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TITLE: An Herbal Derivative as the Basis for a New Approach to Treating Post-Traumatic Osteoarthritis

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14. ABSTRACT 14. ABSTRACT Osteoarthritis (OA) is a painful disease that causes the progressive destruction of joint structures, and is the most common cause of disability among military service members who are removed from active duty for medical reasons. In preliminary work with a small number of animals, we have found that the natural product derivative halofuginone (HF) shows promise with respect to reducing cartilage damage in the destabilized medial meniscus (DMM) mouse model of PTOA. HF inhibits glutamyl- prolyl-tRNA synthetase (EPRS), the enzyme responsible for charging tRNAs with the amino acid proline. The goal of this grant is to test the hypothesis that EPRS inhibitors will provide the basis for a new therapeutic strategy for PTOA. We report here: 1) Preliminary analysis of a study of efficacy of the EPRS inhibitors HF and its less toxic derivative HFol, on PTOA in mice, using the DMM model; 2) Data examining the transcriptomics of HF effects on responses to cytokines in synoviocytes. 3) preliminary characterization of an in vitro chondrocyte system in which chondrocytes retain strong responsiveness to cytokines.						
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INTRODUCTION Osteoarthritis (OA) is a painful disease that causes the progressive destruction of joint structures, and is the most common cause of disability among military service members who are removed from active duty for medical reasons. The progressive period in PTOA provides a target for therapeutic intervention. In preliminary work with a small number of animals, we have found that the natural product derivative halofuginone (HF) shows promise with respect to reducing cartilage damage in the destabilized medial meniscus (DMM) mouse model of PTOA. HF inhibits glutamyl- prolyl-tRNA synthetase (EPRS), the enzyme responsible for charging tRNAs with the amino acid proline. Low-level inhibition of EPRS triggers a metabolic sensor, a stress signal that initiates a sustained adaptive response across affected tissues. The goal of this grant is to test the hypothesis that EPRS inhibitors, acting to suppress a multi-cellular cytokine-driven tissue destructive program, will provide the basis for a new therapeutic strategy for PTOA. The Aims of this grant are to: 1) Characterize the therapeutic timing and functional effects of HF, or novel related EPRS inhibitors, on PTOA in mice, using the DMM model; 2) to examine the early time course of cellular and molecular responses to EPRS inhibitor treatment in the DMM mouse model, as well as in ex vivo in chondrocytes and synoviocytes. We believe that these studies both will establish the molecular and cellular basis for the benefit of a new drug class for PTOA treatment, and provide tools to evaluate different therapeutic strategies (e.g. novel compounds, delivery methods) prior to the appearance of joint pain or dysfunction.; 3) To develop and apply tools for testing the efficacy of EPRS inhibitors following drug delivery to the joint in DMM mice.

KEYWORDS: Post Traumatic Osteoarthritis (PTOA), Halofuginone (HF), tRNA synthetase inhibitor, chondrocyte, MMP, GCN2, Destabilized Medial Meniscus (DMM) model.

ACCOMPLISHMENTS In the first year of the grant, we have undertaken in vivo experiments in the DMM model to test the efficacy of the EPRS inhibitors HF and Hfol, and in vitro experiments to identify relevant cell-based models for understanding the mechanism of EPRS inhibitor action on tissue destruction in OA. We have made good progress towards both goals, and are in the process of analyzing data from the first set of in vivo studies of HF/Hfol action in the DMM model of OA.

A) Major activities: i. In vivo analysis and comparison of the efficacy of systemic HF and Hfol in the DMM mouse model of PTOA. ii. Analysis of RNAseq characterization of effects of HF on tissue damage responses in primary human synoviocytes. iii. Identification and characterization of an experimental system for the study of tissue damage responses in primary chondrocytes.

B) Specific objectives: i. to assay the effects of systemic EPRS inhibitor treatment on multiple parameters of PTOA associated changes in cartilage and bone gene expression, histology, and structure in the DMM model; ii: to use transcriptomic analysis to identify markers and mechanisms of HF action in synoviocytes and chondrocytes; iii. to establish a culture chondrocyte system for studying cytokine induction of tissue remodeling genes, and inhibition by HF.

