AWARD NUMBER: W81XWH-19-1-0222

TITLE: Bone-Targeted Delivery of TGF-Beta Receptor Inhibitor as a Novel Treatment for Osteoarthritis

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REPORT DATE: July 2020

TYPE OF REPORT: Annual report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
July 2020	Annual report	15JUN2019 - 14JUN2020

4. TITLE AND SUBTITLE

Bone-Targeted Delivery of TGF-Beta Receptor Inhibitor as a Novel Treatment for Osteoarthritis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-19-1-0222

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)
Johns Hopkins University
School of Medicine
Office of Research
Administration
Miller Research Building,

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Background: Extreme physical demands contribute to the high incidence of osteoarthritis (OA) in the military population. OA is the most common degenerative joint disorder and the leading cause of physical disability. There is no effective disease-modifying treatment for OA except joint replacement surgery. Additionally, we are lacking fundamental treatment of OA pain although pain is the most prominent symptom of OA and the common reason that military personnel are discharged from service. The prognosis with only analgesia is not satisfactory as it may adversely contribute to the progression of the disease when the primary perception of the joint pathology is blunted. Therefore, there is a tremendous need to develop new therapies to attenuate OA degeneration and alleviate OA pain, thereby improving the quality of life of military soldiers.

It has recently been revealed that excessive activation of TGFβ1 leads to aberrant subchondral bone remodeling and contributes to the onset of joint articular cartilage degeneration. Restoring the structural integrity and mechanical property of the subchondral bone by inhibiting TGFβ activity was effective in preventing OA progression and cartilage degeneration in OA animal models. However, TGFβs are multifunctional cytokines that are involved in a range of biological processes. There are significant concerns that systemic inhibition of TGFβ signaling may lead to a failure in the maintenance of tissue homeostasis of other organs, particularly articular cartilage, where TGFβ serves as a major anabolic factor. Thus, it is of great importance to develop a novel strategy that can retain the efficacy of the TGFβ inhibitor while improving its safety in the therapeutic applications.

On another aspect, we have found that abnormally elevated osteoclastogenesis substantially contributes to OA pain. Specifically, osteoclasts secrete specific neuronal growth factors that induce axonal extrusion and innervation in the subchondral bone which is associated with joint pain. Utilizing the $DMP1^{Cre}/RankL^{f/f}$ mice, in which osteoclasts are substantially decreased, we found that nociceptive innervation in subchondral bone was decreased and pain was reduced. However, subchondral bone pathology and articular cartilage degeneration were only partially improved. Therefore, combinational usage of an osteoclast inhibiting factor with a TGFβ inhibitor has potential to serve as a novel therapy to rescue OA pathology and alleviate OA pain. To reach the purpose of both osteoclast inhibition and subchondral bone tissue-specific TGFβ inhibition, we have developed a new drug (ALN-LY conjugate) that links a TGFβ inhibitor LY2109761 (LY) to an osteoclast inhibitor, Alendronate (ALN), through a metabolically hydrolyzable linker. LY is a selective inhibitor of TGFβ receptor that is being tested in a variety of clinical trials. Alendronate (Aln) is a bisphosphonate drug for osteoporosis patients that inhibits osteoclasts. Additionally, Aln has a high bone affinity that will confer bone-targeted delivery and sustained release of the TGFβ receptor inhibitor.

Hypotheses: ***Hypothesis 1*****:** ALN-LY conjugate can effectively rescue subchondral bone abnormalities and attenuate AC degeneration by specifically inhibiting TGFβ signaling in bony tissue. ***Hypothesis 2*****:** ALN-LY conjugate can alleviate OA symptom by suppressing subchondral bone turnover and consequent nociceptive innervation.

