Author's response to reviews

Title: A novel 11beta-hydroxysteroid dehydrogenase type1 inhibitor CNX-010-49 improves hyperglycemia, lipid profile and reduces body weight in diet induced obese C57B6/J mice with a potential to provide cardio protective benefits

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Author's response to reviews: see over
Dear Editor,

We are submitting the revised version of our manuscript number MS: 2003182285120792. All the necessary changes that are raised by the reviewers have been incorporated (dated 12th June 2014). Also please find below where we have explained the changes that have been addressed in the revised manuscript in detail.

Regards

M.R. Jagannath
Chief Scientific Officer
Major compulsory revisions:

1. The authors did not measure of CNX-010-049 concentration in the blood plasma.

   (a) How did the authors arrive at the 30 mg/kg bid dose (60 mg/kg/d total) for the DIO mouse study?

   Pharmacokinetic profile at 15mg has provided a Cmax of ~450ng. Predicted Cmax at 30mg could be ~1000ng. By considering it plasma protein binding (~90%), we expect free drug concentration in the plasma will be ~100ng (~250nM). The IC50 of CNX-010-49 for mouse isoform is 64nM; we expected 30mg dose should be optimal. This was further confirmed from the ex vivo inhibition study which is presented as Table 1 in the manuscript.

   (b) How did the authors determine at a T1/2 of 7 hours (stated in Discussion, page 21, paragraph 2) without measuring plasma CNX-010-049?

   Pharmacokinetic profile has been performed in Swiss albino mice for CNX-010-49. Please find the PK profile for your reference only. T1/2 of 7 hours mentioned in the paper is from this study.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Route</th>
<th>Kel (1/hr)</th>
<th>Half life (hr)</th>
<th>C_{e}/C_{nov} (ng/ml)</th>
<th>AUC_{ivf} (hr*ng/mL)</th>
<th>Vd_{ss} (ml/kg)</th>
<th>Cl (ml/hr/kg)</th>
<th>T_{max} (hr)</th>
<th>AUC_{0-24h} (hr*ng/mL)</th>
<th>MRT_{last} (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNX-010-49</td>
<td>Intravenous (1 mg/kg)</td>
<td>0.07</td>
<td>0.94</td>
<td>1247</td>
<td>255</td>
<td>3945</td>
<td>-</td>
<td>71</td>
<td>545</td>
<td>0.29</td>
</tr>
<tr>
<td>CNX-010-49</td>
<td>Oral (15 mg/kg)</td>
<td>0.110</td>
<td>7.4</td>
<td>446</td>
<td>877</td>
<td>-</td>
<td>0.25</td>
<td>545</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2. The authors do not measure plasma or tissue cortisol levels in their 10 week in vivo study. This is a pivotal component to provide evidence that their molecule is actually functioning as they intend it to in vivo, and as a way to explain all of their their other findings. The rationale for not measuring cortisol levels in these animals should be included in the discussion.

Since there are many reports available where, they have demonstrated elevated tissue specific glucocorticoids from both T2DM humans and rodents [references in the paper are 13, 18, 19, 20] including transgenic models. Now we have included this information under the discussion part: Page number21 –Paragraph 2–line number7-9.

3. The data presented in Table 1 is potentially very important to provide evidence that orally administered CNX reaches target tissues at sufficient concentrations to inhibit 11B-HSD1 function. It is unclear how many animals were used in this single dose study. Details of the
actual ex vivo experiment are sparse. There is also no standard error of the means reported in the table.

We have modified the ex vivo experiment methodology along with the number of animals used in the study (n=4 per time point). We have now represented the % inhibition along with the SEM (Table 1) in the revised manuscript.

4. There are several issues with the reported statistical analysis of the data. (a) For all histograms, it is unclear whether the significance stars represent significant differences from untreated controls or differences between ‘+’ CNX treated groups and ‘−’ CNX treated groups. The most meaningful comparisons would be between the ‘+’ and ‘−’ CNX treated groups.

As suggested, we have modified the significance representation for all the histograms in the revised manuscript.

(b) In experiments with 3 separate treatment groups, the appropriate statistical analysis would be 1-way ANOVA followed by an appropriate post-hoc test rather than individual Student’s t-tests between untreated controls vs. the + and − CNX treatment groups.

