DAMP/Toll-like Receptor Signalling Attenuation with Mosedipimod: Effect *in vitro* and in Two Animal Models of Nonalcoholic Steatohepatitis (NASH)

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ABSTRACT

Background and Aims: There are no currently approved pharmacotherapies for nonalcoholic steatohepatitis (NASH). There is increasing evidence of an important role of DAMP/PAMP activation of Toll-like receptors (TLRs) in initiation of the fibro-inflammatory cascade in NASH. Attenuating TLR signalling represents an attractive pharmacological strategy to prevent and reverse hepatic fibrosis. The acetylated diacylglycerol 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG, chemical name mosedipimod), is an orally active synthetic mono-acetyl-diglyceride. Mosedipimod has been shown to reduce TLR mediated signalling by accelerating clearance of DAMPs, such as lipopolysaccharide, via IRF3 phosphorylation-induced acceleration of TRAM / TRIF association with TLR4.

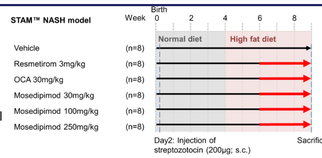
Method: We studied the effects of mosedipimod on molecular, metabolic and histologic facets of NASH in two nutrient-based murine models: a high-fat, high-fructose (HFHF) model of steatosis and inflammation, and the STAM™ model of fibrosing NASH. Effects of mosedipimod on palmitic acid-induced TLR4 signalling were also studied *in vitro*. In the STAM™ model, effects of mosedipimod were compared to two agents in advanced clinical trials, obeticholic acid (OCA) and resmetirom (MGL-3196).

Results: Mosedipimod significantly mitigated HFHF diet-induced hepatic steatosis, reducing lipogenesis-associated signalling, as measured by mRNA expression levels of ChREBP, SREBP-1c and FAS (p<0.05-0.001), indicating attenuation of *de novo* lipogenesis by mosedipimod. Mosedipimod also reduced surface level expression of TLR4 and decreased TNF-α, IL-6 and MIP-2 levels (p<0.05-0.001). Mosedipimod treatment significantly decreased hepatic inflammation (as measured by F4/80), NAFLD activity score (NAS) and fibrosis (Sirius red surface area %, see figure) on endo of treatment liver biopsies (p<0.05-0.001). Mosedipimod demonstrated similar effects to OCA and resmetirom in reducing hepatic steatosis, inflammation, NAS and fibrosis compared with the vehicle group.

Conclusion: Mosedipimod mitigates the HFHF diet-induced hepatic injury and inflammatory cytokine production by modulating DAMP/PAMP/TLR4-dependent signalling pathways. Mosedipimod also prevents the histological and metabolic effects of NASH to a degree comparable to OCA and resmetirom in a widely utilized preclinical model. Taken together these data identify mosedipimod, through a TLR4 signalling dependent mechanism, as a potential therapy for NASH that merits clinical investigation.

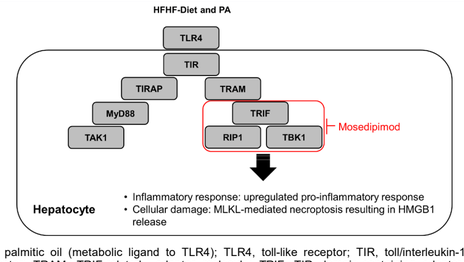
IN VIVO EXPERIMENTAL DESIGN

1. STAM™ Model
- C57BL/6J mice
- Low-dose Streptozotocin (STZ)/high-fat diet-induced NASH model



2. High-Fat High-Fructose (HFHF) Diet-fed Model
- ICR male mice (8-week-old)
- HFHF Diet: Fat 45%,carbohydrate 35%, protein 20%