Specific Aims: Aim 1: We will evaluate safety and efficacy of ALN-LY conjugate in the post-traumatic OA mouse model. We will inject ALN-LY conjugate systemically into the mice and harvest all important organs and tissues. The concentration of the conjugate will be determined by HPLC (high-performance liquid chromatography) assay in blood and all organs to determine the tissue distribution. In OA mouse model, we will treat the mice with the conjugate, solo ALN, LY and vehicle right after anterior cruciate ligament transection (ACLT) or sham surgery. We will examine the treatment efficacy by evaluating joint pathologies using microCT scan and immunohistological staining. The subchondral bone structure, morphology and metabolic status of articular cartilage will be determined at 4 and 8 weeks post-surgery. Aim 2: We will determine the effect of the conjugate in alleviating joint pain and improving functional outcome in the post-traumatic OA mouse model. The nociceptive innervation into subchondral bone and pain behavior will be determined in the same mice utilized in Aim1. Nociceptive axons growth in the subchondral bone will be identified by immunofluorescence staining of CGRP. Pain behavior and joint morbidity will be measured by Von Frey test, gait analysis and voluntary wheel running activity assay. To more specifically prove that OA pain is alleviated by preventing subchondral bone innervation, we will test the effect of ALN-LY conjugate in a pain reporter mouse to directly measure dorsal root ganglion neuronal activation in the post-traumatic OA mouse model.

Impact: Since there is a lack of a disease-modifying drug on the market for OA, the success of the work described in this proposal will facilitate and accelerate therapeutic development of this disease. Developing effective disease-modifying therapy for OA could greatly reduce the necessity of joint replacement surgery and medical expenditure. This proposed study directly impacts the FY18 PRMRP Area of Encouragement: "Basic and translational research to identify treatments to mitigate and/or reverse osteoarthritis, particularly in the knee, hip, ankle, and shoulder".

Military Relevance: The proposed research project, by bone-targeted delivery of TGFβ inhibitor, holds the promise of reducing the burden of OA suffered by military Service members, Veterans, and their family members and caregivers.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	17	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

Military personnel is highly susceptible to Osteoarthritis (OA) because of the work environment and frequent battlefield injuries. There is no effective disease-modifying treatment for OA except joint replacement surgery. Particularly, we are lacking fundamental treatment of OA pain although pain is the most prominent symptom of OA and the common reason that military personnel are discharged from service. The structural and functional integrity of articular cartilage highly relies on its biochemical and biomechanical interplay with the subchondral bone. The impaired ability of subchondral bone to dissipate load articular cartilage affects the homeostasis of articular cartilage. We have demonstrated that high levels of TGFβ in subchondral bone initiates uncoupled subchondral bone formation and promote the degeneration of articular cartilage. Inhibition of TGFβ signaling successfully improved the structure of subchondral bone and attenuated cartilage degeneration. However, TGFβ is a well-known anabolic factor for articular cartilage and has a broad spectrum of functional activity on other organs/tissues throughout the body. The potential detrimental effect on other organs/tissues hinders the process of TGFβ inhibitor being developed as an OA drug. For the perspective of OA pain, we found that abnormally elevated osteoclastogenesis substantially contributes to OA pain. In the present study, we are aiming to test whether combinational usage of an osteoclast inhibiting factor with a TGFβ inhibitor has potential to serve as a novel therapy to rescue OA pathology and alleviate OA pain. To reach the purpose of both osteoclast inhibition and subchondral bone tissue-specific TGFβ inhibition, we developed a new drug (ALN-LY conjugate) that links a TGFβ inhibitor LY2109761 (LY) to an osteoclast inhibitor, Alendronate (ALN), through a metabolically hydrolyzable linker. LY is a selective inhibitor of TGFβ receptor that is being tested in a variety of clinical trials. We designed experiments to test ***1)*:** Whether ALN-LY conjugate can effectively rescue subchondral bone abnormalities and attenuate AC degeneration by specifically inhibiting TGFβ signaling in bony tissue. ***2)*:** whether ALN-LY conjugate can alleviate OA symptom by suppressing subchondral bone turnover and consequent nociceptive innervation. Since there is a lack of a disease-modifying drug on the market for OA, the success of our work will facilitate and accelerate therapeutic development of this disease.

