INVESTIGATIONAL DRUG CLM-022, A POTENT INHIBITOR OF NLRP3 INFLAMMASOME-MEDIATED PYROPTOSIS, AS A POTENTIAL TREATMENT FOR ACUTE AND CHRONIC INFLAMMATORY LIVER DISEASES

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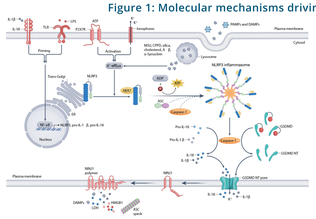
BACKGROUND & AIM

- Inflammation is a common element in the pathogenesis of most chronic liver diseases leading to fibrosis, cirrhosis and liver failure. Due to the close connection with the intestine via the portal circulation, the liver is constantly exposed to assorted gut-derived microbial particles identified as pathogen-associated molecular patterns (PAMPs), which activate resident immune cells. In addition to the intestinal- or virus-derived PAMPs, hepatic innate immune cells are also activated by damage-associated molecular patterns (DAMPs), which are released from injured parenchymal and nonparenchymal cells[1].
- Inflammation is characterized by activation of innate immune cells, production of pro-inflammatory cytokines and pyroptosis which is a lytic form of inflammatory regulated cell death. Pyroptosis is regulated by inflammasomes which are intracellular multiprotein complexes also expressed in both parenchymal and non-parenchymal cells of the liver. In response to cellular danger signals, inflammasomes activate caspase-1, release IL-1β and IL-18[2] (figure 1).

Figure 1: Molecular mechanisms driving NLRP3 inflammasome activation



Legend The canonical NLRP3 pathway involves a two-step process of priming and activation. In the priming step, stimulation of cytokine receptors and pattern recognition receptors (PRRs) results in nuclear factor-κB (NF-κB)-mediated transcriptional upregulation of NLRP3, pro-IL-1β and pro-IL-18. During NLRP3 activation, pathogen-associated molecular patterns (PAMPs) are thought to promote translocation of inactive NLRP3 to subcellular organelles such as the dispersed trans-Golgi network. In response to the activation signal, NLRP3 forms a complex with NEK7 and recruits ATP, apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1 to assemble the NLRP3 inflammasome, which activates caspase-1. Active caspase-1 cleaves pro-IL-1β and pro-IL-18 into their mature forms and also cleaves gasdermin D (GSDMD), allowing GSDMD N-terminal region (GSDMD NT) to form pores in the plasma membrane through which IL-1β, IL-18 and ion fluxes are released.

- **The aim of this work was to investigate the effect of CLM-022, an investigational NLRP3 inhibitor, on inflammasome-driven inflammation and pyroptosis.**
- **To assess the in vitro activity of CLM-022 on inflammasome activation, LPS-primed THP-1 macrophages were stimulated with nigericin. NLRP3 complex assembly, cleaved-gasdermin D production, IL-1β release and pyroptotic cell death were assessed by Western blot, ELISA, ATP & LDH measurement.**
- **For in vivo studies, acute liver failure and inflammation were triggered through galactosamine (GalN) and lipopolysaccharide (LPS) injection in mice.**

METHODS

In vitro Inflammasome induction and inhibition

- THP-1 cells were differenciated with 100ng/mL of PMA during 72H. THP-1 macrophages were primed overnight with 500ng/mL of LPS and activated with 10µM of nigericin. CLM-022, MCC950 (used as pharmacological reference compound for inflammasome inhibition) or vehicule were added 1H before activation with nigericin or post activation 30 minutes after nigericin.
- IL-1β detection was measured with HTRF IL-1 β (Revvity) 2 hours after activation with nigericin. Pyroptotic cell death was induced in THP-1 cells with the same protocol and assessed with CellTiter-Glo and LDH realease was measured in the supernatant through the LDH-Glo cyotoxicity assay (Promega).

Gasdermin analysis

- After inflammasome induction, THP-1 cells were lysed with HEPES-KOH - NP40 Buffer with a cocktail of protease inhibitors. Protein extracts were analysed with a Jess system (Bio-Techne) for GSDMD and cleaved-GSDMD (Cellsignaling #39754 and #36425, respectively) and β-actin for normalisation (Bio-Techne #MAB8929).