MECHANISM OF ACTION

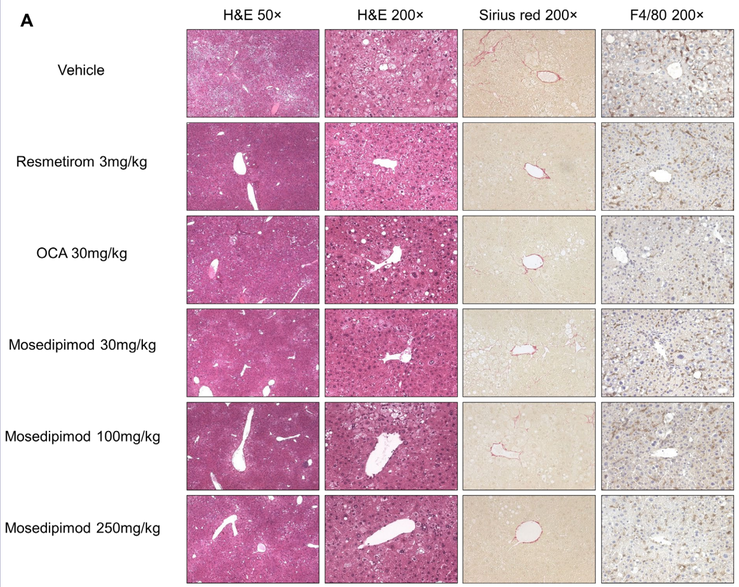


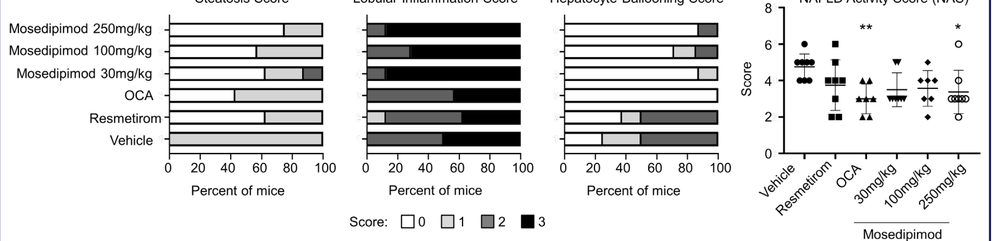
PA, palmitic oil (metabolic ligand to TLR4); TLR4, toll-like receptor; TIR, toll/interleukin-1 receptor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adapter-inducing IFN-β; TIRAP, TIR domain-containing adapter protein; MyD88, myeloid differentiation factor 88; TBK, TANK-binding kinase; RIP1, receptor-interacting protein 1.

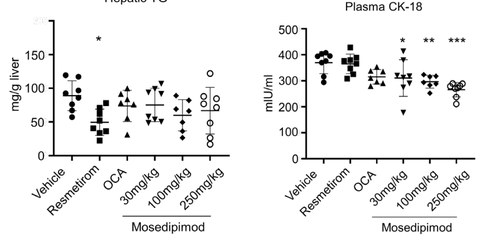
RESULTS

In STAM™ model of NASH (from SMC Lab, Japan)

1. Mosedipimod reduces NAFLD activity score, hepatic fibrosis, macrophage infiltration into liver, and hepatic apoptosis



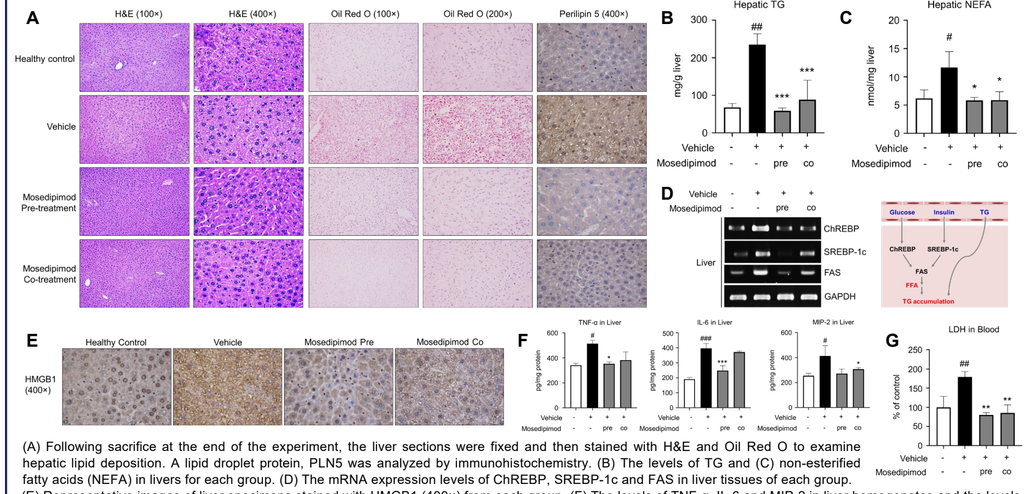




(A) Representative Hematoxylin & Eosin (H&E; 50× and 200×)-, Sirius red (200×)-, and F4/80 (200×)-stained liver sections from each group. (B) Comparison of fibrotic areas and (C) inflamed areas from mouse liver specimens from each group. (D) Individual components of the NAS value, including steatosis, lobular inflammation, and hepatocellular ballooning scores, are shown for each cohort with the total NAS value. (E) The levels of hepatic triglyceride (TG) and (G) the plasma level of cytokeratin-18 (CK-18), a marker of hepatocyte apoptosis, for each group. Data are expressed as mean ± standard deviation (SD). Symbols annotate *P<0.05, **P<0.01, and ***P<0.001 compared to the vehicle group. For NAS, statistical analyses were performed using Dunnett's Multiple Comparison Test. For other data, statistical analyses were performed using Bonferroni Multiple Comparison Test; NAFLD, Non-alcoholic fatty liver disease.

