FXR agonism protects against liver injury in a rat model of intestinal failure-associated liver disease

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Handling editor:

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Review timeline:

1st editorial decision
Date:29-Aug-2017

Ref.: Ms. No. JCTRes-D-17-00009
FXR agonism protects against liver injury in a rat model of intestinal failure-associated liver disease
Journal of Clinical and Translational Research

Dear authors,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you resubmit your work.

Your revision is due by Sep 28, 2017.

To submit a revision, go to http://jctres.edmgr.com/ and log in as an Author. You will see a menu item called Submission Needing Revision. You will find your submission record there.

Yours sincerely,

Rowan van Golen
Associate Editor
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1: In this study, the group tested that intact enterohepatic circulation is critical in recovering liver functions. To study consequences of chronic loss of bile, rats underwent external biliary drainage (EBD) or sham surgery for seven days, and the prophylactic potential of the FXR agonist INT-747 was assessed.

Some concerns are raised about this study:
1. Fig 1 and 2. If FXR activation is known to suppress inflammation and bile acid production, why did the Int747 treated sham mice show higher inflammation index and then the EBD mice show higher or unchanged IL-6 expression?
2. Fig4. Why did not Int747 treatment induce classical FXR target genes, including Bsep and Shp and suppress Cyp7a1 in the liver? Why was C4 not altered in vehicle treated EBD mice?
3. If FXR is the key, then FXR deficiency should be used in the animal model to prove that FXR is the mediator for the liver failure following loss of enterohepatic circulation of bile acids.

Reviewer #2: The manuscript presents a study on the effect of an FXR synthetic agonist, INT-747, on liver pathology markers and FXR-Fgf15 axis in an animal model of intestinal failure-associated liver disease (IFALD). The hepatoprotection effect of FXR agonists like GW4064 was previously described in rat models of hepatic cholestasis, e.g. Liu et al., J. Clin. Invest.(2003) 112:1678. The novelty of this project is to apply the hepatic protective features of FXR activators for the treatment of hepatic cholestasis symptoms associated with intestinal disease.
The study subject is interesting and potentially applicable for medical purposes, and the results showing the positive effects of INT-747 in reducing the serum biochemistry markers of hepatic injury due to intestinal failure are convincing. However, the manuscript comes short in characterizing the signaling mechanism that underlines these effects. Some suggestions include: repeat of some experiments for which the data showed the right trend but there was no significance found, with a larger number of animals to resolve the problem of data significance; ii) solving other major concerns which are listed below.

Major concerns:
- The plots presented for estimation of inflammation and fibrosis in Fig. 1 need to be supported by representative pictures which had been used for scoring. Also the IHC images of CK19-stained cholangiocytes in Fig. 1 have very low resolution, need to be replaced with better quality pictures.
- In Fig 2B, in the first bar plot for hepatic IL-6 mRNA expression, the white bars of sham + veh and sham + INT-747 are missing; usually mRNA fold change is calculated by RT-qPCR based

on ratios of various treatments normalized to a control, like the level of IL-6 mRNA in this case in sham + veh, which would be considered 1 after being compared to the expression of a housekeeping gene . Lower or higher than 1 values of the control (sham+veh) in mRNA expression plots indicate possible wrong calculations. Please explain or make corrections. The same problem in 2A plot for Mrp3.

- In Fig. 4C, because there are obvious variations in the amount of the protein loaded per lane, please provide the quantification of the blot in order to assess the effect of INT-747 on Cyp7a1 protein expression.

- Under Results, on lines 249-250, the authors note that despite clear effects of INT-747 on FXR activation, the gene expression of the tested transporters was not affected. The expression of any gene can be regulated at mRNA, protein or functional level, for example a gene which is highly expressed at mRNA level may be up or down regulated at protein level by decreased or increased ubiquitination and degradation of the protein in proteosome; another level of regulation may be at functional level, i.e. a protein may be highly expressed but post-translationally modified (by phosphorylation, acetylation, etc.) so that its function may be modulated. For this paper, at least a western blot analysis of the studied genes needs to be done to determine whether the FXR agonist affects the expression at protein level, for Mrp2, Mrp3.

