EMBO Molecular Medicine

Tranilast directly targets NLRP3 to treat inflammasome-driven diseases

Yi Huang, Hua Jiang, Yun Chen, Xiaqiong Wang, Yanqing Yang, Jinhui Tao, Xianming Deng, Gaolin Liang, Huafeng Zhang, Wei Jiang, Rongbin Zhou

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Editorial Decision:	6 February 2018
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Editor: Céline Carret

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 14 December 2017

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript.

You will see from the set of comments pasted below that overall the referees are positive about the paper. This said, in order to increase clarity and conclusiveness, you are strongly encouraged to do the following: compare MCC950 to TR to show how it performs (ref.1), test TR effect on pyroptosis (ref.2), and provide details and clarifications throughout including full western blots, expand discussion and proofread the text (ref2 and 3). During our cross-commenting exercise, it became clear that all referees support the requested drugs comparison, and I really hope you'll be able to perform it.

We would welcome the submission of a revised version within three months for further consideration and would like to encourage you to address all the criticisms raised as suggested to improve conclusiveness and clarity. Please note that EMBO Molecular Medicine strongly supports a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

I look forward to receiving your revised manuscript.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

In this work Huang et al describe Tranilast as a specific NLRP3 inhibitor, and provides mechanistic data as well as in vivo data demonstrating a strong effects in mouse models of known NLRP3-dependent diseases. The work is of very high quality, and the conclusions are supported by the data. However, as the data stand now, it is difficult to critically assess how potent Tranilast is compared to other known NLRP3 antagonists, e.g. MCC950. This should be tested, both in vitro and in vivo.
Referee #1 (Remarks for Author):

Very nice piece of work. My only point (which I think is important to be able to put the data in context with the rest of the field), is that more data - both in vitro and in vivo - should be provided where Tranilast is compared directly to other known NLRP3 antagonists.

Referee #2 (Remarks for Author):

In this manuscript, the authors report that Tranilast (TR), an old anti-allergic clinical drug, specifically inhibits NLRP3 inflammasome activation in macrophages by inhibiting the assembly of NLRP3 inflammasome. They show that TR directly binds to NLRP3 and prevents is oligomerization. With in vivo experiments, the author further report that TR has remarkable preventive or therapeutic effects on mouse models of NLRP3 inflammasome-related human diseases as gouty arthritis, cryopyrin-associated autoinflammatory syndrome and type 2 diabetes. Finally they show that TR is also efficient ex vivo using mononuclear cells from patients with Gout. Hence, as a direct NLRP3 inhibitor, TR appears as a promising component for treating NLRP3-driven diseases.

The data are very convincing and the conclusions raised by the authors are well supported by their results. My only concern is that the authors have not investigated the impact of TR on pyroptosis. Indeed, NLRP3 inflammasome activation triggers caspase-1 processing and this inflammatory caspase not only promotes the maturation of Il-1b and IL-18 but also cleaves gasdermin D to promote pyroptosis and ensuing the release of the pro-inflammatory cytokines. As TR prevents NLRP3 inflammasome activation, a decrease in pyroptosis/cell death is expected in TR-pretreated cells after exposure to nigericin, ATP, MSU and alum. This should be shown. Likewise, an inhibition in the gasdermin D cleavage is expected. In the case of cLPS, in contrast, the pre-treatment with TR should have a minor effect on gasdermin D cleavage and ensuing pyroptosis as in this condition, NLRP3 inflammasome activation is a consequence of the K+ potassium efflux triggered by the pyroptosis after the cleavage of gasdermin D by caspase-4 (in human) or caspase-11 (in mouse).

Other comments:
In Figure 5H, the authors show a full WB of caspase-1 in cell extract where pro-caspase-1 and the different isoforms as well as the p20 mature form can be seen. I do not understand why in the other figures, in the "input", the authors only show the pro-caspase-1 given that after strong signals as exposure to nigericin, the p20 caspase-1 can be seen in cell extract. Likewise, for pro-IL-1b, after priming in BMDMs, several isoform of IL-1b are detected in cell extracts and after NLRP3 inflammasome activation, the mature p17 IL-1b is very often detected.

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This is a study of significant novelty and translational value. Yet, the study lacks essential experimental information throughout. Also, the English used needs polishing.

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Huang et al. have investigated the effect of Tranilast (TR), an old anti-allergic clinical drug, in inflammasome activation. They found that TR directly inhibits NLRP3 activation by binding to the NACHT domain of NLRP3 and suppressing the assembly of the fully functional NLRP3 inflammasome. This seems to be specific for NLRP3 as the activation of AIM2 and NLRC4 inflammasomes is not affected. They also show that in experimental animal models in vivo TR suppresses gouty arthritis, cryopyrin-associated autoinflammatory syndromes and type 2 diabetes. Overall, this is an interesting and novel study. As inhibitors suppressing NLRP3 activation of clinical applicability are actively being sought, this study has also significant translational potential.

