Combination of sivelestat and N-acetylcysteine alleviates the inflammatory response and exceeds standard treatment for acetaminophen-induced liver injury

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Abstract
Hepatocyte death during acetaminophen (APAP) intoxication elicits a reactive inflammatory response, with hepatic recruitment of neutrophils and monocytes, which further aggravates liver injury. Neutrophil elastase (NE), secreted by activated neutrophils, carries degradative and cytotoxic functions and maintains a proinflammatory state. We investigated NE as a therapeutic target in acetaminophen-induced liver injury (AILI). C57BL/6 mice were administered a toxic dose of APAP, 2 h prior to receiving the NE inhibitor sivelestat, N-acetylcysteine (NAC), or a combination therapy, and were euthanized after 24 and 48 h. Upon APAP overdose, neutrophils and monocytes infiltrate the injured liver, accompanied by increased levels of NE. Combination therapy of NAC and sivelestat significantly limits liver damage, as evidenced by lower serum transaminase levels and less hepatic necrosis compared to mice that received APAP only, and this to a greater extent than NAC monotherapy. Lower hepatic expression of proinflammatory markers was observed in the combination treatment group, and flow cytometry revealed significantly less monocyte influx in livers from mice treated with the combination therapy, compared to untreated mice and mice treated with NAC only. The potential of NE to induce leukocyte migration was confirmed in vitro. Importantly, sivelestat did not impair hepatic repair. In conclusion, combination of NE inhibition with sivelestat and NAC dampens the inflammatory response and reduces liver damage following APAP overdose. This strategy exceeds the standard of care and might represent a novel therapeutic option for AILI.

KEYWORDS
acute liver failure, acute liver injury, hepatotoxicity, paracetamol

1 INTRODUCTION

Drug-induced liver injury, and more specifically acetaminophen (APAP) overdose, is the leading cause of acute liver failure (ALF) in the United States and Europe.1 APAP is a widely used analgesic and antipyretic drug that is considered safe in therapeutic doses, but may cause severe liver damage, ALF, and death at supratherapeutic doses.2 The antioxidant N-acetylcysteine (NAC) represents the only antidote; however, treatment should be started in time, as the benefit of NAC decreases with the time passed between the moment of overdose and treatment.1,3 If NAC and supportive therapy fail to allow spontaneous recovery, liver transplantation may be the only curative option.1 As such, there is a need to develop novel medical therapies that restrict liver damage and prevent the progression to liver failure, which may reduce the necessity of transplants and APAP-related deaths.

Over the last few years, research focused on understanding the immunologic mechanisms underlying acetaminophen-induced liver injury (AILI) and the potential to modulate the inflammatory...
response as a therapeutic strategy. N-acetyl-p-benzoquinone imine (NAPQI), the metabolite of APAP, induces hepatocyte death during APAP hepatotoxicity, with subsequent release of damage-associated molecular patterns (DAMPs). Upon activation by DAMPs, resident macrophages produce a variety of proinflammatory cytokines and chemokines, which stimulate the recruitment of neutrophils and monocytes into the liver. Products of these cells amplify liver damage, but may also promote resolution. As a result, the exact role of infiltrating immune cells in AILI is not completely understood. For example, neutrophils represent the earliest and major increase in hepatic leukocytes during AILI; however, whether they contribute to the severity of liver damage is controversial. In this sense, further investigation of the association between infiltrating neutrophils, monocytes, and resident macrophages may provide new insights into the pathophysiology of AILI and help to identify new therapeutic targets.

Neutrophil elastase (NE) is a serine protease that is stored in azurophil granules in neutrophils, as well as to lesser extent in monocytes, and is released upon exposure to inflammatory stimuli. It degrades the extracellular matrix, which further facilitates immune cell migration. In addition to its proteolytic action, NE causes organ damage by direct cytotoxicity and by inducing the release of proinflammatory mediators. The therapeutic potential of sivelestat, a highly specific NE inhibitor, has been studied in different pathologies mediated by acute inflammation, mainly in the field of pulmonology. Both preclinical and clinical studies indicate beneficial effects in acute lung injury, with less severe lung damage and improved clinical outcome. This has resulted in the approval of sivelestat for the treatment of acute lung injury in Japan and South Korea. Also, very recently, NE inhibition, both by using knockout mice and using sivelestat, has been shown to successfully reduce portal hypertension in two mouse models.

In this study, we investigated the contribution of NE in AILI in mice, and evaluated whether targeting NE with sivelestat holds therapeutic potential.