- The comments in the Results, on lines 284-287 (that despite the clear effects of INT-747 on serum biochemistry and histopathology tests, FXR activation by INT-747 had no significant effect on FXR-FgF15 axis), are opposite to the statement in the Discussion section, on lines 293-298, concluding that the FXR activator INT-747 appears to re-instate normal feedback regulation of BA synthesis via the intestinal FXR/Fgf15 axis. Please explain or make corrections, according to the results. Since the data demonstrate that FgF15, Cyp7a1 and Shp, the major target genes of FXR, are reversed to the almost normal level in sham-operated animals treated with vehicle, at mRNA level, one should conclude that that the treatment with the FXR activator contributes to the re-instating of FXR-Fgf15 axis. The data can be improved to significance by adding a number of experimental animals.

Minor cocerns
- Quantification of fibrosis as detected with Sirius Red staining of liver sections, should be performed by image analysis with ImageJ instead of scoring on a scale from 0 to 4.
- In Fig. 2A, add Y-axis title.
- In Fig. 4A and B, add Y-axis title.

********Authors response********
Dear Editor,

Please find the revised version of our manuscript "FXR agonism protects against liver injury in a rat model of intestinal failure-associated liver disease" (JCTRes-D-17-00009).

We greatly appreciated the opportunity to resubmit our work, and would like to express our gratitude to the Reviewers for their critical feedback and recognizing the potential for clinical translation of our findings. We addressed the comments raised by the Reviewers to the best of our abilities, but were unable to generate new insights into the molecular mechanisms underlying the protective effect of FXR agonism in our IFALD model.

The original experiments were conducted several years ago, and materials sampled at that time have largely been consumed for a multitude of analyses. Remaining sample quantities are insufficient for meaningful analysis (and sound comparisons between groups) of factors put forward by the reviewers. We considered the request for new animal experiments outside the scope of a revision, as it would require obtaining new ethical approval, a process that will require at least 10 months since the instalment of a new Dutch Law on animal experiments has come into effect.

We are convinced that our observations are of interest to the readership of JCTR and scientific community in general. Faced by above limitations, we hope that follow-up experiments will take our initial observations to the next level, address the questions that we cannot answer yet, and pave the way for exploring translatability.

We are looking forward to hearing from you.

Yours sincerely,

Prof. Steven W.M. Olde Damink, MD MSc PhD

We are indebted to both reviewers for their critical and constructive feedback.

Reply to Reviewer #1:

Comment 1: Fig 1 and 2. If FXR activation is known to suppress inflammation and bile acid production, why did the Int747 treated sham mice show higher inflammation index and then the EBD mice show higher or unchanged IL-6 expression?

Reply: Both groups of sham-operated rats (mice were not used in this study) had similar histological inflammation scores. Thus, INT747 does not result in inflammation in rats with an intact enterohepatic circulation, and in fact reduced the inflammation that resulted from continuous external biliary drainage (**Fig. 1B**). As correctly pointed out by the reviewer, this is not reflected by reduced hepatic *Il6* mRNA levels in INT747-treated rats with continuous external biliary drainage.

Lack of obvious (long-lasting) transcriptional effects following INT747 administration (once daily as a bolus) appears to be a general pattern in this study, and may relate to the time interval of 12 hrs between last dosing and sacrifice. INT747 is administered in unconjugated form, and like other hydrophobic bile salts, is postulated to follow a nuclear route after uptake by the liver (ref: http://www.agialpress.com/journals/nurr/2016/101207.). By activating FXR, nuclear INT747 elicits a transcriptional response that aims to prevent bile salt toxicity, amongst others by promoting bile salt conjugation and accordingly aqueous solubility. INT747 is conjugated prior to secretion in bile and undergoes enterohepatic circulation (predominantly in its conjugated form) in the sham-operated animals with intact biliary anatomy. In subsequent rounds of hepatic transit, conjugated OCA is postulated to follow a non-nuclear route for rapid re-secretion in bile. Transcriptional responses elicited by INT747 in the liver may thus be of limited duration, i.e. only during first passage through the liver, in our experimental set-up. Functional consequences of transient FXR activation may persist for a longer period, as reflected in improved inflammatory scores and biliary fluid output (Fig 1B, Fig 4).

Above explanation for apparent transcriptional inertness of INT747 in our model, has been included in the manuscript in line 317 – 333.

Comment 2. Fig 4. Why did not Int747 treatment induce classical FXR target genes, including Bsep and Shp and suppress Cyp7a1 in the liver? Why was C4 not altered in vehicle treated EBD mice?