Specific comments

1. Often, information on essential experimental details is lacking in both the text and Fig. legend,

making difficult the assessment of the data. For example, in Fig. 1, there is no information provided about the duration of LPS stimulation in the various situations, the timing of supernatant or cell extract collection for ELISA etc. In Fig. 2 and 3 the timing of treatment with nigericin is not mentioned. This goes throughout the manuscript. This is essential information and should be part of the text and Figure legends.

2. In lines 127-128, the authors state that they searched for 'NLRP3 inhibitors in clinical drugs and found TR can directly...'. If the include that as an approach they used in this work, they should also have the data in.

3. The word 'priming' as used in lines 180-191 to describe the results of Fig. 1 is inappropriate and confusing. LPS induces 'priming' of the inflammasome anyway, and that is independent of the addition of TR before or after LPS stimulation. Thus, TR treatment at 3h post LPS stimulation should not be affecting priming (much) while treatment before should.

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6. The Discussion is short and should be expanded

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1st Revision - authors' response 23 January 2018

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Very nice piece of work. My only point (which I think is important to be able to put the data in context with the rest of the field), is that more data - both in vitro and in vivo - should be provided where Tranilast is compared directly to other known NLRP3 antagonists.

Reply: Thanks very much for the suggestion. We have compared the activity of TR with MCC950 and the data were shown as appendix Fig.S6A-C in the revised manuscript. The results showed that although the *in vitro* inhibitory activity of TR on MSU-induced IL-1b secretion was around 100-500 times less potent than MCC950 (Appendix Fig S6A), its *in vivo* activity on MSU-induced peritonitis was only around 5-10 times less potent than MCC950 (Appendix Fig S6B, C).

Referee #2 (Remarks for Author):

In this manuscript, the authors report that Tranilast (TR), an old anti-allergic clinical drug, specifically inhibits NLRP3 inflammasome activation in macrophages by inhibiting the assembly of NLRP3 inflammasome. They show that TR directly binds to NLRP3 and prevents is oligomerization. With in vivo experiments, the author further report that TR has remarkable preventive or therapeutic effects on mouse models of NLRP3 inflammasome-related human diseases as gouty arthritis, cryopyrin-associated autoinflammatory syndrome and type 2 diabetse. Finally they show that TR is also efficient ex vivo using mononuclear cells from patients with Gout. Hence, as a direct NLRP3 inhibitor, TR appears as a promising component for treating NLRP3-driven diseases.

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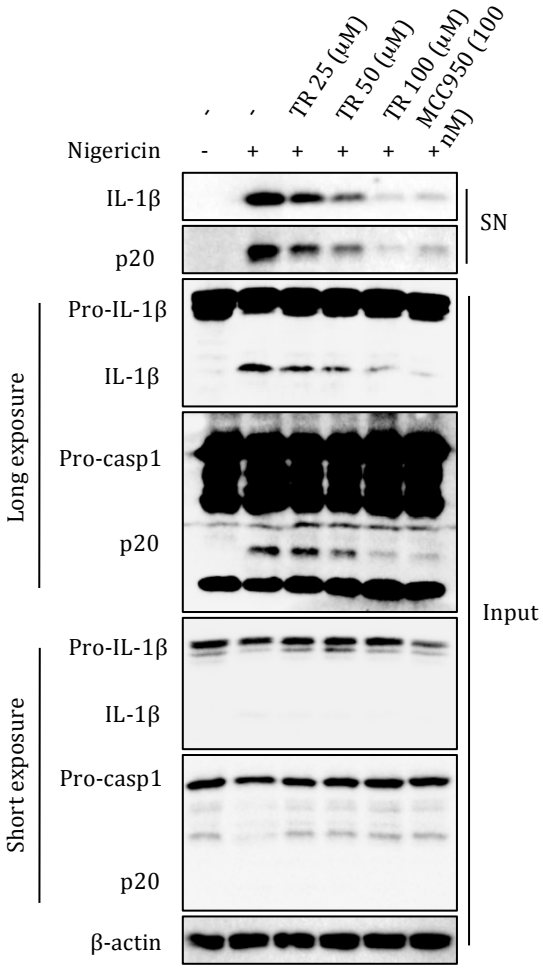
Reply: Thanks very much for the suggestions. In the revised manuscripts, we provided new data showing that TR could block nigericin-induced pyroptosis and Gsdmd activation (Appendix Fig S1A, S1E), but could not block cLPS-induced pyroptosis and Gsdmd activation (Appendix Fig S1E, S1F).