KEY WORDS

Alendronate
Articular cartilage
Nociceptive innervation
Osteoarthritis
Osteoclast
Pain
Subchondral bone
TGF-beta

ACCOMPLISHMENT

Major tasks:	**Completion status**
1. Local IACUC Approval and ACURO Approval	Completed (Aug 15th, 2019)
2. ACLT/Sham surgery and pharmaceutical interventions after surgery	Completed (March 30th, 2020)
3. Tissue specimen collection and specimen processing	Completed (April 30th, 2020)
4. Evaluation of treatment efficacy in reversing joint pathologies	Completed (June 15th, 2020)
5. Evaluation of functional outcome after treatment	Completed (May 30th, 2020)
6. Determine tissue distribution of conjugate	~30% of completion
7. Immunofluorescence staining of CGRP and IB4	~50% of completion
8. ACLT/Sham surgery in Pirt-GCaMP3 mice and drug treatment	~5% of completion
9. Examine the evoked activity of L4 DRG neurons	in preparation

Accomplishment under above goals:

We first tested if the conjugate can successfully inhibit TGF-β signaling *in vitro*. We treated human MSCs with the conjugate and found that the TGF-β signaling was effectively inhibited as evidenced by a significant reduction of pSMAD2/3 (**Fig. 1**). Then we examined the efficacy in preventing the development and progression of OA in anterior cruciate ligament transection (ACLT) mouse model. We randomized 3-month-old

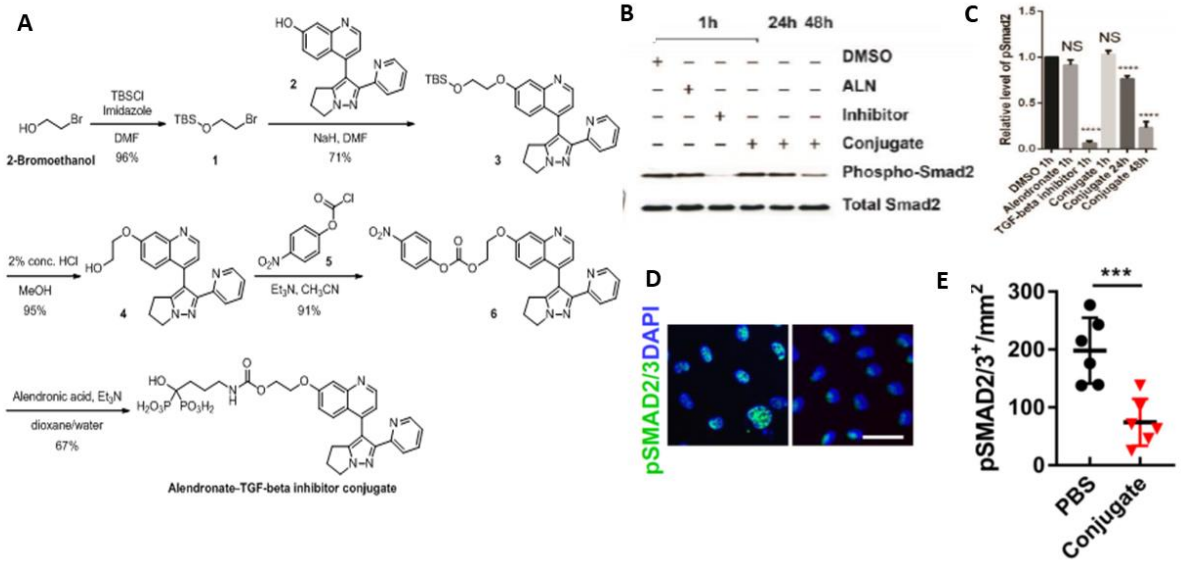

Fig 1. The ALN-LY conjugate inhibits TGF-β signaling. (A) Synthesis and chemical structure of the conjugate. **(B)** Western-blot analysis of pSmad2 in the cell lysate of mesenchymal stem cells. **(C)** Quantitative analysis of the Western blot in (B). Data are presented as relative level of pSmad2/Smad2 ratio, normalized to the intensity of DMSO treated group. **(D)** Immunofluorescence staining of pSmad2 in mesenchymal stem cells, left: PBS treated group, right: conjugate treated group. **(E)** Quantitative analysis of immunofluorescence staining in (D).