ASC complex analysis

- Bone marrow-derived dendritic cells (BMDCs) were treated with LPS (100ng/mL) for 3 hours to induce NLRP3 inflammasome activity, and then CLM-022, MCC950 or vehicule were added for 30 minutes. After, nigericin was added and cells were harvest after 30 minutes. Cells were lysed with HEPES-KOH - NP40 buffer with a cocktail of protease inhibitor and ASC (>48kDa - ADI-905-173-100, Enzo Life Science) oligomerization was assessed with Western blot analysis after pellets treatment with 2mM of disuccinimidyl suberate to induce proteins cross-linking.

CLM-022 p.o. administration of GalN/LPS mice

- C57BL/6 male mice (8-10-week-old) received an i.p. co-injection of D-galactosamine (GalN, 700 mg/kg) and lipopolysaccharide (LPS E. coli, 25 µg/kg) diluted in phosphate buffered saline (PBS). Mice received oral administration with CLM-022 (1 - 3mpk or 10mpk) or vehicle 1h prior to GalN/LPS injection (n=8-10/group). Mice were terminated 5 hours after GalN/LPS injection and blood was collected to measure serum ALT and AST using a Daytona Rx automate (Randox Laboratories), and cytokines were measured by Luminex (Luminex 100/200) for IL-6 and an ELISA for IL-1β (#MLB00C Bio-Techne).

RESULTS

CLM-022 ACTIVITY ON NLRP3 BLOCKS COMPLEX ASSEMBLY, REFLECTED BY LOSS OF ASC OLIGOMERS

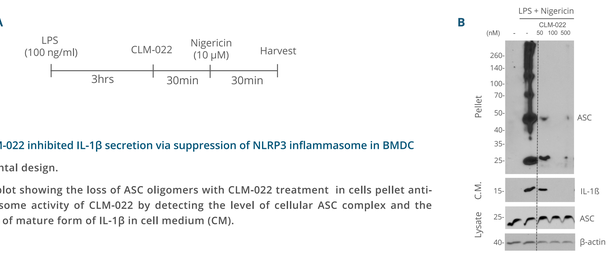


Figure 2: CLM-022 inhibited IL-1β secretion via suppression of NLRP3 inflammasome in BMDC

A. Experimental design.

B. Western blot showing the loss of ASC oligomers with CLM-022 treatment in cells pellet anti-inflammasome activity of CLM-022 by detecting the level of cellular ASC complex and the inhibition of mature form of IL-1β in cell medium (CM).

CLM-022 INHIBITS IL1 B PRODUCTION INDUCED BY INFLAMMASOME ACTIVATION

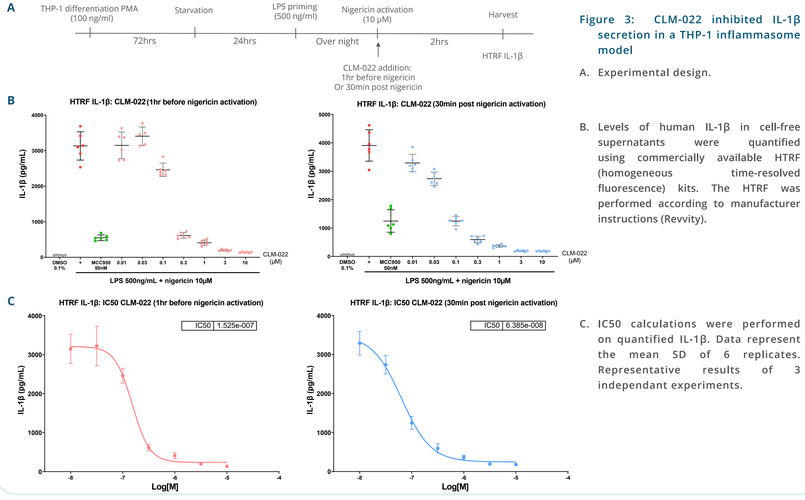


Figure 3: CLM-022 inhibited IL-1β secretion in a THP-1 inflammasome model

A. Experimental design.

B. Levels of human IL-1β in cell-free supernatants were quantified using commercially available HTRF (homogeneous time-resolved fluorescence) kits. The HTRF was performed according to manufacturer instructions (Revvity).

C. IC50 calculations were performed on quantified IL-1β. Data represent the mean SD of 6 replicates. Representative results of 3 independant experiments.