Other comments:
In Figure 5H, the authors show a full WB of caspase-1 in cell extract where pro-caspase-1 and the different isoforms as well as the p20 mature form can be seen. I do not understand why in the other figures, in the "input", the authors only show the pro-caspase-1 given that after strong signals as exposure to nigericin, the p20 caspase-1 can be seen in cell extract. Likewise, for pro-IL-1b, after priming in BMDMs, several isoform of IL-1b are detected in cell extracts and after NLRP3 inflammasome activation, the mature p17 IL-1b is very often detected.

Reply: Thanks very much for the suggestion. In our experiences, the caspase-1 P20 and IL-1b p17 were weak in the cell extracts of BMDMs when stimulated with NLRP3 activators (please see the data below), so we only showed the pro-caspase-1 and pro-IL-1b in the "input". Indeed, most publications in this filed showed the data in a similar way.



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Specific comments

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Reply: Thanks very much for the suggestions. Some details have been described in methods sections of the original manuscripts. For example, we have provided the information about inflammasomes stimulation:" For induction of inflammasome activation, 5×10^5 macrophages were plated overnight in 12-well plates and the medium was changed to Opti-MEM (1% FBS) in the following morning, then the cells were primed for 3 h with ultrapure LPS (50 ng/ml) or Pam3CSK4 (400 ng/ml). After that, TR were added into the culture for another 30 min and then the cells were stimulated for 4 h with MSU (150 μg/ml), Alum (300 μg/ml), S. typhimurium (multiplicity of infection (MOI)) or for 30 min with ATP (2.5 mM) or nigericin (3 μM). Cells were transfected with poly A:T (0.5 μg/ml) for 4 h or LPS (500 ng/ml) for overnight through the use of Lipofectamine2000 according to the manufacturer's protocol (Invitrogen)."

In the revised manuscripts, we added some lacking information in figure legends or Methods.

2. In lines 127-128, the authors state that they searched for 'NLRP3 inhibitors in clinical drugs and found TR can directly...'. If they include that as an approach they used in this work, they should also have the data in.

Reply: Thanks for the comments. We revised the sentence as "In this study, we showed that TR directly bound to NLRP3 and inhibited NLRP3 inflammasome assembly and the subsequent caspase-1 activation and IL-1b production" in the revised manuscript.

3. The word 'priming' as used in lines 180-191 to describe the results of Fig. 1 is inappropriate and confusing. LPS induces 'priming' of the inflammasome anyway, and that is independent of the addition of TR before or after LPS stimulation. Thus, TR treatment at 3h post LPS stimulation should not be affecting priming (much) while treatment before should.

Reply: Thanks very much for the comment. We revised the sentences as " we then examined whether TR inhibited NLRP3 inflammasome activation via regulating the expression of NF-kB-dependent NLRP3 or pro-IL-1b expression. When BMDMs were stimulated with TR after 3-hour LPS treatment, TR had no effect on LPS-induced NLRP3, pro-IL-1b expression, TNF-a or IL-6 production (Fig 1C, D and appendix Fig S1B-D), suggesting that TR-induced NLRP3 inflammasome inhibition was not caused by the downregulation of NLRP3 or pro-IL-1b expression at this condition".

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Reply: Thanks for the comments. The NLPR3, ASC and b-actin WB at the bottom (labelled on the left as 'input') are extracts before IP. We corrected the labels on the top as "IgG" and "anti-NEK7 or ASC" In Fig.2B, C.

In The Fig.3, streptavidin beads (not antibodies) were used to pulldown, so in my opinion, they are not classical "Immunoprecipitation" and "Pulldown" seems to be better than "IP" in these figures.

5. How do the authors envisage a small molecule such as TR inhibiting NLRP3 oligomerization? Also, how can that be specific for NLRP3 but not other inflammasomes? Moreover, how can a small molecule such as TR inhibit protein interactions driven by the interaction of large protein surfaces?

Reply: It is a good question. Honestly we don't know the detailed mechanism of how TR inhibits NLRP3 oligomerization. We have discussed this issue in the revised manuscript:
"An interesting question is that how a small molecule such as TR can inhibit NLRP3 oligomerization. Previous results have shown that the ATPase activity of NLRP3 NACHT domain is essential for the oligomerization of NLRP3 (Duncan et al, 2007). Moreover, several inhibitors, including parthenolide, Bay 11-7082, INF39, 3,4-methylenedioxy-β-nitrostyrene and CY-09 have been reported to inhibit NLRP3 inflammasome activation by suppressing the ATPase activity of NLRP3 (Cocco et al, 2017; He et al, 2014; Jiang et al, 2017; Juliana et al, 2010), so it is possible that TR might inhibit the ATPase activity of NLRP3 to block its oligomerization. However, our data showed that TR had no effects on its ATPase activity. Another possibility is that TR might target the interfaces of NLRP3-NLRP3 interaction. Although the protein-protein interaction (PPI) interfaces are generally flat and large (roughly 1,000–2,000 A^2 per side) and are different with the deep