For all the in vivo experiments which have 3 separate groups, we have used one-way ANOVA followed by Dunnett’s test. Student’s t-test was used for the cell based experiments. In fact one-way ANOVA also showed a similar statistical significance in the cell based experiments. Now we have mentioned that one-way ANOVA followed by Dunnett’s test is used for all the statistical analysis in the revised manuscript.

(c) Fig 5A, B:
   (i) Please state the statistical analysis used for comparison of serum glucose levels between the treatment groups at the various time points.
   (ii) It is unclear what the stars represent.
   (iii) The # symbol is not defined

Statistical analysis used for serum glucose is one-way ANOVA followed by Dunnett’s test. We have represented the symbols according to the treatment group in the legends of revised manuscript.

(d) Fig 6: Please state the statistical analysis used for 6E. 1 way ANOVA followed by Dunnett’s test does not appear to be appropriate.
Every respective time point comparison between the groups was carried out independently. Hence in figure 6E, we used one-way ANOVA followed by Dunnett’s test for the statistical analysis.

(e) Fig 7A, 8A:
(i) Please state statistical analysis used. 1 way ANOVA followed by Dunnett’s test does not appear to be appropriate.

Every respective time point comparison between the groups was carried out independently. Hence we used one-way ANOVA followed by Dunnett’s test for the statistical analysis.

(ii) The # symbol is not defined

We have represented the symbols according to the treatment group in the legends of revised manuscript.

**Minor compulsory revisions:**

1. It would be instructive if the authors reported the molecular weight of CNX-010-049. As recommended, we have mentioned the molecular weight of CNX-010-49 (MW=408.5) under introduction - Page number 7–Paragraph 3 –line number 6 in the revised manuscript.

2. Figure 1E – G: it is unclear what concentration of CNX-010-49 was used in the experiments that these histograms are representing.

   We have mentioned the concentration of CNX-010-49 (100µM) used in the Figure E-G under figure legend 1. Also the concentration is mentioned under the result section.

3. Numbers of animals or numbers of replicate experiments should be described in all figure legends.

   We have mentioned the number of animals or the replicates used under each figure legend.

4. What was the composition of the vehicle in the in vivo study?
We have used 0.5% Carboxy methyl cellulose as a vehicle to dose CNX-010-49 in our in vivo study. This is mentioned under materials and methods section - Page number 11–Paragraph 1–line number 1 in the revised manuscript.

5. Please provide more detail on what the specific bases for determining which treatment groups HFD animals were assigned to in the in vivo study.

After 11 weeks of diet intervention (High Fat Diet), the HFD fed animals were randomized / assigned to either vehicle (HFD control) or CNX-01-49 treatment groups based upon body weight, glucose AUC during OGTT and fasting blood glucose. This is mentioned under materials and methods section - Page number 11–Paragraph 1–line number 1-3 in the revised manuscript.

**Additional Minor points:**

6. Pg. 5, paragraph 2: minerallocorticoid has two l’s in it.

Suggested changes have been made in the revised manuscript

7. Pg. 6: paragraph 2: Increased ALT levels are used as a biomarker of hepatic injury – not clear what the point of mentioning it in that paragraph is.

This sentence is removed in the revised manuscript

8. Pg. 6: paragraph 4: “Till date” – should be “to date”.

Suggested changes have been made in the revised manuscript

9. Pg. 7: paragraph 2: “One can attribute this lack of unpredictable efficacy...” this sentence is difficult to interpret and is a major sentence for explanation of the rationale for the development of their molecule.

The sentence has been modified in the revised manuscript. Introduction -Page number7–Paragraph 2–line number 3-5 in the revised manuscript.
10. Pg. 7; paragraph 3: “...we screened and selected the compounds that have shown more reductase activity than dehydrogenase activity...”. I think that the intention was to say that the selected compounds that have shown more inhibition of reductase than dehydrogenase function

Suggested changes have been made in the revised manuscript

11. pg. 8 para. 2: (methods) “...2 days of post confluence cells... were treated cell culture media”

We have modified the sentence under method section. Materials and methods-Page number8–Paragraph 2–line number 3 in the revised manuscript.