C57BL/6J (WT) mice into 6 groups: sham-vehicle, ACLT-vehicle, ACLT-ALN (100 μg/kg ALN), ACLT-ALN-LY (100 μg/kg), ACLT-LY (100 μg/kg), ACLT-ALN/LY compounds (100 μg/kg LY and 100 μg/kg ALN). All mice received the intraperitoneal injections of above-mentioned drugs three times a week for 1 month, or until sacrifice at 2 weeks or 4 weeks or 8 weeks after surgery. The structure of subchondral bone was determined by micro-CT analysis, cartilage degeneration was evaluated by the OARSI grading system. Immunohistological staining was performed to examine the status of angiogenesis, bone remodeling and nociceptive innervation in the subchondral bone. The function outcome was determined by pain behavior tests (Von Frey, voluntary wheel running activity, and gait analysis).

As expected, the articular cartilage degeneration was attenuated with a weekly injection of conjugate 100ug/kg in ACLT mice compared with the other groups, with a significant improvement of the OARSI score (**Fig. 2**). Safranin O-Fast green staining showed that ACLT induced loss of proteoglycan in articular cartilage observed in the ACLT-vehicle group was not observed in the ALN-LY treatment group at 8 weeks post-surgery. The

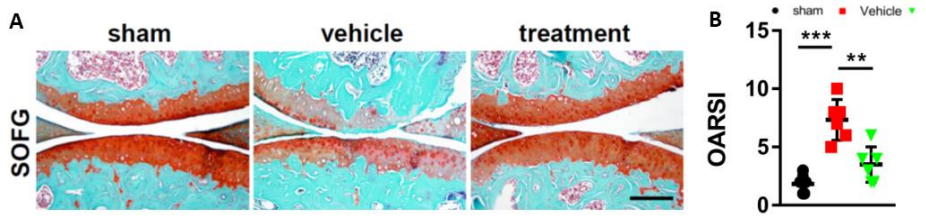

Fig 2. ALN-LY conjugate treatment prevented ACLT induced cartilage degeneration in OA mouse model. (A) Representative image of Safranin O Fast green staining. Vehicle: PBS, treatment: conjugate. **(B)** Quantitative analysis of (A) using OARSI grading system.

OARSI score of the ACLT-ALN-LY group was significantly lower than that of the ACLT-vehicle group. The ALN, LY, or ALN/LY treated group slightly decreased the OARSI score relative to that of the ACLT-vehicle group but didn't achieve a statistical difference.

In concurrent with the cartilage protection, the subchondral bone microarchitecture was improved in µCT analysis of ACLT mice treated with the conjugate compared with the other groups (**Fig. 3**). The significant alteration of subchondral bone structure in the vehicle-treated ACLT mice was observed at 1-month post-surgery by micro-CT analysis. The subchondral bone structure was substantially improved in the ALN-LY treated mice as evidenced by decreased total tissue volume (TV), lowered trabecular pattern factor (Tb.pf) and decreased thickness of subchondral bone plates (SBP Th), as compared to that of the vehicle-treated mice post ACLT. The sole treatment of ALN, LY, or ALN/LY did not show a significant beneficial effect in reversing the ACLT induced pathological changes in the subchondr