C) Significant results: Objective i (Principal effort, Li Lab) : We have now completed a study of 32 mice (4 treatment groups, 8 mice each) over 16 weeks treatment with HF and the novel EPRS inhibitor HFol (reasons for inclusion of HFol are provided below) in the DMM mode of PTOA. We have generated fixed or frozen joint tissue for analysis of tissue histology and gene expression. Because of the time intensive nature of these analyses they are currently still in progress. Whole animal examination of body weight and bone volume (by micro-CT) indicate that HF causes some decrease in both (undesirable outcomes potentially reflecting toxicity), while HFol treatment showed no body weight or bone volume decreases, consistent with reduced toxicity of HFol relative to HF. Preliminary histological analysis of joints indicates that both HF and HFol reduce joint destruction in DMM PTOA. We have not analyzed enough the samples to determine statistical significance. Our plan over the next month is to do blinded histological and molecular analysis of tissue sections and than evaluate treatment efficacy for both HFol and HF. Detailed description of study:

Objective: To determine whether HF and HFOL can prevent/delay the progression of the articular cartilage degeneration induced by destabilization of the medial meniscus (DMM).

Rationale: In our previous studies, we found that it takes about 16 weeks for mice to develop a typical OA knee joint after DMM surgery. Therefore, we plan to use 16 weeks after DMM surgery as the end point to characterize mouse knee joints for evidences of articular cartilage degeneration.

Methods and results:

Experiment 1. Treatment of mice with HF or HFOL

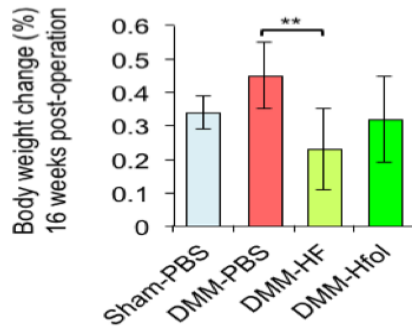


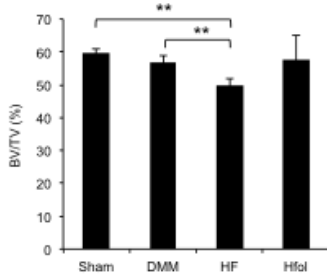
Fig.1. Body Weight changes following DMM Surgery and 16 week treatment with HF or HFol

There are four groups of mice: sham surgery treated with PBS, DMM surgery treated with PBS, DMM surgery treated with HF and DMM surgery treated with HFOL. There are 8 mice in each group. Mice (C57BL/6j) at the age of 10 weeks old were subject to the surgery. Two weeks after the surgery, mice were treated either with PBS or HF (0.2 mg/kg body weight) or HFOL (1 mg/kg body weight) every other day. At 8 weeks after the surgery, one set of four groups of mice was euthanized for the collection of knee joints. Another set of four groups of mice was kept alive to the age of 16 weeks after the surgery and then the mice were euthanized for the collection of knee joints.

Experiment 2. Measurement of mouse body weights

We measured the body weight of the mice during the drug treatment.

Body weight changes (%) were calculated. There was no difference in body weight gain among the groups at 8 weeks following the surgery (**Fig.1**). However, the DMM/HF group showed a significantly less body weight gain compared with that of the DMM/PBS group, $p < 0.01$, at 16 weeks after the surgery. There was no difference between DMM/HFol and DMM/PBS, $p > 0.05$



	BV/TV (%)	p-value	
		vs Sham	vs DMM
Sham	59.67	-	-
DMM	56.67	0.0840	-
HF	49.96	0.0040	0.0056
Hfol	57.52	0.6167	0.8443

Fig.2. Bone Volume changes following DMM Surgery and 16 week treatment with HF or Hfol. Top: sample CT analysis; bottom: tabulation of data

Experiment 3. Measurement of bone volume in epiphysis of tibia in mice by micro-computed tomography (μ CT)

A high-resolution desktop micro-tomographic imaging system (μ CT40, Scanco Medical AG, Brüttisellen, Switzerland) was used to assess trabecular bone microarchitecture, total and bone volumes, and mineral densities of the tibial epiphysis (**Fig.2**). Scans were acquired using a 10μ m isotropic voxel size, 70 kVp,

114 mAs, 200 ms integration time, and were subjected to Gaussian filtration and segmentation. Images and results were shown below.

