression of Phlpp1 and Phlpp2 mRNA and protein expression in vitro and in vivo. These data advance our understanding of how Phlpp inhibitors function within chondrocytes and provide a basis towards understanding their safety and activity in vivo.

Declaration of competing interest

None of the authors have any conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found at https://doi.org/10.1016/j.ocarto.2021.100190.

Contributions

- Conception and design: ELT, EWB, JJW, SRW
- Analysis and interpretation of the data: ELT, EWB, SAB, JJW, JMR
- Drafting of the article: ELT, JJW
- Critical revision of the article for important intellectual content: ELT, JJW, JMR
- Final approval of the article: ELT, JJW
- Obtaining of funding: JJW
- Collection and assembly of data: CAT, ELT, EWB, EZ, SRW

References

[1] E. Sebbag, et al., The world-wide burden of musculoskeletal diseases: a systematic analysis of the World Health Organization Burden of Disease Database. Ann. Rheum. Dis. 78 (6) (2019) 844–848.
[2] U.S.B.o.D. Collaborators, et al., The state of US health, 1990-2016: burden of diseases, injuries, and risk factors among US States. J. Am. Med. Assoc. 319 (14) (2018) 1444–1472.
[3] Arthritis and Related Conditions. The Burden of Musculoskeletal Diseases in the United States Bone and Joint Decade, 2008, p. 6 (Chapter 4).
[4] J. Murphy, C.G. Helmick. The impact of osteoarthritis in the United States: a population-health perspective: a population-based review of the fourth most common cause of hospitalization in U.S. adults, Orthop. Nurs. 31 (2) (2012) 85–91.
[5] R. Bitton, The economic burden of osteoarthritis, Am. J. Manag. Care 15 (8 Suppl) (2009) S230–S235.
[6] W. Zhang, et al., Current research on pharmacologic and regenerative therapies for osteoarthritis, Bone Res. 4 (2016) 15040.
[7] C.H. Evans, V.B. Kraus, L.A. Setton, Progress in intra-articular therapy, Nat. Rev. Rheumatol. 10 (1) (2014) 11–22.
[8] E. Sireci, et al., Discovery of small molecule inhibitors of the PHI domain leucine-rich repeat protein phosphatase (Phlpp) by chemical and virtual screening, J. Med. Chem. 53 (19) (2010) 6899–6911.
[9] S.M. Hwang, et al., Phlpp inhibitors block pain and cartilage degradation associated with osteoarthritis, J. Orthop. Res. 36 (5) (2018) 1487–1497.
[10] E.W. Bradley, et al., Deletion of the PHI-domain and leucine-rich repeat protein phosphatase 1 (Phlpp1) increases fibroblast growth factor (Fgf) 18 expression and promotes chondrocyte proliferation, J. Biol. Chem. 290 (26) (2015) 16272–16280.
[11] E.W. Bradley, et al., Phlpp1 facilitates post-traumatic osteoarthritis and is induced by inflammation and promoter demethylation in human osteoarthritis, Osteoarthritis Cartilage 24 (6) (2016) 1021–1028.
[12] N.A. Warfel, A.C. Newton, Pleckstrin homology domain leucine-rich repeat protein phosphatase (Phlpp): a new player in cell signaling, J. Biol. Chem. 287 (6) (2012) 3610–3616.
[13] T. Gao, F. Furnari, A.C. Newton, Phlpp: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth, Mol. Cell. 18 (1) (2005) 13–24.
[14] T. Gao, J. Brognard, A.C. Newton, The phosphatase Phlpp controls the cellular levels of protein kinase C, J. Biol. Chem. 283 (10) (2008) 6300–6311.
[15] J. Brognard, et al., Phlpp and a second isoform, Phlpp2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms, Mol. Cell. 25 (6) (2007) 917–931.
[16] J. Liu, et al., Phlpp-mediated dephosphorylation of S6K1 inhibits protein translation and cell growth, Mol. Cell Biol. 31 (24) (2011) 4917–4927.
[17] M. Qiao, et al., Mtll is an interacting protein that mediates Phlpp-induced apoptosis, Mol. Cell 38 (4) (2010) 512–523.
[18] A.K. O’Neill, M.J. Niederst, A.C. Newton, Suppression of survival signalling pathways by the phosphatase Phlpp, FEBS J. 280 (2) (2013) 572–583.
[19] S.R. Weaver, et al., Pleckstrin homology (PH) domain and leucine rich repeat phosphatase 1 (Phlpp1) suppresses parathyroid hormone receptor 1 (Pthrh1) expression and signaling during bone growth, J. Bone Miner. Res. (2021).
[20] M. Gouset, et al., Primary culture and phenotyping of murine chondrocytes, Nat. Protoc. 3 (8) (2008) 1253–1260.
[21] E.W. Bradley, L.R. Carpio, J.J. Westendorf, Histone deacetylase 3 suppression increases PH domain and leucine-rich repeat phosphatase (Phlpp1) expression in chondrocytes to suppress Akt signaling and matrix secretion, J. Biol. Chem. 288 (14) (2013) 9572–9582.
[22] A. Mansoor, N. Mahabadi, Volume of distribution, in: StatPearls, Treasure Island (FL), 2020.
[23] C.J. Murray, et al., Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010, Lancet 380 (9859) (2012) 2197–2223.
[24] I. Tachmazidou, et al., Identification of new therapeutic targets for osteoarthritis through genome-wide analyses of UK Biobank data, Nat. Genet. 51 (2) (2019) 230–236.
[25] C. Zhang, et al., Phlpp1 is associated with human intervertebral disc degeneration and its deficiency promotes healing after needle puncture injury in mice, Cell Death Dis. 10 (10) (2019) 754.
[26] S. Miyamoto, et al., Phlpp-1 negatively regulates Akt activity and survival in the heart, Circ. Res. 107 (4) (2010) 476–484.
[27] B. Chen, et al., Phlpp1 gene deletion protects the brain from ischemic injury, J. Cerebr. Blood Flow Metabol. 33 (2) (2013) 196–204.
[28] T.C. Jackson, et al., Pharmacological inhibition of pleckstrin homology domain leucine-rich repeat protein phosphatase is neuroprotective: differential effects on astrocytes, J. Pharmacol. Exp. Therapeut. 347 (2) (2013) 516–528.
[29] Y.A. Wen, et al., Loss of Phlpp protects against colitis by inhibiting intestinal epithelial cell apoptosis, Biochem. Biophys. Acta 1852 (10 Pt A) (2015) 2013–2023.
[30] J.B. Catterall, et al., Protein modification by deamidation indicates variations in joint extracellular matrix turnover, J. Biol. Chem. 287 (7) (2012) 4640–4651.
[31] J.B. Catterall, et al., Asparatic acid racemization reveals a high turnover state in knee compared with hip osteoarthritic cartilage, Osteoarthritis Cartilage 24 (2) (2016) 374–381.
[32] G. Reyes, et al., Pleckstrin homology domain leucine-rich repeat protein phosphatases set the amplitude of receptor tyrosine kinase output, Proc. Natl. Acad. Sci. U. S. A. 111 (38) (2014) E957–E965.
[33] X. Li, J. Liu, T. Guo, beta-TrCP-mediated ubiquitination and degradation of PHLPP1 is positively regulated by Akt, Mol. Cell Biol. 29 (23) (2009) 6692–6705.
[34] X. Li, et al., The deubiquitination enzyme USP46 functions as a tumor suppressor by controlling PHLPP-dependent attenuation of Akt signaling in colon cancer, Oncogene 32 (4) (2013) 471–478.