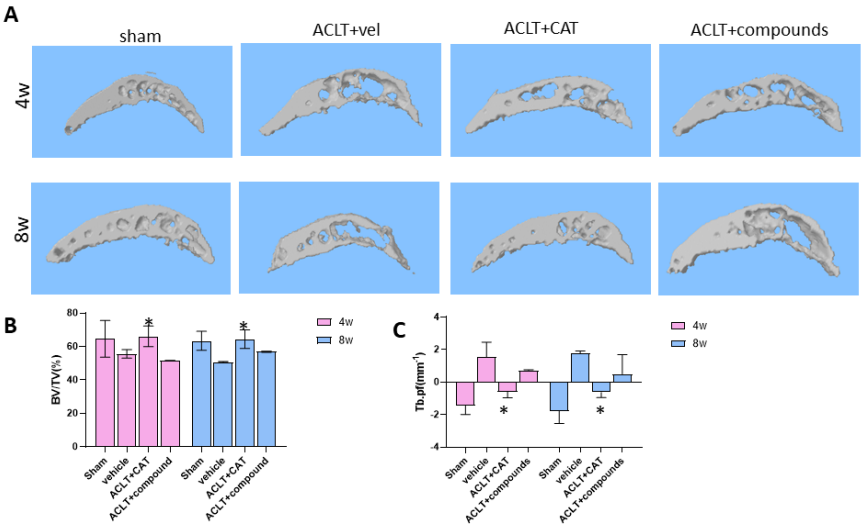

Fig 3. ALN-LY conjugate treatment prevented ACLT induced subchondral bone deterioration in OA mouse model. (A) Representative image of 3D reconstructed µCT images. Vel: PBS, CAT: conjugate, compounds: ALN+LY. **(B-C)** Quantitative analysis of (A).

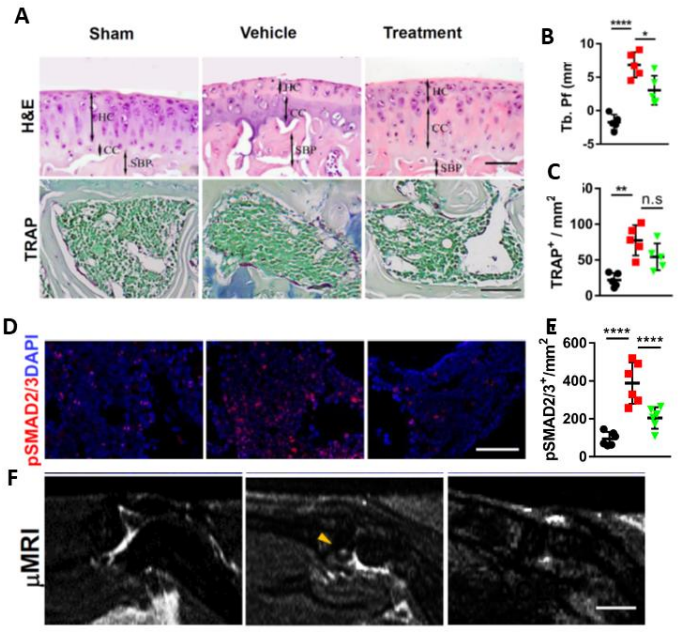

Fig 3. ALN-LY conjugate treatment inhibited TGFβ signaling in the subchondral bone in OA mouse model. (A) Representative image of HE staining (top) and trap staining (bottom) in knee joint section of mice received sham or ACLT surgery. Vehicle: PBS treated ACLT mice, treatment: conjugate treated ACLT mice. **(C)** Quantitative analysis of trap staining in (A). (**D-E**) Immunofluorescence staining of pSmad2/3 in subchondral bone sections. (**F**) MRI analysis of mice post sham (left) or ACLT surgery treated with PBS (middle) or conjugate (right).

Consistent to the *in vitro* findings, the phosphorylation of Smad2/3 in subchondral bone marrow cells was effectively inhibited by the conjugate (**Fig. 4D, E**). The number of $TRAP^+$ osteoclastic cells were slightly decreased but not reach statistical significance when compared to vehicle treated group (**Fig. 4A, C**). As a result, the BML in tibial subchondral bone was significantly reduced in the conjugate treated group (**Fig. 4F**) further indicating that coupling of the osteoclast bone resorption and osteoblastic bone formation were restored.

