Small molecule inhibitors of Dishevelled-CXXC5 interaction are new drug candidates for bone anabolic osteoporosis therapy

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(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 04 September 2015

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

Although all Reviewers are globally positive on your manuscript, Reviewers 2 and 3 do raise some issues that require further action. I will not dwell into much detail, but I would like to highlight the main points.

Reviewer 2 is satisfied that the main conclusions are quite well supported by the experimental data, but does feel that further histomorphometric data to demonstrate how much new osteoblast surface is generated following treatment is required to lend more conclusive support to your conclusions. This Reviewer also suggests a profiling approach to gain further insight into the potential pathways being triggered by the compounds.

Reviewer 3 suggests that KY-02327 has the potential to inhibit estrogen production thereby suppressing bone mass and would thus like you to verify blood estrogen levels after treatment. S/he also notes the discrepancy between its efficacy in vitro and in vivo and would like to attempt to clarify the reasons for this.

I would like to add that I personally concur with Reviewer 2's (and in part also Reviewer 3's) suggestion to improve English usage, clarity and readability for readers unfamiliar with both areas covered in the manuscript (bone regulation and Wnt signaling).

In conclusion, while publication of the paper cannot be considered at this stage, we would be
pleased to consider a revised version of your manuscript with the understanding that the Reviewers' concerns must be addressed with additional experimental data where appropriate and that acceptance of the manuscript will entail a second round of review.

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

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

The authors have developed a small molecule inhibitor of the interaction between disheveled-CXXC5. Using a combination of in vitro, cell culture and in vivo experiments they have demonstrated that they can increase osteoblast differentiation and bone formation. Importantly, they have used an in vivo bone loss model (ovariectomy) to demonstrate that the small molecule inhibitor can be given orally to increase bone formation. The inhibitor is at least as active as recombinant PTH.

It is my opinion that this is an important advance in the treatment of bone loss/osteoporosis. There is currently no oral therapy available increase of bone formation. Currently available oral drugs inhibit bone resorption and do not increase bone formation.

Referee #1 (Remarks):

This is a well done study that demonstrates the development and utility of an orally administered agent that increases bone formation. The reviewer has no objections to any of the methods used, the statistical analysis thereof and the conclusions drawn by the authors.

Referee #2 (Comments on Novelty/Model System):

The authors have used a variety of techniques to examine the ability of small molecules to disrupt the interaction of disheveled protein and a disheveled binding protein (CXXC5) on the Wnt/catenin signaling pathway and ultimately bone formation in several in vitro and in vivo models. The fact that the authors have found small molecules that appear to work through the release of the negative regulation Wnt/catenin signaling is impressive although the structure of the lead molecule might render it unstable.

While the authors have demonstrated an increase in bone volume, I would want to also see bone histomorphometric data on the amount of active osteoblast surface in the bones of animals treated with the compounds - this would provide further evidence to support the comments made in the manuscript concerning the linkage between activation of the Wnt/catenin pathway, osteoblast recruitment and osteoblast activity (i.e., bone formation). The narrative in the introduction and additional sections needs some attention to help the reader (expert or non-specialist) understand the subject matter and the context for the study. The use of an array of techniques - including a molecular structure/function examination - may make the paper eligible for publication in EMBO Mol Med.

Referee #2 (Remarks):

Interesting paper that uses a variety of in vitro and in vivo models to assess the ability of small molecules to disrupt a protein/protein interaction that negatively regulates the Wnt/catenin pathway (and bone formation). The experiments appear to have been appropriately designed and well controlled. The authors describe a series of results - including a molecular structure/function analysis of the mechanism of disruption by the aforementioned small molecules. The in vivo model data is compelling and I would want to the authors to use bone histomorphometry to show how much new osteoblast surface is created following treatment and how much of that surface would be considered as an active bone formation surface - this would add further support to the comments made in the introduction regarding the impact of the Wnt/catenin signaling on osteoblast activity and bone formation. Additionally, have the authors considered submitting the compounds to a profiling service such as BioSeek or Genometry's L1000 assay to gain further information about
target and off-target engagement of these molecules? That profiling data could add further weight to the specificity of the pathway involvement and potentially give some insight into the toxicity induced by some of the compounds - this would also give another set of important reference information for the primary data.