Reply: This observation is acknowledged by our group and a potential explanation was already discussed above and in the manuscript (line 312 – 314).

The Reviewer pointed out the discrepancy between higher Cyp7a1 protein and unaltered serum C4 levels in vehicle-treated EBD rats (not mice). We were surprised by this finding too. The concept that serum C4 is as a marker for hepatic Cyp7a1 activity was derived from studies in healthy animals and healthy volunteers. Validity of C4 as a marker for bile salt synthesis in conditions that affect the liver is unexplored. It is conceivable that hepatic inflammation encountered in our model influences the uncharted process by which C4 that is formed in the endoplasmic reticulum ends up in the blood compartment. Assay of hepatic Cyp7a1 activity could shed light on this issue, but is not possible with remaining archival materials.

Comment 3. If FXR is the key, then FXR deficiency should be used in the animal model to prove that FXR is the mediator for the liver failure following loss of enterohepatic circulation of bile acids.

Reply: This is an excellent suggestion, and feasible if the study had been performed in mice (where long-term biliary drainage is way more challenging). Although knock-out approaches for rats are increasingly available, we felt that such effort was beyond the scope of a revision.

Reply to Reviewer #2

Major concerns:

Comment 1. The plots presented for estimation of inflammation and fibrosis in Fig. 1 need to be supported by representative pictures which had been used for scoring. Also the IHC images of CK19-stained cholangiocytes in **Fig. 1** have very low resolution, need to be replaced with better quality pictures.

Reply: Representative pictures that served to score inflammation (H&E staining) and bile duct area (CK19 staining) are presented in **Fig 1A**. Fibrosis was scored by the pathologist by microscopic examination of Sirius red stained sections, unfortunately no pictures were recorded at that time. If desired by the Journal, pictures can be made of the original sections and included in Figure 1.

We are aware that the resolution of the CK19 staining in the PDF version of our manuscript is poor, and this is likely due to compression of image files during merging of manuscript files into a single PDF. The original figure panels were submitted in high-resolution quality during manuscript submission.

Comment 2. In Fig 2B, in the first bar plot for hepatic IL-6 mRNA expression, the white bars of sham + veh and sham + INT-747 are missing; usually mRNA fold change is calculated by RT-qPCR based on ratios of various treatments normalized to a control, like the level of IL-6 mRNA in this case in sham + veh, which would be considered 1 after being compared to the expression of a housekeeping gene . Lower or higher than 1 values of the control (sham+veh) in mRNA expression plots indicate possible wrong calculations. Please explain or make corrections. The same problem in 2A plot for Mrp3.

Reply: The comments of the Reviewer regarding the mRNA fold changes of *lL-6* and *Mrp3* has been corrected and recalculated according the median transcript levels of vehicle treated sham rats as controls. Significant differences were similar as in the original manuscript. Note that *Il6* mRNA was almost undetectable in livers of sham-operated rats receiving vehicle, and was marginally expressed in liver of INT747-treated animals undergoing sham surgery. For this reason fold change (relative to median of sham-operated rats receiving vehicle) was not feasible in the initial figure representation.

Comment 3. In Fig. 4C, because there are obvious variations in the amount of the protein loaded per lane, please provide the quantification of the blot in order to assess the effect of INT-747 on Cyp7a1 protein expression.