We also performed a series of behavior tests to determine whether ALN-LY treatment successfully prevents the functional decline of mobility (**Fig. 5**). In CatWalk gait analysis, the ACLT-vehicle group showed decreased maximum intensity, print area and increased swing phase of the ipsilateral limb than the contralateral limb after 8 weeks of ACLT surgery. The disparity between the contralateral and ipsilateral hind limbs was significantly reduced in the ALN-LY group post ACLT as compared to that of the ACLT-vehicle group. No

significant differences in maximum intensity, print area, and swing phase were observed between the hind limbs in ALN-LY group. The assessment of voluntary wheel running activity indicates that the daily well-being of ALN-LY treated mice were remarkably improved than vehicle-treated mice at 8 weeks post ACLT. The total traveled distance, active time, mean speed and maximum speed in the ALN-LY group were significantly greater than ALN, LY, or ALN/LY treated group post ACLT. The secondary mechanical allodynia and hyperalgesia of the mice were measured by Von Frey analysis. We found that the paw withdraw frequency increased significantly at 4weeks and 8 weeks after ACLT relative to the sham-operated group whereas ALN-LY treated group showed significantly decreased paw withdraw frequency compared to the vehicle-treated group. Taken together, ALN-LY substantially attenuated ACLT induced articular cartilage damage and subchondral bone structural alteration. Importantly, ALN-LY showed a promising effect in joint pain relief in OA mouse model. Subsequently, the functional mobility of the mice was substantially improved.