CLM-022 INHIBITS PYROPTOSIS INDUCED BY INFLAMMASOME ACTIVATION

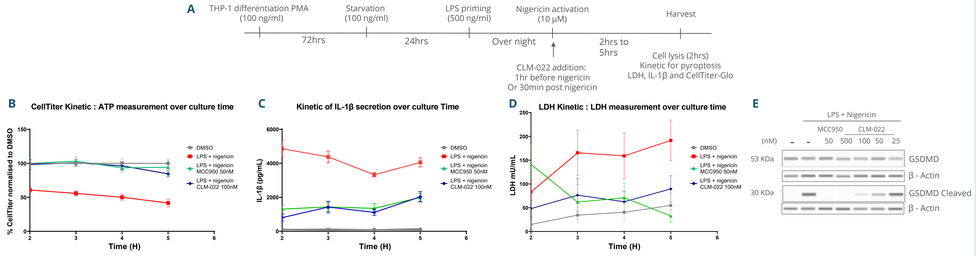


Figure 4: CLM-022 inhibits pyroptosis

A. Experimental design.

B. ATP measurement was measured with CellTiter-Glo luminescent cell viability assay (Promega) directly on treated cells. Results as the mean value ± SD of 6 replicates normalised to DMSO condition.

C. Levels of human IL-1β in cell-free supernatants were quantified using HTRF kits.

D. LDH cytotoxicity measurement kits quantifies cytotoxicity based on the measurement of lactate dehydrogenase (LDH) activity released from damaged cells.

E. Lysate of THP-1 macrophages were loaded at 0,5mg/ml for GSDMD and 0,7mg/mL for cleaved GSDMD. Experiment was conducted throught a Jess system under reducing conditions and using the 12-230kDa separation system.

ORAL ADMINISTRATION OF CLM-022 ALLEVIATES GALN/LPS-INDUCED LIVER INJURY

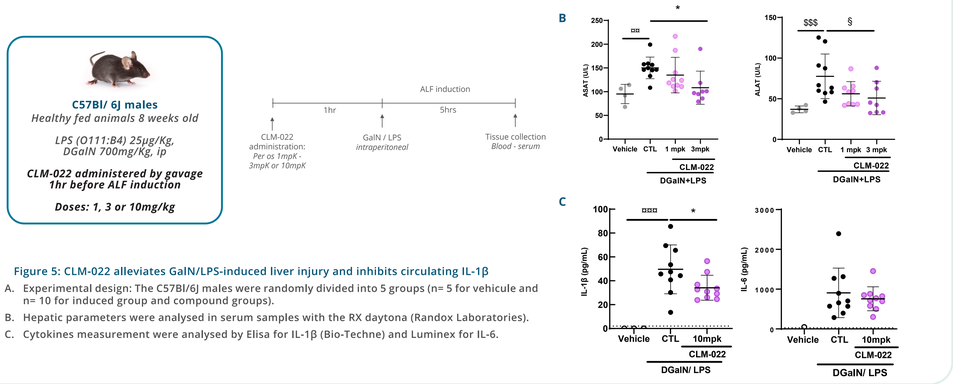


Figure 5: CLM-022 alleviates GalN/LPS-induced liver injury and inhibits circulating IL-1β

A. Experimental design: The C57Bl/6J males were randomly divided into 5 groups (n= 5 for vehicule and n= 10 for induced group and compound groups).

B. Hepatic parameters were analysed in serum samples with the RX daytona (Randox Laboratories).

C. Cytokines measurement were analysed by Elisa for IL-1β (Bio-Techne) and Luminex for IL-6.

CONCLUSION

- **Recent findings have highlighted the potential role of the NLRP3 inflammasome in the pathophysiology of ACLF[3,4], which is consistent with the activities of the NLRP3 inflammasome in transforming a variety of pathogen-associated and damage-associated signals that activate pro-inflammatory caspases, which promote the secretion of IL-1b and IL-18, and pyroptosis, a pro-inflammatory form of cell necrosis.**
- **In the present study, the ability of CLM-022 to inhibit the NLRP3 inflammasome is shown through the inhibition of 1) inflammasome complex formation, 2) IL-1β production, and 3) pyroptosis. As well, oral administration of CLM-022 was shown to decrease inflammatory markers and protect against liver injury in an in vivo model of induced acute liver failure. Taken together, these results are consistent with the potential of investigational drug CLM-022 to treat acute and chronic inflammatory liver diseases, including ACLF.**

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DISCLOSURE

HE, AC, ED ,NSV, VL, GV, MM, DH, SSJ are employees and stock shareholder of GENFIT S.A; BS is scientific adviser of GENFIT S.A.

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