12. Discussion: pg. 22, paragraph 3: “...triglyceride levels were very high in DIO mice which were significantly reduced upon 11B-HSD1 by CNX-010-049 which substantiate the role”. This sentence needs to be revised.

We have now revised the sentences under discussion part. Discussion -Page number22–Paragraph 2–line number 4-5 in the revised manuscript.

13. Include scale bar in fig 8C.

Scale bar has been included in fig 8C

GENERAL REVIEW POINTS

Results
1. Figure 1: this is good to show that the mouse is a relevant species
>IC50 at human 11BHSD1 = 6 nM
>IC50 at mouse 11BHSD1 = 64 nM
>No effect on reduction of dehydrogenase activity
-what are the relative levels of dehydrogenase activity of the enzyme?

11B-HSD1 is a bidirectional enzyme which can act as both a reductase (activating glucocorticoids) and a dehydrogenase (inactivating glucocorticoids). In conditions of excess NADPH, as seen in metabolic overload; 11B-HSD1 functions as a reductase and generates active cortisol while in situations of low levels of NADPH (higher NADP+) it functions as a dehydrogenase and generates inactive cortisone. One would expect dehydrogenase activity
under stress conditions or low glucose levels. This information is present under introduction - Page number 2–Paragraph 2–line number 7-11 in the revised manuscript.

Table 1: They show inhibition of 11-BHSD1 in liver, skeletal muscle, and adipose tissue (38 – 58% vs. control) at time points at 1 and 7 hr after single 30 mg/kg dose.
> No effects observed at 24 hr.

Based on the pharmacokinetics profile data, we did not observe any compound after 10h. Hence there shall be no inhibition after 10h and this is one of the reasons for twice a dosing in our animal experiments.

Figure 2
> They show that the presence of CNX inhibits LPCF evoked gluconeogenesis (production of glucose) in mouse hepatocytes. CNX also inhibits expression of gluconeogenic markers G6PC and PEPCK.
> Potential issue: they demonstrate that the IC50 for mouse 11-BHSD1 inhibition is 64 nM – but they are using 1 uM CNX in these experiments. 15-fold the IC50.
> Wrong statistical assessment (see major points above)
> How does inhibiting 11-BHSD1 influence gluconeogenic activity in mouse hepatocytes? A brief description of the thinking behind why they did this experiment would be helpful.

We agree with reviewer that 1 uM CNX is in excess (15-fold the IC50). By considering the cell permeability and the plasma protein binding, we have used 1µM of CNX-010-49 for all the cell based assays. (We used 10% serum in the cell culture media)

For all the cell based assays, we have used Student’s t-test to assess statistical significance. In fact one-way ANNOVA also showed a similar statistical significance. Now we have mentioned that one-way ANOVA followed by Dunnett’s test is used for all the statistical analysis in the revised manuscript.

It is well established in the literature where 11β-HSD1 controls expression of key gluconeogenic genes PEPCK and G6PC and hence the fasting glucose. We have mentioned the relevant reference in the manuscript [references 39, 40].

Figure 3
Result: They show that CNX inhibits GPCI evoked expression of PDK4, and TRIM63. Also restores mitochondrial copy number?
> Potential issues: Same issue with the 1 uM dose as in Fig. 2
> What is relevance of PDK4?
> Same issue with the stars as in fig 2 (which thing is significantly different from which?)
> Very small error bars: provide the number of replicate experiments.
By considering the cell permeability and the plasma protein binding, we have used higher concentration of CNX-010-49 for all the cell based assays. (We used 10% serum in the cell culture media)

Glucocorticoid receptors are known to transcribe PDK4 and TRIM63 genes in skeletal muscle cells. The relevant references are mentioned in the manuscript [references 42, 43, 44].

We have modified the representation of significance in the revised manuscript along with the replicates (n=6).

Figure 4 a, c, d
Result: CNX inhibits adipocyte differentiation and hypertrophy. Also inhibits isoproteneol + cortisone induced adipocyte lipolysis

>Potential issues:
Statistical analysis should be 1 way ANOVA with appropriate statistical test. Describe whether the ‘treatment groups’ are different from each other.
Very small error bars: provide the number of replicate experiments.
>Fig. 4a: what is CDM?