Reply: For estimation of Cyp7a1 protein quantity, 4-6 liver homogenates per group were
analyzed by immunoblotting. To avoid introduction of bias, samples belonging to the four
distinct groups were loaded in adjacent wells of the PAA gel. A representative part of the blot
immunostained for Cyp7a1, and the corresponding part following reprobing with beta-actin antibodies, are
shown in Fig 1C. The actual semi-quantification of Cyp7a1 protein per group is depicted below. We felt
that there was no added value in including this graph as a panel in Figure 4.
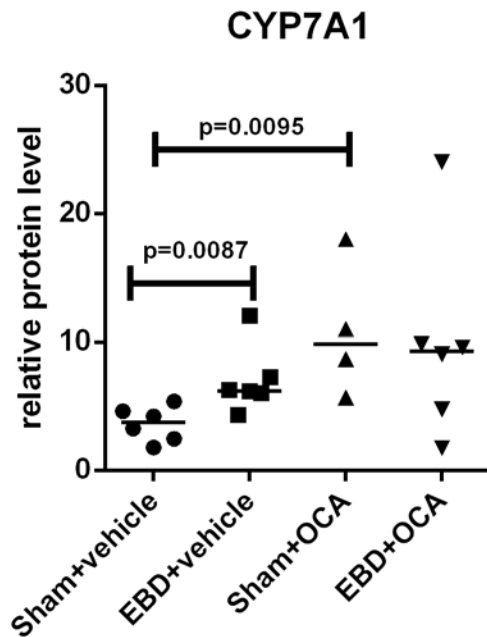
Comment 4. Under Results, on lines 249-250, the authors note that despite clear effects of INT-747 on
FXR activation, the gene expression of the tested transporters was not affected. The expression of any gene
can be regulated at mRNA, protein or functional level, for example a gene which is highly expressed at
mRNA level may be up or down regulated at protein level by decreased or increased ubiquitination and
degradation of the protein in proteosome; another level of regulation may be at functional level, i.e. a protein
may be highly expressed but post-translationally modified (by phosphorylation, acetylation, etc.) so that its
function may be modulated. For this paper, at least a western blot analysis of the studied genes needs to be
done to determine whether the FXR agonist affects the expression at protein level, for Mrp2, Mrp3.
Reply: We agree with the reviewer that there are many levels at which regulation of gene expression/protein
function can take place. Since FXR is a ligand-activated transcription factor, altered expression of FXR-
regulated genes, however, is the obvious read-out for FXR activation. We do acknowledge the general lack
of transcriptional effects following INT747 administration, and as detailed in our reply to reviewer#1, we
propose that is related to the time interval between last dosing and sacrifice (12 hrs), combined with
postulated ineffectivity of enterohepatically circulating INT747 after its first passage across the liver.

Comment 5. The comments in the Results, on lines 284-287 (that despite the clear effects of INT-747 on
serum biochemistry and histopathology tests, FXR activation by INT-747 had no significant effect on FXR-
FgF15 axis), are opposite to the statement in the Discussion section, on lines 293-298, concluding that the
FXR activator INT-747 appears to re-instate normal feedback regulation of BA synthesis via the intestinal
FXR/Fgf15 axis. Please explain or make corrections, according to the results. Since the data demonstrate
that FgF15, Cyp7a1 and Shp, the major target genes of FXR, are reversed to the almost normal level in
sham-operated animals treated with vehicle, at mRNA level, one should conclude that that the treatment

with the FXR activator contributes to the re-instating of FXR-Fgf15 axis. The data can be improved to significance by adding a number of experimental animals.

Reply: In line 284-287 is stated that Fxr agonism had no effect on expression of prototypical Fxr target genes in the liver including *Bsep* and *Shp*. However, in line with the description in the discussion section, line 282-283 explains that INT-747 restored *Fgf15* expression and prevented induction of *Cyp7a1*.

The original study was conducted several years ago, and enlargement of group sizes is no option given inherent differences between experimental conditions (housing, animals, microbial status, chow composition etc.) in the historical and present-day setting.

Minor concerns

Comment 1: Quantification of fibrosis as detected with Sirius Red staining of liver sections, should be performed by image analysis with ImageJ instead of scoring on a scale from 0 to 4.

Reply: Sirius red staining was performed using liver sections counterstained with H&E, interfering with image analysis with ImageJ or other software. Instead, sections were scored by a veterinarian pathologist blinded to experimental group allocation.

Comment 2: In Fig. 2A, add Y-axis title, and In Fig. 4A and B, add Y-axis title.
Thanks for pointing out these omissions. Corrections have been made.

2nd editorial decision
Date: 15-Oct-2017

Ref.: Ms. No. JCTRes-D-17-00009R1
FXR agonism protects against liver injury in a rat model of intestinal failure-associated liver disease
Journal of Clinical and Translational Research

Dear authors,

The resubmitted version of the above-mentioned manuscript has been (re)reviewed by an external referee and the JCTR editorial board. I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

You will receive the proofs of your article shortly, which we kindly ask you to thoroughly review for any errors.

Thank you for submitting your work to JCTR.

Kindest regards,

Rowan van Golen
Associate Editor
Journal of Clinical and Translational Research

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