cavities that typically bind small molecules (~300–500 A^2)(Fuller et al, 2009; Hwang et al, 2010), not all residues at the PPI interface are critical(Arkin et al, 2014). Indeed, at least some PPIs might have small-molecule-sized "hot spots" that are essential for the interaction and can dynamically adjust to bind a small molecule(Arkin et al, 2014). In the last decade, more than 40 PPIs have now been targeted and several inhibitors have reached clinical trials(Labbe et al, 2013). So, TR might bind to a "hot spot" of NLRP3 that is critical for NLRP3-NLRP3 interaction and then block its activation. Future studies need to identify the residues of NLRP3 NACHT domain that are responsible for TR binding and clarify the detailed mechanism of how TR blocks NLRP3-NLRP3 interaction by using biochemical and structural approaches."

6. The Discussion is short and should be expanded

Reply: Thanks very much for the suggestion and the discussion has been expanded in the revised manuscript.

7. English needs polishing.

Reply: Thanks very much, we have modified the text in the revised manuscript.

2nd Editorial Decision 6 February 2018

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending a few final amendments.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

This work is of high academic and technical quality, and the findings are novel.

Referee #1 (Remarks for Author):

The authors have addressed my only major point as requested. Although the comparison, did not come out in favour of Tranilast, and think the total data package is now so strong that the work deserves to be presented for the scientific audience.

Referee #2 (Remarks for Author):

The referees' comments have been addressed in a satisfactory manner. I therefore recommend acceptance for publication

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Names: Rongbin Zhou and Wei Jiang
Journal Submitted to: EMBO Mol Med
Manuscript Number: EMM-2017-08689

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes were selected on the basis of preliminary results to ensure an adequate power.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample sizes were selected on the basis of preliminary results to ensure an adequate power.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No exclusion of data points was used.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Mice were randomized into different groups and randomized prior to treatment.
For animal studies, include a statement about randomization even if no randomization was used.	Male C57BL/6J mice with similar plasma glucose levels and body weights were randomized into different groups.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Group allocation was performed at random,
4.b. For animal studies, include a statement about blinding even if no blinding was done	The researchers were not blinded to the treatment groups when performing experiments.
5. For every figure, are statistical tests justified as appropriate?	Yes, statistical analysis were performed with the unpaired t-test for two groups or two-way ANOVA or a generalized Wilcoxon test
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	All values are expressed as the mean and s.e.m.
Is there an estimate of variation within each group of data?	Yes, statistical analysis were performed with the unpaired t-test for two groups or two-way ANOVA or a generalized Wilcoxon test
Is the variance similar between the groups that are being statistically compared?	Yes, statistical analysis were performed with the unpaired t-test for two groups or two-way ANOVA or a generalized Wilcoxon test

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Anti-β-actin (1:5000, P30002) and Anti-DYKDDDDK-Tag mAb was from Abmart. Anti-human pro-IL-1β (1:1000, 60136-1-Ig), anti-TRPV2 (1:1000, 15991-1-AP) and anti-HPGDS (1:1000, 22522-1-AP) were from Proteintech. Anti-mouse IL-1β (1:1000, AF-401-NA) was from R&D Systems. Anti-mouse caspase-1 (p20) (1:1000, AG-20B-0042) and anti-NLRP3 (1:1000, AG-20B-0014) were from Adipogen. Anti-human caspase-1(1:1000, 2225) was from Cell Signaling. Anti-ASC (1:500, sc-22514-R) and anti-NEK7 (1:500, SC-50756) were from Santa Cruz. Anti-human cleaved IL-1β (1:1000, A5208206) was from Sangon Biotech. Anti-Flag (1:2000, F2555) or anti-VSV (1:2000, V4888) were from Sigma.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	THP-1, HEK-293T, L929 cells and iBMDMs were not authenticated but routinely tested for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	6-week-old male C57BL/6J mice used in the studies were obtained from Model Animal Research Center of Nanjing University. Nlrp3-/- mice were described previously (Martinon et al, 2006). LysM-cre mice (B6.129P2-Lyz2tm1(cre)Ifo/J) and Nlrp3A350VneoR mice were from Jackson Laboratory. All animals were housed under 12-hr light/dark cycle at 22-24°C with unrestricted access to food and water for the duration of the experiment except during fasting tests.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were approved The Ethics Committee of University of Science and Technology of China.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	All studies were performed in compliance with ARRIVE guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The synovial fluid was obtained from 4 patients with gout and knee effusions. To use these clinical materials for research purposes, prior patients' written informed consents and approval from the Institutional Research Ethics Committee of Anhui Provincial Hospital were obtained (Approval No. 20160167).
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	The synovial fluid was obtained from 4 patients with gout and knee effusions. To use these clinical materials for research purposes, prior patients' written informed consents. Human data experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA