EFFICACY OF NITAZOXANIDE (NTZ) IN PATHOGEN-ASSOCIATED MOLECULAR PATTERNS (PAMPs)-INDUCED DISEASE MODELS

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BACKGROUND & AIM

- Nitazoxanide is an FDA approved anti-parasitic drug that is currently under development for the treatment of Acute-on-Chronic Liver Failure (ACLF), a severe syndrome affecting patients with acutely decompensated cirrhosis[1]
- We previously demonstrated that NTZ alleviates systemic inflammation and organ damage in disease models of ACLF[2-4]

Protection from encephalopathy in patients with cirrhosis[5,6]

Anti-microbial activity against anaerobic bacteria (Gram⁻ and Gram⁺)[7-10]

Rationale for developing NTZ in ACLF

Protection from systemic inflammation, hepatic and renal injury in ACLF rats[2-4]

As systemic inflammation, triggered by bacteria or Pathogen-Associated Molecular Patterns (PAMPs), is a primary driver of acute-on-chronic liver failure (ACLF)[4], we aim to evaluate:

1. NTZ efficacy in two rodent models of PAMPs-induced diseases:

- **A model of sterile endotoxemia in rats, leading to a rapid deterioration of hepatic function due to LPS-induced systemic inflammation**
- **A model of sepsis due to the leakage of intestinal microbiota obtained by cecal ligation and puncture (CLP) surgery in mice, to mimic the translocation of enterobacteria, PAMPs and toxins from the gut, a recognized trigger of systemic inflammation in patients with ACLF**

2. The efficacy of TZ, the active metabolite of NTZ, to counteract cytokine release induced by a wide range of PAMPs

METHODS

Evaluation of NTZ on endotoxemia in healthy rats

Male Sprague Dawley rats received a single intraperitoneal injection of 1 mg/kg LPS (Escherichia coli O111:B4). NTZ (30, 100 or 300 mg/kg) or a control vehicle (Ctrl) was administered by oral gavage concomitant to LPS injection. Serum was collected at 3h and 5h for measurement of cytokines levels by Luminex and serum hepatic markers by Daytona plus automate

Evaluation of NTZ in sepsis mice

Sepsis was induced through CLP surgery in C57BL/6 male mice. Briefly, under anesthesia, an abdominal incision was performed, and the caecum was tightly ligated at half the distance between distal pole and the base of the caecum. The caecum was punctured once through-and-through and replaced in its original position within the abdomen, which was closed with sutures and wound clips. NTZ (100 mg/kg/day) or vehicle was orally administered 3.5h post-CLP, then BID (2x 50 mg/kg/day) for 6 days. Survival was monitored over 7 days

Evaluation of NTZ in macrophages

Murine Raw264.7 macrophages were treated for 6h with TLR agonists kit (Mouse TLR1-9 agonist kit, InvivoGen) as described in Table 1. Six µM of tizoxanide (TZ, active metabolite of NTZ), 3 µM TAK-242 (TLR4 antagonist) or a vehicle were added on cells 1h before stimulation with TLR agonists. Concentration of inflammatory cytokines IL-6, TNFα and IFNβ was measured in the supernatant using Luminex Mouse kit (Thermo Scientific)

TABLE 1: LIST OF TLR AGONISTS USED FOR IN VITRO STUDIES

Agonist	TLR	Final concentration
PAM3CSK4	TLR1/2	100 ng/ml
HKLM	TLR2	10^{+8} c/ml
Poly(I:C)HMW	TLR3	100 ng/ml
LPS-EK	TLR4	10 ng/ml
FLA-ST	TLR5	100 ng/ml
FSL-1	TLR6/2	10 ng/ml
ODN1826	TLR9	5 µM

STATISTICS

- Endotoxemia model: Scatter plots show mean ±SD. For data following a normal distribution: ###: p<0.001 (two-tailed Student T test), *: p<0.05; **: p<0.01; ***: p<0.001 (Dunnett's multiple comparisons test). For non-normally distributed variables: $: p<0.05 (two-tailed Mann-Whitney), §§: p<0.01 (Dunn's multiple comparisons test)
- Sepsis model: Survival curves were compared using Gehan-Breslow-Wilcoxon test (**: p<0.01)
- Cellular model: Bar graphs show mean ±SD. To compare inhibition % of cytokines in macrophages, a one-sample Wilcoxon test was used ($: p<0.05; $$: p<0.01; $$$: p<0.001)

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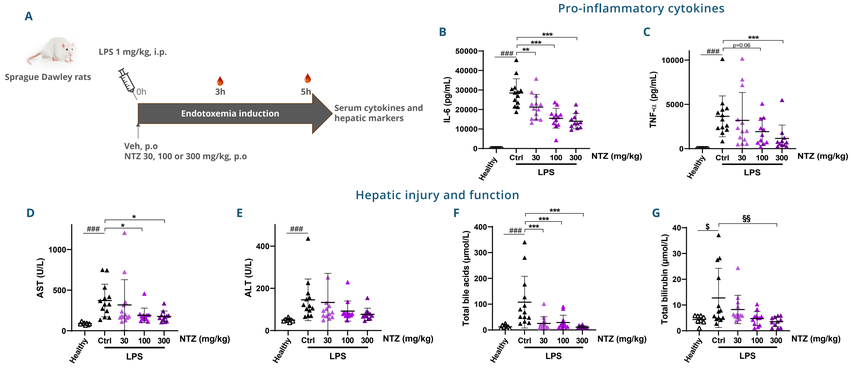
RESULTS