As indicated in the image and the figure, the bone volume is reduced in DMM/HF group, compared with that in the sham ($p=0.0040$) and DMM ($p=0.0056$) groups

As indicated in the image and the figure, the bone volume is reduced in DMM/HF group, compared with that in the sham ($p=0.0040$) and DMM ($p=0.0056$) groups

Experiment 4. Histology analysis of mouse knee joints

For knee joints from 8 weeks after the surgery, all of the samples were embedded in paraffin and 10 of them were sectioned and stained by Safranin O/Fast green, n=2 in Sham/PBS, n=3 in DMM/PBS, n=3 in DMM/HF and n=2 in DMM/HFOL. For knee joints from 16 weeks after the surgery, 25 of the samples were embedded in paraffin and the rest of them are in the process to be embedded.

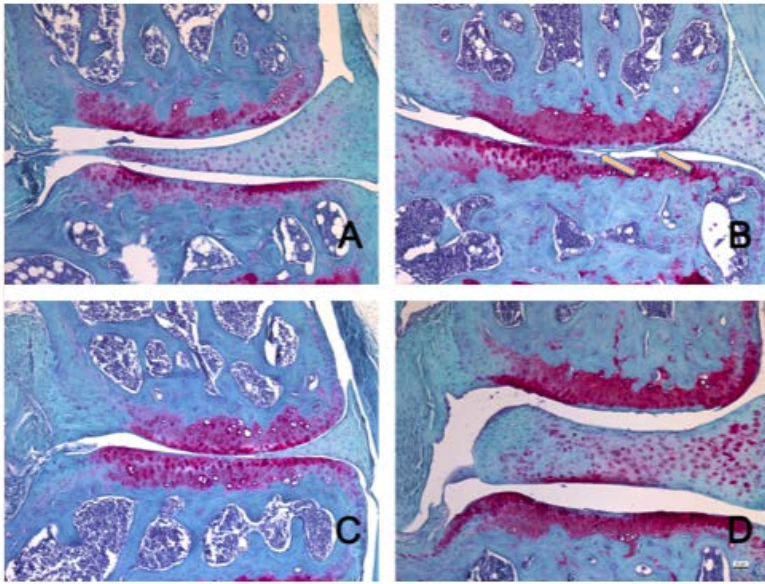


Fig.3. Sample histology of knee joint following DMM Surgery and 16 week treatment with HF or Hfol. A) Sham surgery; B) DMM surgery, vehicle treatment. Arrows mark sites of fibrillation reflecting cartilage damage. C) HF treated or D) Hfol treated DMM mice. No fibrillation noted in treated limbs.

For histology analysis, Knee joints were decalcified in Morse's solution. For each knee joint, 6 μ m thick serial sagittal sections were cut. Every tenth section was collected for Safranin O/Fast green staining, see the figure below (Fig.3).

We do not observe overt morphological changes with the exception of slightly localized proteoglycan degradation in the sham mice (A). Fibrillations are seen in DMM/PBS mice (B). There are no fibrillations in DMM/HF and DMM/HFOL groups. This indicates that HF or HFOL treatment may be able to delay the progressive process of articular cartilage degeneration induced by DMM. Obviously, the examination of more samples is needed to confirm this observation. We plan to do so in the following year.

Objective ii (Principal effort, Whitman Lab): We have completed analysis of global transcriptomic (RNAseq) analysis of the effects of HF on gene expression in primary human synoviocytes in the presence or absence of TNF. We find the HF selectively inhibits a subset of TNF responses involved in tissue remodeling. A summary of this analysis is provided in **figure 4**. The data represent the global pattern of effects of HF on TNF induced transcriptional responses, and show that HF selectively inhibits the most robust responses to TNF, and has a particularly dramatic and selective effect on Matrix Metalloproteinases (MMPs). We are currently generating for comparable analysis in chondrocytes.

Objective iii. (Principal effort, Whitman Lab) We have tested a broad set of established and primary chondrocytes from several sources, and found that most show very weak cytokine responses that make them unsuitable for the intended analyses. We have found, however, that primary chondrocytes freshly derived from the mouse tibial plateau show very robust induction of tissue destruction responses in response to the cytokine IL1 β (**Fig.5**). We are now optimizing and characterizing these responses, and testing the efficacy of HF and HFol for inhibition of cytokine responses in these cells.