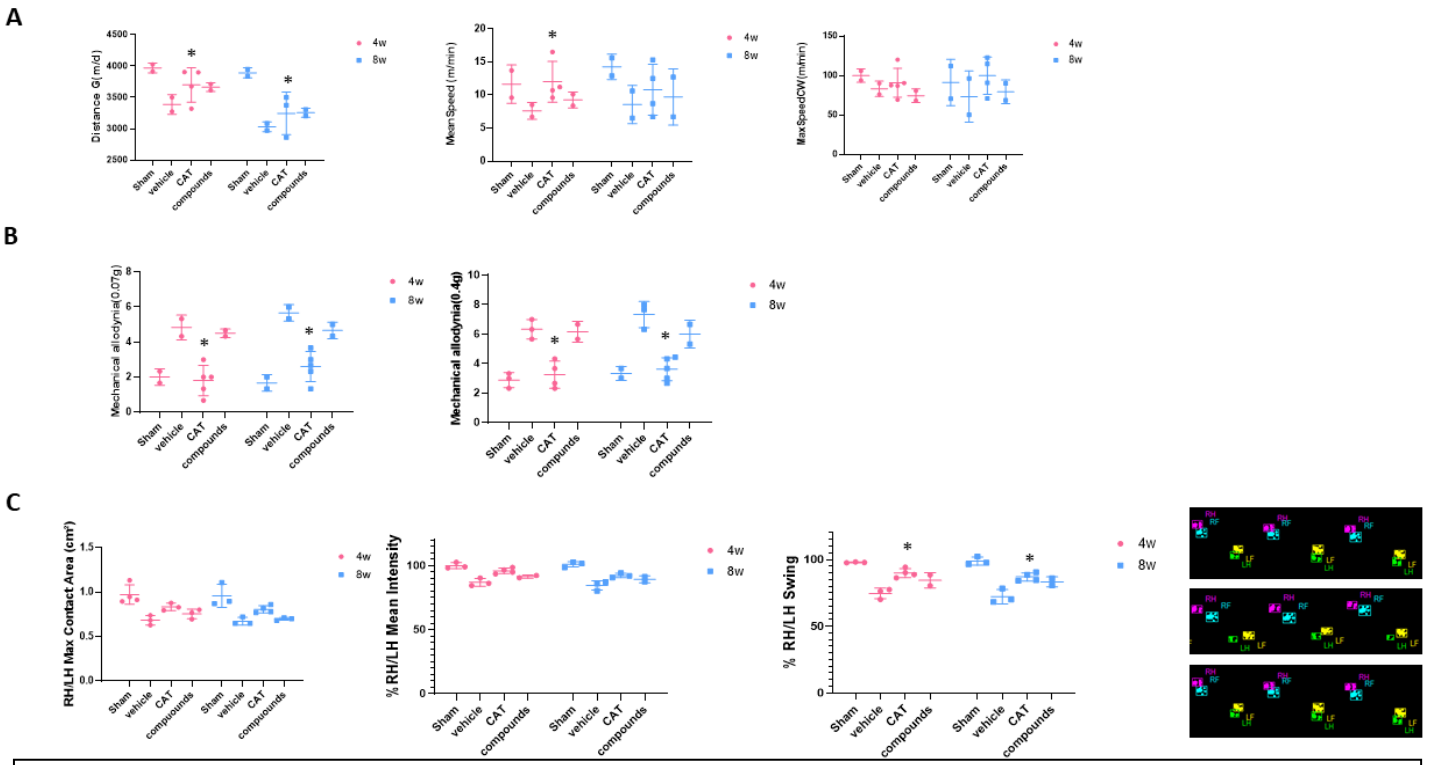

Fig 5. Conjugate ameliorate joint pain in OA mouse model. (A) Auto wheel running test at 4 weeks and 8 weeks post-surgery. **(B)** Von Frey test at 4 weeks and 8 weeks post-surgery. **(C)** Gait analysis at 4 weeks and 8 weeks post-surgery. Sham: sham surgery, vehicle: PBS treated ACLT mice, CAT: conjugate treated ACLT mice, compound: ALN+LY treated ACLT mice.

Opportunities for training and professional development:

Nothing to report.

Results disseminated to communities of intest:

Nothing to report.

Plan for next reporting period:

In the next stage of study, we will mainly focus on determining the effect of the conjugate in alleviating joint pain and improving functional outcome in OA mouse model. We will increase the sample size in the behavioral test to determine the effect of the conjugate in alleviating joint pain and improving functional outcome in the post-traumatic OA mouse model. Pain behavior and joint morbidity will be measured by Von Frey test, gait analysis and voluntary wheel running activity. Nociceptive axons growth will be identified by immunofluorescence staining of CGRP and in subchondral bone. To more specifically prove that OA pain is alleviated by preventing subchondral bone innervation, we will test the effect of ALN-LY conjugate in a pain reporter mouse (Pirt-GCaMP3 mice) to directly measure dorsal root ganglion neuronal activation in the post-

traumatic OA mouse model. The L4 DRG neuron activity (indicated by Ca^{2+} sensitive fluorescence) will be measured under a confocal microscopy.

IMPACT

The impact on the development of the principal discipline of the project:

Since there is a lack of a disease-modifying drug on the market for OA, the success of the work described in this proposal will facilitate and accelerate therapeutic development of this disease. Developing effective disease-modifying therapy for OA could greatly reduce the necessity of joint replacement surgery and medical expenditure. The proposed research project, by bone-targeted delivery of TGFβ inhibitor, holds the promise of reducing the burden of OA suffered by military Service members, Veterans, and their family members and caregivers.

The goal of the proposed study is to determine the therapeutic efficacy of the conjugate in rescuing osteoarthritic joint pathologies as well as alleviating symptoms. The results are expected to provide a strong technological and theoretical foundation for future clinical trials. Moreover, because of the time limitation, we are not able to substantially investigate the toxicology and side-effect of the conjugate in the proposed study. The detailed pharmacokinetic profile and toxicology study are necessary for the next step drug development. Finally, at the late stage OA when large cartilage defect already develops, a combination of multiple therapeutic approaches may be needed. The unique pharmacological characteristic of the conjugate makes it be a promising drug candidate to be tested in improving biomechanical microenvironment for AC when combined with cartilage regeneration techniques. The proposed project will enable us to generate preliminary data, optimize hypothesis and experimental design for the next step of investigations.