For all the cell based assays, we have used Student’s t-test to assess statistical significance. In fact one-way ANNOVA also showed a similar statistical significance. Now we have mentioned that one-way ANOVA followed by Dunnett’s test is used for all the statistical analysis in the revised manuscript.

We have observed very small standard error of means in the entire cell based experiments where we have used 6 replicates for each treatment group.
CDM corresponds to complete differentiating media and now we have mentioned in the methods as well as in the figure legend.

Figure 5:
Results: Fasting glucose levels are decreased in High fat diet CNX treated animals compared to non-CNX treatd HFD animals starting at ~8 weeks of treatment.
Also, at the end of the treatment period, fasting glycerol and Free fatty acid concentration levels were decreased in CNX treated animals.

Potential issues:
Panel A: what was the statistical analysis done in this figure? 1 way ANOVA with Dunnett’s post test would be correct for C and D, but not A and B (perhaps 2 way repeated measures ANOVA?).
Panel B: symbols include ‘#’. Unclear what this means

Since every respective time point comparison between the groups was carried out independently, we used one-way ANOVA followed by Dunnett’s test for the statistical analysis.
Yes, we have used one-way ANOVA for figure C-D
We have represented the symbols according to the treatment group in the legends of revised manuscript.

Figure 6:
Results: HFD fed animals have increased fasting and fed insulin levels. CNX treated have a smaller increase in insulin.
Potential issues:
Very difficult to read the symbols in panel E.
Statistical analysis for A and B should be 1 way ANOVA.
Statistical analysis for 6E? Student’s t-test could perhaps be used in 6 F and G.
Relevant statistical comparison is between the 2 right-most bars.

HFD control animals are hyperinsulinemic which an indicator of insulin resistance. Compared to HFD control, CNX-010-49 reduced the hyperinsulinemia by improving insulin sensitivity.
Symbols are modified so that one can appreciate.

Yes, we have used one-way ANNOVA for panel A-B.

Since every respective time point comparison between groups was carried out independently, we used one-way ANOVA followed by Dunnett’s test for the statistical analysis.

For figure 6 F-G was with Student’s t-test. In fact one-way ANNOVA also showed a similar statistical significance. Now we have mentioned that one-way ANOVA followed by Dunnett’s test is used for all the statistical analysis in the revised manuscript.

Figure 7:
Results: Serum TG levels increased in HFD fed animals. CNX treated animals had a smaller increase in serum triglycerides
>Figure 7B: there is no effect of CNX on liver TG levels. Shows that reduced plasma Triglycerides is not due to accumulation in liver
Potential issues:
>Explain statistical analysis used in panel A

Since every respective time point comparison between the groups was carried out independently, we used one-way ANOVA followed by Dunnett’s test for the statistical analysis in panel A.

Figure 8:
Results: CNX increases brown fat phenotype and thermogenesis
CNX decreases adipocyte size
CNX also has decreased circulating leptin levels at the end of the treatment period.
Potential issues:
> Explain statistical analysis used in panel A
> Explain what the # symbol means in panel A, D, and E
> Fig. 8B: what are the symbols representing?

Scale bar in fig 8C

Since every respective time point comparison between the groups was carried out independently, we used one-way ANOVA followed by Dunnett’s test for the statistical analysis in Figure A.

We have represented the symbols according to the treatment group in the legends of revised manuscript.

Symbols are incorporated in the figure 8B.

Scale bar has been included in fig 8C

Figure 9:
Cardiovascular risk factors?
> They need to further describe the relevance of the biomarkers used.
IL-6 and PAI-1, and Fetuin A.

Potential issues
> Panels A-C: should use 1 way ANOVA with this data
> What do stars mean. Comparison should be between the two right-most bars

For figure 9 A-C was with Student’s t-test. In fact one-way ANOVA also showed a similar statistical significance. We have changed this in the figure legend.

We have changed the representation in the revised manuscript.

The relevance of each biomarker has been mentioned briefly in the discussion part and the relevant references were also given [references 54-59, last paragraph under discussion].