In HFHF Diet-fed mice model (from KRIBB, Korea)

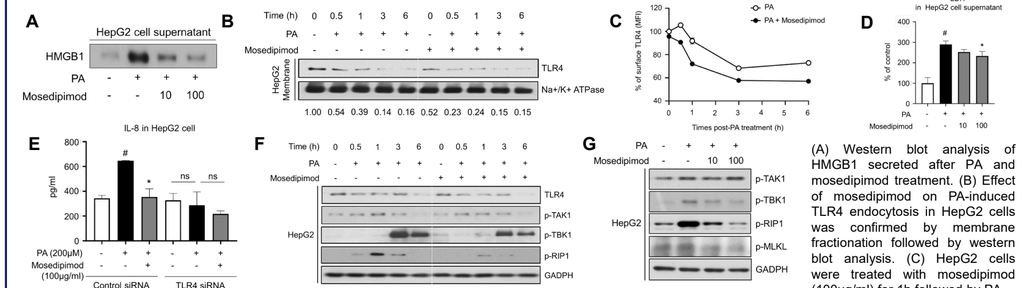
2. Mosedipimod attenuates hepatic steatosis, DAMP, and pro-inflammatory cytokines by attenuating *de novo* lipogenesis in liver



(A) Following sacrifice at the end of the experiment, the liver sections were fixed and then stained with H&E and Oil Red O to examine hepatic lipid deposition. A lipid droplet protein, PLN5 was analyzed by immunohistochemistry. (B) The levels of TG and (C) non-esterified fatty acids (NEFA) in livers for each group. (D) The mRNA expression levels of ChREBP, SREBP-1c and FAS in liver tissues of each group. (E) Representative images of liver specimens stained with HMGB1 (400×) from each group. (F) The levels of TNF-α, IL-6 and MIP-2 in liver homogenates and the levels of lactate dehydrogenase (LDH) (G) in plasma samples from each group. Symbols indicate *P<0.05, **P<0.01, and ***P<0.001 compared to the vehicle group.

In Vitro Study Results (from KRIBB, Korea)

3. Mosedipimod mitigates DAMP release through modulation of TLR4/TRIF-mediated pro-inflammatory signaling pathway



(A) Western blot analysis of HMGB1 secreted after PA and mosedipimod treatment. (B) Effect of mosedipimod on PA-induced TLR4 endocytosis in HepG2 cells was confirmed by membrane fractionation followed by western blot analysis. (C) HepG2 cells were treated with mosedipimod (100μg/ml) for 1h followed by PA (200μM) for the indicated time. TLR4 surface staining was measured by flow cytometry and line graphs indicate mean fluorescence intensity (MFI) of TLR4 over time. (D) The levels of lactate dehydrogenase (LDH) secreted after mosedipimod treatment (10, 100μg/ml), treated 1hr before PA (200μM) treatment. (E) Effect of TLR4 gene silencing on PA-induced IL-8 production in HepG2 cells using siRNA specific for TLR4. The levels of IL-8 in the HepG2 supernatants were measured by ELISA after PA and mosedipimod treatment for 24h in TLR4 gene-silenced cells. (F) Effect of mosedipimod on PA-induced phosphorylation of TLR4 downstream signaling molecules. The cells were treated with mosedipimod (100μg/ml) for 1h followed by PA (200μM) for the indicated time and (G) based on mosedipimod's dose-dependent response. Data are expressed as mean ± SD. Symbols represent *P<0.05, **P<0.01, and ***P<0.001 compared to vehicle.

CONCLUSION

- **In various NASH murine models, mosedipimod ameliorates hepatic steatosis, fibrosis, and apoptosis by quickly removing DAMPs.**
- **Mosedipimod significantly reduces NAS value based on key histological parameters, including steatosis and hepatocyte ballooning.**
- **Mosedipimod is as equivalent or better than drug candidates for NASH in Phase 3 Clinical Trials (OCA and resmetirom) in terms of mitigating NASH symptoms (reduced liver fibrosis, plasma CK-18 fragments, and inflammation area).**
- **Mosedipimod regulates TRIF-dependent RIP1 and TBK1 activation and attenuates the TLR4/TRIF signaling-mediated pro-inflammatory cytokine production and DAMP release.**
- **Collectively, mosedipimod may be a potent therapeutic candidate to resolve NASH symptoms and to prevent progression to liver fibrosis.**