ASK1 INHIBITOR SRT-015 REDUCES SYSTEMIC INFLAMMATION WHILE PROMOTING IMMUNE HOST DEFENSE MECHANISMS IN PRECLINICAL *IN VITRO* AND *IN VIVO* MODELS RELATED TO ACLF

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BACKGROUND & AIM

- Acute-on-Chronic Liver Failure (ACLF) is characterized by multiple organ failures and high short-term mortality. ACLF is closely associated with the presence of intense systemic inflammation and impaired immune defense responses against pathogens, such as decreased neutrophil degranulation and phagocytosis, as well as oxidative stress[1]
- Apoptosis-signal-regulating kinase 1 (ASK1) is a key mediator of the inflammatory response, activated by reactive oxygen species (ROS) produced after recognition of pathogen associated molecular patterns (PAMPs). Its phosphorylation results in activation of JNK and p38 that regulate cell death and immune response[2]
- SRT-015 is a novel investigational ASK1 inhibitor. Preclinical studies have shown its anti-apoptotic and anti-inflammatory activities *in vitro*, as well as its ability to reduce liver injury and counteract systemic inflammation in acute liver failure models and improve survival in septic mice[3,4]

• The aim of this study was to investigate the effects of SRT-015 on systemic inflammation in a new model of ACLF that closely mimics the human condition[5], as well as on human leukocyte functions *in vitro*

METHODS

Evaluation of SRT-015 in ACLF mice

Male C57BL/6J mice received intraperitoneal (i.p.) injections of CCl4 two times per week for 13-17 weeks. A control group of mice receiving i.p. injections of vehicle (olive oil) (n=10) was also included. Once mice receiving CCl4 developed ascites, they were randomly divided in three groups: one group was sham operated (CCl4+Sham, n=6) and the other two underwent cecal ligation and puncture (CLP) surgery and subsequent divided into the CCl4+CLP placebo group (n=9) and the CCl4+CLP SRT-015 treated group (n=10). SRT-015 (20 mg/kg) was administered by oral gavage at -19h, -0.5h, and +20h relative to CLP. Mice were terminated 24h post-CLP and blood was collected to measure cytokines using MAGPIX system (Luminex Corp., Austin, TX) using a custom-made Milliplex Mice Expanded Cytokine Magnetic MCYTOMAG-70K (Merck Millipore, Burlington, MA)

Evaluation of SRT-015 in human blood cells after LPS stimulation *ex vivo*

Blood from 4 healthy volunteers was collected in lithium heparin tubes at the Établissement Français du Sang (EFS, France) and stored at room temperature until use (<20 hours). Whole blood was incubated 4 hours with lipopolysaccharide (LPS Klebsiella pneumoniae, 50 ng/ml, Sigma-Aldrich). A dose range of SRT-015 (0.47 - 0.94 - 1.88 - 3.75 - 7.5 - 15 - 30 µM) was added 15 min after LPS. Secretion of TNFα, IL-6 and IL-1β was determined by measuring their concentration in the whole blood using ProcartaPlex immunoassay for Luminex (Thermo Fisher Scientific)

Evaluation of SRT-015 effect on isolated human PBMC after LPS stimulation

Human peripheral blood mononuclear cells (PBMC) were isolated from 6 healthy volunteers by Ficoll-Paque density gradient. The cells were resuspended at a concentration of 3 × 10^6 cells/mL and stimulated with lipopolysaccharide (LPS Escherichia coli O111:B4, 10 ng/mL, Sigma-Aldrich) for 3 hours. SRT-015 (2 and 10 µM) was added to the cells 30 min after the addition of LPS. Total RNA was isolated using TRIzol reagent and RT-qPCR was performed (Applied Biosystems). Gene expression levels were normalized to β-actin as an endogenous control and expressed relative to a calibrator sample (vehicle-treated cells). Raw data were standardized to allow comparisons across different ranges, and results were expressed as the percentage of mRNA expression