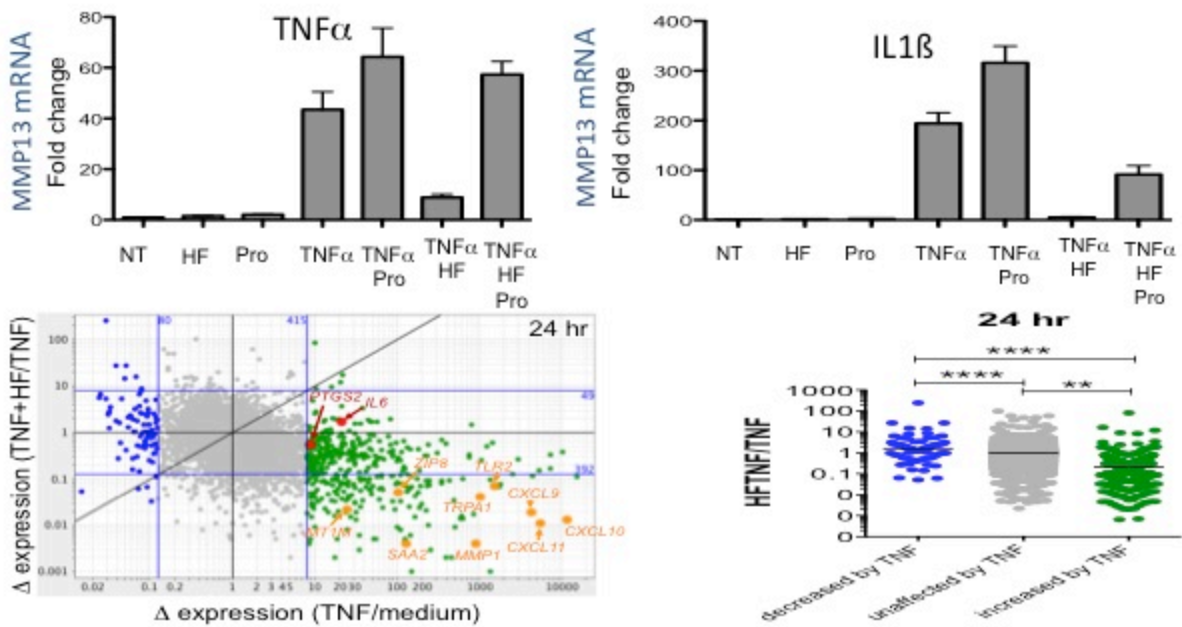


Fig.4. HF inhibits cytokine responses in primary FLS. A) HF inhibition of MMP13 induction. Human RA-FLS were pre-treated with 200 nM HF and/or 2 mM proline (Pro) for 16 hours, and treated with TNF α or IL1 β for 6 hours. Transcript levels of MMP13 were quantified by qRT-PCR and normalized to phosphoglycerate kinase 1 (PGK1) levels. Results are representative of three independent experiments. **B. RNA-seq analysis of HF effects on TNF responses.** *Left* – selective regulation of TNF-inducible pro-inflammatory gene expression in fibroblast-like synoviocytes (FLS), determined by RNA-seq. after 24 hr culture in media alone, TNF α , or TNF α plus HF. Data are presented as fold-change/fold-change plot; x-axis shows the change (D) in normalized gene expression in TNF α -treated vs. untreated FLS (TNF/medium); y-axis shows D gene expression in FLS treated with TNF α plus HF vs. TNF α alone (TNF+HF/TNF). Blue- and green-highlighted genes are reduced or increased by TNF α treatment (10-fold), respectively. Examples of TNF α -inducible genes that are not affected (highlighted red) or suppressed (highlighted orange) by HF treatment are indicated by arrowhead and text. *Right* – opposing effects of TNF α and HF on FLS gene expression. Effects of HF treatment on the expression of genes that are strongly decreased (≥ 10 -fold, blue), not affected (within 10-fold, grey), or strong induced (≥ 10 -fold, green) by TNF α stimulation after 24 hr (compared to cells cultured in media alone). Data are presented as fold-change in TNF α + HF- vs. TNF α only-treated cells.