The paper needs a re-write of certain passages (introduction as an example) to improve the grammar and establish a greater clarity of background context and to define the aims of the study for readers who are familiar with the concepts of bone regulation and turnover but who may not be aware of the nomenclature and terms that describe the individual regulators of Wnt/catenin signaling (i.e., CXXC5 is a disheveled binding protein).

Referee #3 (Comments on Novelty/Model System):

In the ovariectomized mice model the compound KY-02327 has very modest effect in restoring bone loss. Yet the same compound has more profound effect in vitro (Fig 3). This may be may because canonical WNT Signaling has been shown to inhibits FSH (follicle stimulating hormone) mediated steroidogenesis in rodent granulosa cells. This means that compound KY-02327 has potential to inhibit estrogen production and suppress bone mass. Therefore, it would seem logical to estimate blood estrogen concentration in the injected animals and controls.

In vitro cell studies could be improved by looking at the expression of a number of key downstream osteogenic transcripts, such as osterix, collagen and osteocalcin.

Referee #3 (Remarks):

The work presented is an extension of previously published concept but it has obvious translational implications. I highlight some of the main points about the manuscript:

1. Canonical WNT Signaling has been shown to inhibits FSH (follicle stimulating hormone) mediated steroidogenesis in rodent granulosa cells. This means that compound KY-02327 has potential to inhibit estrogen production and suppress bone mass. Therefore, it would seem logical to estimate blood estrogen concentration in the injected animals and controls.

2. In addition to effect on alkaline phosphatase it would be informative to see effect of KY-02327 the on MC3T3E1 cells proliferation and expression of key transcripts, such as collagen 1a and osteocalcin.

3. In the ovariectomized mice KY-02327 has very modest effect in restoring bone loss. Yet the same compound has more profound effect in vitro (Fig 3). Could this be due to explained by in vivo inhibition of steroidogenesis as explained in 1 above?

4. Manuscript length could be reduced by at least one third by avoiding repletion and omitting methods given in the results.

5. The number of replicates of in vitro cell experiments should be given.

RESPONSE TO REVIEWERS

REVIEWER #1
We greatly appreciate the reviewer’s interest and comments on our work.

REVIEWER #2
1. The reviewer requested bone histomorphometry analysis for the in vivo experiments.
As the reviewer requested, we performed bone histomorphometric analyses on KY-02327- or PTH-treated ovariectomized (OVX) mice. We quantified the number of osteoblasts and the calcine double-labelled surface on the bone. Both the number of osteoblasts and the calcine double-labelled surface were increased approximately 2-fold by KY-02327 treatment. This result was similar to that observed with PTH treatment (Alexander et al., J Bone Miner Res. 2001, 16:1665-73). We provided these new data in Fig. 6, B and C and described the results (page 13, line 16-18) in the revised manuscript.

2. The reviewer suggested to evaluate possible off-target effects and toxicity of KY-02327. KY-02327 exhibited a specific inhibitory effect on the Dvl-CXXC5 interaction in vitro, and the KY-02327-Dvl PDZ complex structure was determined, suggesting that KY-02327 may specifically inhibit protein-protein interaction. However, to address the reviewer's concern of off-target effects further, we monitored the transcriptional regulation of target genes of major cell signaling pathways in mouse embryonic fibroblast cells after 12 hours of treatment with KY-02327 (Table S2, Fig. S5 and page 14, line 11-17). KY-02327 significantly increased the mRNA levels of Wnt/b-catenin target genes (Fig. S5A). However, the target genes of other pathways were unaffected by the treatment of KY-02327 (Fig. S5B-J). These results show the specificity of KY-02327 on the Wnt/b-catenin pathway.