Evaluation of SRT-015 effect on neutrophil degranulation

Polymorphonuclear leukocytes (PMNs) were isolated from 5 healthy donor blood samples by Ficoll-gradient. After 30 min of resting, SRT-015 (2 and 10 µM) was added to the cells 5 min before addition of phorbol 12-myristate 13-acetate (PMA). After 2 hours of incubation, degranulation was assessed by quantification of the myeloperoxidase (MPO) release with Human ELISA kit (Abcam) and activity by using the Neutrophil MPO Activity Assay kit (Cayman Chemical, ann Arbor, MI). Raw data were standardized to allow comparisons across the different conditions

Evaluation of SRT-015 effect on neutrophil phagocytosis

Polymorphonuclear leukocytes (PMNs) were isolated from 7 healthy donor blood samples by Ficoll-gradient. After 30 min of resting, cells were incubated with opsonized fluorescent-labeled Escherichia coli (Thermo Fisher Scientific) in the presence of vehicle or SRT-015 (2 and 10 µM) for 2 hours. Phagocytic activity was quantified by measuring the fluorescence intensity of internalized E. coli using the FLUOstar Optima plate reader (Ortenberg). Raw data were standardized to allow comparisons across the different conditions

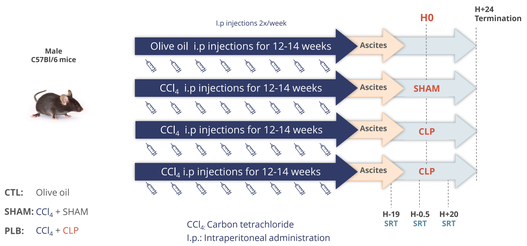
Statistical analyses

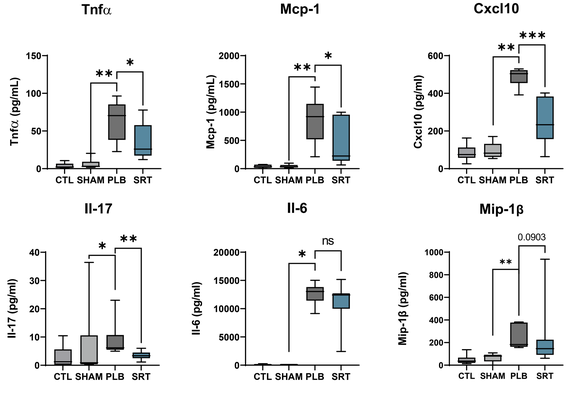
Normal distribution of the data was evaluated, and parametric or non-parametric tests were applied accordingly (GraphPad Prism). Statistical tests are indicated for each figure

RESULTS

SRT-015 ALLEVIATES SYSTEMIC INFLAMMATION IN ACLF MICE

EVALUATION OF SRT-015 IN CCl_4+CLP MICE



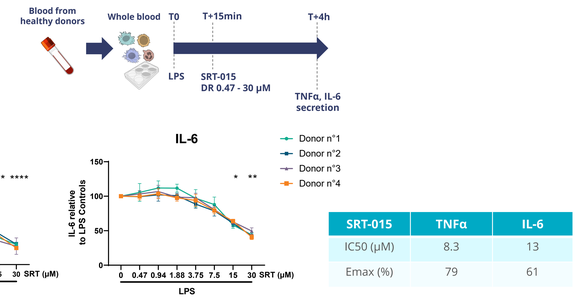


*Serum Tnfa, Mcp-1, Cxcl10, Il-17, Il-6 , and Mip-1β levels in control mice (CTL), CCl_4/SHAM-operated mice (SHAM), CCl_4/CLP-operated placebo-treated (PLB), and CCl_4/CLP-operated SRT-015-treated mice (SRT). *: p<0.05; **: p<0.01; ***: p<0.001 for comparison between groups using Mann-Whitney test*

→ Mice that received oral administration of SRT-015 had significant decreases in circulatory pro-inflammatory cytokines and chemokines compared to the placebo group