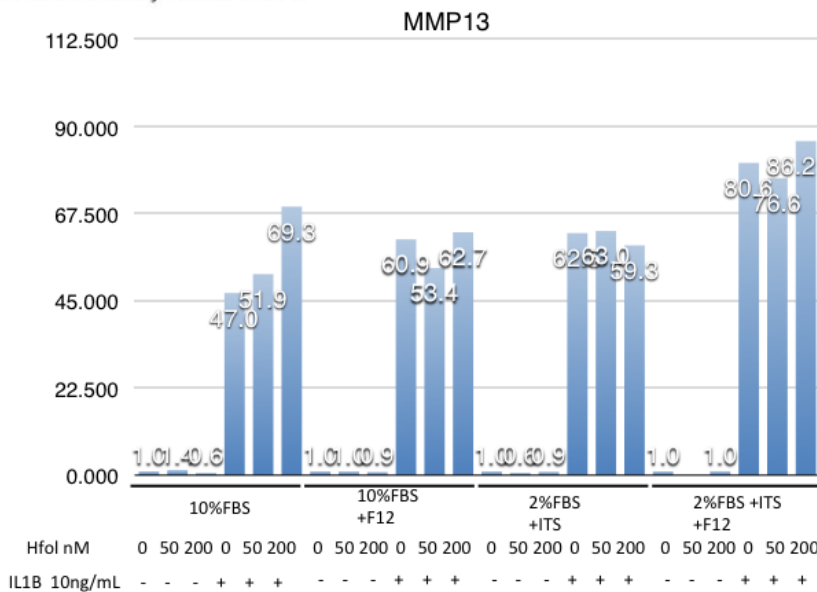


Fig.5. Testing conditions for IL1 β stimulation of primary chondrocytes. Primary chondrocytes were harvested from 4 week old mouse tibial plateau and analyzed for IL1 β induction of gene expression associated with joint destruction. 50-70 fold induction seen; little effect of Hfol at these doses, will try higher. Similar induction seem with other markers of tissue destruction.

Training and professional development:

Fan Jie is currently a visiting PhD student from West China University of Chengdu, China. She has been working on this project since the beginning. She has carried out the experiments and joined the discussion and interpretation of the results. During this training, she will learn how to design and perform experiments to test a hypothesis. She will also learn how to interpret results from experiments. We anticipate that she will obtain sufficient results from the experiments for her thesis defense in the summer of 2017..

Yeonjin Kim is a postdoctoral fellow whose training is enhanced by learning about PTOA models and mechanisms, through mentoring with PIs and seminars at HSDM on joint biology.

Dissemination of results to communities of interest? Nothing to report.

Plans for the next reporting period to accomplish the goals: We will complete analysis of existing dataset as described in SOW. If HFol proves to be equal or more efficacious than HF in the DMM model, we will continue to test timing of efficacy as described in SOW, if Hfol is less efficacious we will continue with HF. We will continue to examine molecular markers in synoviocytes and chondrocytes that are modulated by HF/HFol. as well as potential mechanisms underlying inhibition as described in SOW. We plan to prepare a manuscript on HF/HFol efficacy in DMM during the next reporting period.

IMPACT: Our establishment of the selective action of HF on tissue remodeling responses in synoviocytes (objective 2) provides important proof of principle that this class of compounds can have selective therapeutic effects in vivo. We are in the planning stages of a manuscript to report these findings, but this will await more extensive analysis of in vivo data and chondrocyte studies.

CHANGES/PROBLEMS. The use of EPRS inhibitors other than HF, such as Hfol, was a sub-aim described in our grant proposal, but was not a priority in the original SOW. Our reason for modifying the initial animal study of DMM to include a comparison with HFol was that in a toxicity study performed for us at a Contract Research Organization (Charles River Labs) subsequent to the SOW submission but prior to the start of the grant, we found that HFol was ~20 fold less toxic than had been previously reported for HF. Since the goal of the current grant is to develop therapeutics feasible for use in humans, the therapeutic characterization of an EPRS inhibitor with substantially reduced toxicity relative to HF was clearly consistent with the stated objectives of the project. Since our pilot experiments in the DMM model were performed with HF, however, it was also essential to compare efficacy of Hfol to HF rather than to simply switch to HFol. The timeline of the in vivo studies has been somewhat delayed by the necessity to train new personnel to participate in study execution, but this is now completed and analyses are well underway.

The difficulties in identifying cytokine responsive cultured chondrocyte populations has delayed progress in the SOW timeline regarding the identification of novel transcriptional markers of EPRS inhibitor action in vitro. We believe this problem is now solved with the identification of primary mouse chondrocytes from the tibial plateau as cytokine responsive. The short usable lifespan of these cells, and requirement for mice of a specific age (4-6 weeks) as source, has significantly slowed the pace of these experiments compared to what was originally planned, in addition to being more labor intensive. These issues do not entail any major revision to the long term goals of the project.