Impact on other disciplines:

Nothing to Report.

Impact on technology transfer:

Nothing to Report.

Impact on society beyond science and technology:

Since there is a lack of an effective drug on the market for OA, the success of the work described in this proposal will facilitate and accelerate therapeutic development of this disease and reduce the necessity of joint replacement surgery. The bone-targeted delivery system synergistically combines the beneficial effect of both bisphosphonate and TGFβ inhibitors and minimizes the possible negative effect of TGFβ inhibitors. The proposed strategy can efficiently stabilize the structure of subchondral bone and thereby prevent further damage of articular cartilage. It also holds promise to promote cartilage regeneration by providing a healthier microenvironment.

Chronic pain is the most prominent symptom of OA and primary reason for patients seeking medical intervention. Available therapies (e.g., NSAIDs, steroids, or desensitization of peripheral nerves) were reported to have an effect on alleviating mild to moderate joint OA pain. However, pure analgesia likely contributes to OA progression in the long run as the primary perception of the pathological changes within the joint is blunted. Thus, treating OA pain by targeting its cause would be the fundamental solution. Heavily innervation of perivascular sensory nerves fibers was observed in osteoarthritic subchondral bone indicating that subchondral bone is one of the primary sources of OA pain. This has been supported by the evidence that removal of subchondral bone leads to immediate pain relief in patients who receive total knee replacement and the close relationship between OA joint pain subchondral bone marrow lesions. The bone turn-over rate and osteoclast activity are dramatically increased in OA subchondral bone. It has been found that the enlarged osteoclasts population strongly promotes sensory innervation in osteoarthritic subchondral bone by secreting netrin-1, a nerve axon guidance and cell migration inducer. The bisphosphonate component in this conjugate can effectively inhibit osteoclast formation and therefore conceivably suppress the nerve axon growth in OA subchondral bone. Moreover, as the one of the primary inducer of angiogenesis in OA subchondral bone, excessive TGFβ likely promote nociceptive innervation indirectly. It is expected that this conjugate has a synergistic effect of TβRI inhibitor and bisphosphonate in reversing OA pathology and relieving OA pain.

Apart from the general impact on civilian OA, the present proposal has an immediate impact on the mission of the Department of Defense. Developing effective disease-modifying therapy for OA could greatly reduce the necessity of joint replacement surgery and medical expenditure.

CHANGES/PROBLEMS

Some of the in vivo experiments can't be completed on time as proposed due to the pandemic of COVID-19. We will request NCE to accomplish the project.

Our university has been closed since March 2020. During that period, no animal experiment can be conducted and we were requested to euthanize non-essential research animals and only some key breeders were allowed to be maintained. The majority of our core facilities were closed. My project is impacted because all of my proposed experiments in the project are in vivo experiments.

When the school reopens, we will resume the breeding and expending of transgenic mice, which will approximately take 4-5 months. The OA animal model will be conducted in 12-week old mice. Post-surgery experiments, tissue processing, and assessment took another 4-5 months. Two-three months are requested for additional or repeated experiments due to unexpected accidents or death of animals. Thus one-year no-cost extension will be necessary for accomplishing this project.

PRODUCTS

We had submitted a conference abstract to 2020 MHSRS. Due to COVID-19 pandemic, the conference is canceled and the abstract is available on-line through the homepage of the MHSRS website.

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PARTICIPANTS&OTHER COLLABORATING ORGANIZATIONS:

Name	Project Role	Nearest person month worked	Contribution to Project	Funding Support
Gehua Zhen	PI	8.4	Overall scientific management, animal surgery, immune-staining.	NIH/NIAMS R01 AR071432
Qiaoyue Guo	Graduate Student	12	Animal surgery, behavior test, CT analysis, HPLC	None

Has there been a change in the active other support of the PI or key personnel since the last reporting period?

Nothing to Report.

What other organization were involved as partners?

Nothing to Report.

SPECIAL REPORTING REQUIREMENTS

N/A

APPENDIX

N/A