We also assessed the possible toxicity of KY-02327 using conventional in vitro tests, including the Ames test, a CYP inhibition assay, and hERG binding assays. KY-02327 did not show significant toxicity in these genetic (Table S3), hepatic (Table S4), and cardiac (Table S5) tests. We have provided these data (Table S3-S4) and described the results in the revised manuscript (page 14, line 18- page 15, line 6).

In addition, we did not observe any significant changes in the weight of OVX mice treated with KY-02327 (Fig. S6A). Organs from drug-treated animals did not show significant histological abnormalities (Fig. S6, B-D and page 15, line 7-10).

Finally, treatment with KY-02327 did not significantly alter the proliferation of various types of normal or tumor cells (Fig. S6E and page 15, line 10-13). Overall, KY-02327 did not cause significant toxicity in vivo or in vitro. However, further chemical synthesis of analogs to develop a compound optimized for pharmacological characteristics and functionality will be required in the process of drug development.

3. The reviewer requested to improve English usage, clarity, and readability for readers unfamiliar with bone mass regulation and Wnt signaling. We have added an extra explanation of bone mass regulation (page 3, line 5-10) and Wnt signaling (page 4, line 16–page 5, line 4). Further, the English usage of the revised manuscript is improved.

REVIEWER #3
1 & 3. The reviewer raised a concern with inhibition of estrogen production by KY-02327 treatment. He or she suggested that the inhibition could be explained by the difference between in vivo and in vitro effects of KY-02327.
Recent studies have shown that both canonical and non-canonical Wnt pathways regulate granulosa cells and steroidogenesis in the ovary (Abedini et al., 2015; Stapp et al., 2014). In our OVX mouse experiments, however, we completely removed both ovaries, meaning that no granulosa cell existed and no estrogen production occurred in these animals. Therefore, Wnt-mediated regulation of estrogen production would have no effect on our animal experiments. However, we will carefully consider this point in the development of KY-02327 and its derivatives as a human therapeutic agent.

2. The reviewer requested to show the effects of KY-02327 on proliferation and differentiation of MC3T3E1 cells.
The effect of KY-02327 on proliferation of MC3T3E1 was monitored by MTT assay. The results showed that KY-02327 did not significantly affect the proliferation of MC3T3E1 cells (Fig. S6E). Additionally, the proliferation of murine primary osteoblasts (OB) was unaffected by KY-02327 treatment (Fig. S6E). Meanwhile, quantitative real-time PCR analyses on KY-02327-treated MC3T3E1 cells showed that mRNA levels of collagen 1a (Col1a) and osteocalcin (OCN) increased
after KY-02327 treatment in a dose-dependent manner (Fig. 5, D and E). These results are described in the revised manuscript (page 13, line 6-10).

4. The reviewer suggested to reduce the manuscript length by avoiding repletion and omitting methods given in Results.
As the reviewer requested, we have edited our manuscript to make it more concise.

5. The reviewer pointed that the numbers of replicates of cell experiments should be given.
As the reviewer requested, we have provided the replicate numbers for all cell experiments in the figure legends of the revised manuscript.

References

2nd Editorial Decision

Dear Prof. Choi,

Thank you for the submission of your manuscript to EMBO Molecular Medicine and please accept our apologies for the unusual delay, due also to the concomitant holiday season.

We have now heard back from the two Reviewers whom we asked to evaluate your manuscript.

As you will see the Reviewers are now satisfied with your manuscript and I am thus prepared to accept your manuscript for publication pending my following editorial requests:

1) Please note the comment by Reviewer 2. I agree with him/her that the manuscript needs some work to improve delivery, comprehension and impact of your work. Your manuscript features much valuable data including structural biology and should read as a comprehensive analysis of molecular structure function together with the molecules, but it is indeed lacking cohesion and fluency. Some transition statements in the narrative to link the various approaches in a more cohesive manner would enormously help.

2) As per our Author Guidelines, the description of all reported data that includes statistical testing must state the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the actual P value for each test (not merely 'significant' or 'P < 0.05').