SRT-015 REDUCES LPS-INDUCED PRO-INFLAMMATORY CYTOKINES PRODUCTION FROM HUMAN BLOOD IMMUNE CELLS

EVALUATION OF SRT-015 IN HUMAN BLOOD CELLS AFTER LPS STIMULATION *EX VIVO*

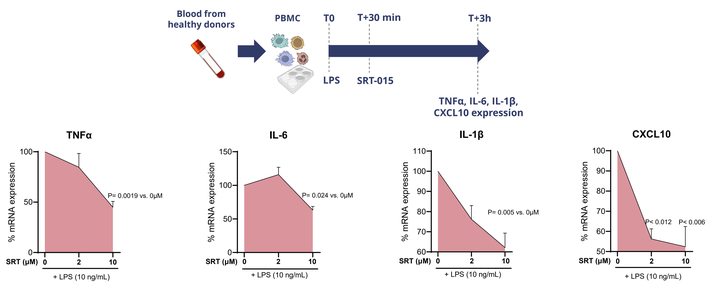


SRT-015	TNFα	IL-6
IC50 (µM)	8.3	13
Emax (%)	79	61

*Pro-inflammatory cytokines (TNFα, IL-6) were measured in whole blood from 4 healthy donors 4h after LPS stimulation. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001 for comparison between the mean of the LPS group and the SRT groups using Kruskal-Wallis test*

→ SRT-015 reduces LPS-induced pro-inflammatory cytokines secretion from human blood immune cells *ex vivo* in a concentration-dependent manner

EVALUATION OF SRT-015 EFFECT ON ISOLATED HUMAN PBMC AFTER LPS STIMULATION

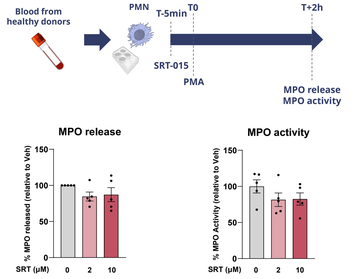


Expression of TNFα, IL-6, IL-1β and CXCL10 was measured in PBMC after LPS stimulation for 3h. P values for the comparison between groups using Kruskal-Wallis followed by Dunn's test are indicated on the graphs

→ SRT-015 reduces LPS-induced pro-inflammatory cytokines and chemokines expression in human PBMC

EFFECT OF SRT-015 ON NEUTROPHIL FUNCTIONS

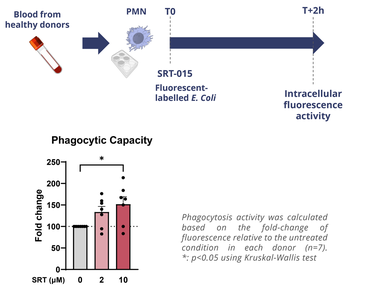
EVALUATION OF SRT-015 EFFECT ON NEUTROPHIL DEGRANULATION



MPO release and activity were calculated relatively to the Vehicle (Veh, SRT 0 µM) condition in each donor (n=5)

→ SRT-015 had no significant effect on neutrophil degranulation

EVALUATION OF SRT-015 EFFECT ON NEUTROPHIL PHAGOCYTOSIS



*Phagocytosis activity was calculated based on the fold-change of fluorescence relative to the untreated condition in each donor (n=7). *: p<0.05 using Kruskal-Wallis test*

→ SRT-015 significantly increased neutrophil bacterial phagocytosis in a concentration-dependent manner

CONCLUSION

- **These data confirm the beneficial effect of SRT-015 on systemic inflammation in a preclinical model of ACLF and show its ability to improve the antibacterial function of neutrophils**
- **These results support the development of the investigational drug SRT-015 for the treatment of patients with ACLF**

REFERENCES

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DISCLOSURE | VL, OH, MC, SD, DH, SSJ are employees and stock shareholder of GENFIT S.A.; BRG, CLV, BJC, BSR, AD, CM performed these studies in a framework of scientific collaboration between EF CLIF-IDIBAPS and GENFIT; RM and JC are consultant for GENFIT S.A.; BS is scientific adviser of GENFIT S.A.