PRODUCTS. Data described above reflect work product that are in preparation for publications

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name: Malcolm Whitman	
Project Role: Principal Investigator	
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked: 5	
Contribution to Project: develop overall project plan, obtain test compounds, establish experimental plan in consultation with other project members, review and analyze data in consultation with other project members.	
Funding Support: DoD, NIH, ABLs, HSDM	

Name: Tracy Keller	
Project Role: Co-Investigator	
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked: 3	
Contribution to Project: plan and direct ex vivo experiments on chondrocytes and synoviocytes	
Funding Support: DoD, NIH, ABLs	

Name: Yefu Li	
Project Role: Principal Investigator	
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked: 5	
Contribution to Project: DMM surgery and oversight of mice, participation in analysis of mice	
Funding Support: DoD, Servier, HSDM	

Name: Lin Xu	
Project Role: Co-Investigator	
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked: 7	
Contribution to Project: DMM surgery and oversight of mice, participation in analysis of mice	
Funding Support: DoD, Servier,	

Name: Fan Jie, PhD	
Project Role: Research Scientist	
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked: 12	
Contribution to Project: Assist on surgery, care for mice and HF injections, tissues harvesting and subsequent analysis of tissues.	
Funding Support: DoD	

Name: Kristen Powers
Project Role: Research Assistant
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 5.0
Contribution to Project: execution of in vitro assays, harvest of primary cells
Funding Support: DoD, NIH, ABL

Name: Yeon Jin Kim, PhD.
Project Role: Postdoctoral scientist
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 6.0
Contribution to Project: Design and execution of experiments on HF effects in vitro, development of Q-PCR assays and execution of transcriptomics.
Funding Support: DoD, NIH

Changes in Active Support

Whitman (PI) 09/17/2015 – 08/31/2019 1.2 Cal. Mos.

NIH-NIAMS R01GM115417-01 \$296,586

Title: The first secreted Tyrosine kinase

The major goals of this project are to investigate the regulation of VLK, the first secreted Tyrosine kinase, within the secretory pathway, identify secreted targets for VLK phosphorylation in cells that express VLK endogenously, and begin to establish how VLK phosphorylation modifies the function of specific secreted or secretory pathway resident proteins.

Program Official: Bernadette Tyree

Email: tyreeb@mail.nih.gov

Phone: 301-594-5032

Fax: 301-480-4543

Whitman (PI) 04-01-2015 – 03-31-2020 3.0 Calendar Mos.

NIH/NIAMS R01 AR066717 \$334,966

Title: Role of the first secreted tyrosine kinase in bone development, homeostasis, and repair

The major goal of this project is to investigate an identified new mechanism for the regulation of proteins that control bone and cartilage matrix homeostasis, with substantial implications for new therapeutic approaches to skeletal disease.

Program Official: Bernadette Tyree

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Fax: 301-480-4543

Whitman (PI) 05-25-2015 - 05-27-2017 1.2 Calendar Mos Allied Bristol Life

Sciences \$117,994

Title: Identification of cellular regulators and biomarkers of fibrosis targeted by AAR and EPRS inhibitors

The major goal of this project is identify mechanisms and biomarkers involved in the development and treatment of lung fibrosis.

Program Official: Satish Jindal, Ph.D. CEO

Allied-Bristol Life Sciences

Email: pipeline@alliedminds.com

Phone: 617-419-1800

Li (PI) 01/11/13 to 12/31/16 3.6 calendar months

Sponsor: Servier, France No Cost Extension

Title: Chondro-protective effects of the deletion of Htra1 and Ddr2 in mouse knee joints against osteoarthritis

The goal of this project is to determine whether removal of a serine protease (high temperature requirement A, HtrA1) and a cell surface tyrosine kinase receptor for native type II collagen (discoidin domain receptor 2, Ddr2) in the articular cartilage of knee joints can delay the progression of osteoarthritis (OA) in mouse models.

Other Organizations

None

Special Reporting Requirements

This is a collaborative award to Dr. Whitman and Dr. Li, reports are filed for both Whitman and Li

Appendices:

If applicable, PDF files for published manuscripts.

None