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

Referee #2 (Remarks):
Please seek out the help of a medical writer who can review and join together more effectively the narrative in the paper. Science is strong and experiments have been well executed but it needs some work to make it flow a lot easier for the reader.

Referee #3 (Comments on Novelty/Model System):
The authors have addressed the points I raised and the manuscript is now in an acceptable form.
EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

Corresponding Author Name: Kang-Yul Choi
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2015-05724

Reporting Checklist for Life Sciences Articles (Rev. July 2015)

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 best laboratory practice and are presented to reflect the results of the experiment 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 labelled 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 authorship 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 (e.g., 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/changed/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 measure[s]: common tests, such as t-test (please specify whether paired or unpaired), simple t-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.e.m.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to use a specific subsection in the methods section for statistics, nongen, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (not applicable). Please fill out these boxes. (Do not worry if you cannot see all your text once you press return)

B. Statistics and general methods

1. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

In vitro and in vivo experiments were performed three times independently. (Fig 1B, 1D, 1F 2A, 2C, 2E, 3A, 3B, 3D, 4A, 4B, 4F, 4G, 5G). Four mice for each treatment group were used for all animal experiments. (Fig 6). Sample size is denoted on each figure legend.

2. For animal studies, include a statement about sample size estimate even if no statistical methods were used.

Sample size of animal experiment is denoted in the figure legend of Fig 6. A statement about sample size estimate is provided in Materials and Methods section (page 26).

3. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?

We didn’t apply any pre-established criteria for inclusion/exclusion of samples during sample analyses.

4. 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.

All the mice were randomly assigned to each experimental groups.

5. For animal studies, include a statement about randomisation even if no randomisation was used.

The statement is provided in Materials and Methods section (page 27).

6. Were any steps taken to minimize the effects of subjective bias during group allocation of and/or when assessing results (e.g., blinding of the investigator)? If yes, please describe.

The investigators were blinded to the group allocation during the treatments and experiments.

7. For animal studies, include a statement about blinding if blinding was done.

The statement is provided in Materials and Methods section (page 29).

8. For every figure, are statistical tests justified as appropriate?

Yes.

9. In the data file, do the assumptions of the tests (e.g., normal distribution) describe which methods used to assess it?

For the evaluation of homogeneous samples, t-tests or non-parametric tests were performed under the assumption that all groups show normal distributions. For the animal experiments, normally each group is assessed by SPSS Descriptive Statistics analysis tool.

10. Is there an estimate of variation within each group of data?

Variance of the data within each group of data is assessed by calculating standard deviations.

11. Is the variance similar between the groups that are being statistically compared?

Homogeneity of variance among groups are assessed by SPSS’s Descriptive Statistics analysis tool.

C. Reagents

- The information can be located.

- Every question should be answered.

- If the question is not relevant to your research, please write NA (not applicable).
D. Animal Models

1. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

2. For experiments involving the collaborators, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

3. We recommend consulting the ARRIVE guidelines (see link list at top right) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. Please confirm compliance.

4. For experiments involving the collaborators, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

5. Identification of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

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.

7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

8. The antibody information is provided in Materials and Methods section (page 20).

9. The information on cell lines are described in Materials and Methods section (page 10).

E. Human Subjects

1. Identify the committee(s) approving the study protocol.

2. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WHIRI Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

3. For publication of patient photos, include a statement confirming that consent to publish was obtained.

4. Report any restrictions on the availability (and/or on the use) of human data or samples.

5. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

F. Data Accessibility

1. Provide accession codes for deposited data. See author guidelines, under '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-genomic data
   e. Proteomics and molecular interactions.

   Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal’s data policy. If not 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 FigureNote (see link list at top right).

2. Access to human clinical and genomic datasets should be provided with all the 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 EGA or EGA or equivalent, where applicable.

3. 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.

G. Dual use research of concern

1. Could your study fall under dual use research? Please check biosecurity guidelines (see link list at top right) and list of select agents and toxins (GPRN-CSS) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.