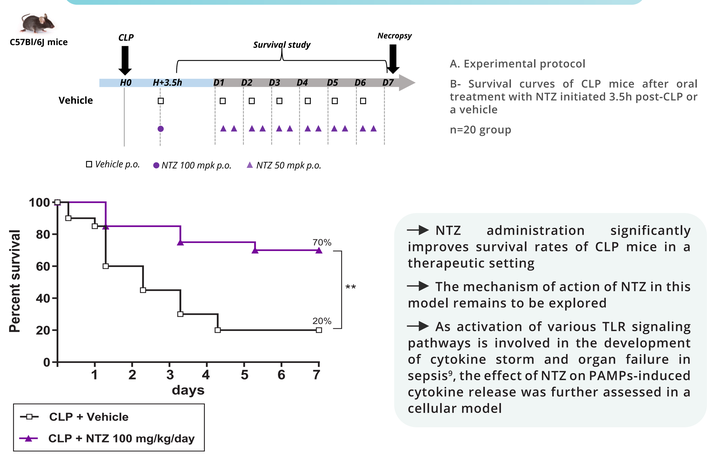
NTZ DOSE-DEPENDENTLY ALLEVIATES LPS-INDUCED SYSTEMIC INFLAMMATION AND IMPROVES HEPATIC FUNCTION IN RATS



A. Experimental protocol
B-C. Serum levels of cytokines IL-6 (A) and TNF-α (B) measured 3h post-LPS
D-E. Serum levels of hepatic injury markers AST (D) and ALT (E) measured at 5h post-LPS
F-G. Serum levels of hepatic function markers TBA (F) and TBIL (G) measured 5h post-LPS
n=8 per control group (healthy rats), n=11-13 per LPS-treated groups

→ NTZ reduces LPS-induced systemic inflammatory markers and protects against endotoxin-induced alterations of hepatic functions in a dose-dependent manner

NTZ IMPROVES SURVIVAL OF MICE WITH SEPSIS



A. Experimental protocol

B- Survival curves of CLP mice after oral treatment with NTZ initiated 3.5h post-CLP or a vehicle

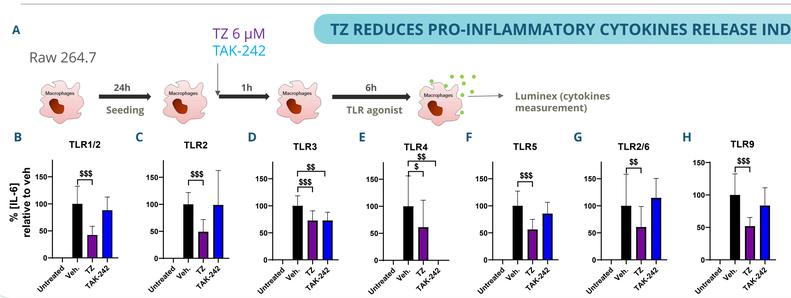
n=20 group

→ NTZ administration significantly improves survival rates of CLP mice in a therapeutic setting

→ The mechanism of action of NTZ in this model remains to be explored

→ As activation of various TLR signaling pathways is involved in the development of cytokine storm and organ failure in sepsis[9], the effect of NTZ on PAMPs-induced cytokine release was further assessed in a cellular model

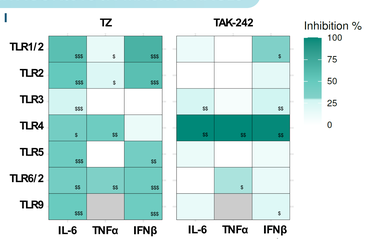
TZ REDUCES PRO-INFLAMMATORY CYTOKINES RELEASE INDUCED BY A LARGE RANGE OF TLR AGONISTS IN MACROPHAGES



A. Experimental protocol

B-H. IL-6 release following macrophages stimulation with PAM3CSK4 (B), HKLM (C), Poly (I:C) HMW (D), LPS E. Coli (E), flagellin FLA-ST (F), FSL-1 (G) or CpG ODN1829 (H) for 6h. n= 3 biologically independent samples

I. Summary of inhibition % of TLR-agonists induced- IL-6, TNFα and IFNβ releases. Significant changes between drug treatment and vehicle are indicated ($: p<0,05, $$: p<0.01, $$: p<0.001), n= 3 biologically independent samples



→ While TAK-242 mostly inhibits LPS-induced pro-inflammatory cytokine release (TLR4 signaling), TZ showed a broader effect on the inhibition of cytokine secretion induced by a large range of PAMPs: agonists of TLR1/2, TLR2, TLR3, TLR4, TLR5, TLR6/2, and TLR9

CONCLUSION

- **Oral NTZ treatment alleviates systemic inflammation and improves survival in endotoxemia and sepsis models, respectively**
- **Combined with its antimicrobial and hepatoprotective properties, the ability of TZ to reduce the inflammatory response induced by a large range of PAMPs further supports the development of NTZ as a new therapeutic approach for ACLF**

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DISCLOSURE

MB, SD, PD, PP, YH, SSJ, DH and VL are employees and stock shareholders of GENFIT S.A.; DD is a consultant for GENFIT S.A.; BS is scientific adviser of GENFIT S.A.

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