2 | MATERIALS AND METHODS

2.1 | Animals

Seven-week-old male C57Bl/6J mice (Janvier Labs, Le Genest-Saint-Isle, France) were housed in open cages in a temperature-controlled room at 20°C with a 12 h dark to 12 h light cycle at the animal facility of the Faculty of Medicine and Health Sciences (Ghent University, Ghent, Belgium). Animals had free access to water and commercial chow (mouse maintenance chow, Carfil Labofood, Belgium). Mice were acclimatized under controlled conditions for 1 wk prior to the experiments. All mice received care in accordance with the “Guide for the Care and Use of Laboratory Animals” and the Belgian national guidelines for animal protection. The Ethical Committee of Experimental Animals at the faculty of Medicine and Health Science, Ghent University, Belgium, approved the experiments (ECD 17/127).

2.2 | APAP-induced liver injury

Mice were i.p. injected with PBS or 300 mg/kg of APAP (Sigma, Diegem, Belgium) dissolved in warmed PBS, after overnight fasting. Two hours after APAP injection, mice received either PBS vehicle, sivelestat (150 mg/kg i.p., dissolved in PBS, Tocris, Abingdon, United Kingdom), NAC (200 mg/kg i.p., dissolved in PBS, Sigma), or the combination of sivelestat and NAC (separate injections), creating five study groups (n = 8 in each group). Dose regimens were based on published protocols. Additional PBS was i.p. injected to correct for fluid administration in mice receiving monotherapy. The experiments were performed 3 times and data are represented as pooled results.

2.3 | Tissue harvesting

Twenty-four or 48 hours after PBS or APAP administration, mice were anesthetized using ketamin (60 mg/kg, Ceva santé animale, Brussels, Belgium) and xylazin (6 mg/kg, Kela, Sint-Niklaas, Belgium). Retro-orbital blood was collected and animals were euthanized by cervical dislocation. The liver was perfused with PBS and a portion was harvested for histology, protein and RNA extraction, and FACS by incubation for 20 min in 1 mg/kg Collagenase A (Sigma) and 10 U/mL Dnase (Roche Diagnostics, Vilvoorde, Belgium) in a heated bath (37°C). Suspensions were filtered and cells were stained with appropriate antibodies.

2.4 | Flow cytometry and sorting

After antigen blockade with Fc block (BD Biosciences, Erembodegem, Belgium), cells were stained with CD45/APC-Cy7, Ly6G/PerCP-Cy5.5, Ly6C/V450, and TIM4/PE from BD Biosciences, F4/80/FITC from Invitrogen (Fisher Scientific, Erembodegem, Belgium), CD11b/PE-Cy7 from Biolegend (London, United Kingdom), for 20° at 4°C in the dark as previously described. Cells were analyzed with a FACSaria III cell sorter (BD Biosciences) and FlowJo software (FlowJo LLC, BD Biosciences). Cells were gated as CD45+ leukocytes and subsequently as CD11b+Ly6G−Ly6C+Tim4+ monocytes, F4/80+CD11bLy6G−Ly6C−Tim4+ Kupffer cells, F4/80+CD11bLy6G−Ly6C−Tim4− monocyte-derived macrophages and CD11b+Ly6C+Ly6G+ neutrophils. These cells were sorted and RNA was extracted using the RNeasy micro kit (Qiagen, Venlo, Netherlands).

2.5 | Biochemical assays

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined by automated procedures (c701 module, Cobas 8000, Roche Diagnostics).

2.6 | Sample handling

Liver tissue for RNA and protein isolation was frozen in liquid nitrogen. RNA was extracted from 20 mg of frozen liver tissue preserved in RNA-later (Ambion, Thermo Fisher Life Technologies, Ghent, Belgium), according to the manufacturer’s guidelines (RNeasy Mini Kit, Qiagen) and measured for purity and quantity by spectrophotometry
(Nanodrop, Thermo Scientific, Waltham, MA, USA). cDNA was made out of one microgram of mRNA by reverse transcription using the iScript cDNA synthesis kit (BioRad, Temse, Belgium) according to the manufacturer’s instructions.

Total protein extract was obtained by homogenizing tissue in RIPA buffer (PBS, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5.5% β-glycerophosphate, 1 M dithiothreitol, and complete protease and phosphatase inhibitors [Roche Diagnostics]). The total protein yield was determined using Bradford reagent (BioRad).

2.7 | Quantitative RT-PCR

Diluted cDNA was subjected to 45 cycles of quantitative PCR amplification using SYBR Green mix (Sensimix, Bioline Reagents Ltd., London, United Kingdom) and 2 µM of each primer. A 2-step program was run on a LightCyclerR 480 (Roche Diagnostics). Melting curve analysis confirmed primer specificities. All reactions were run in duplicate and normalized to reference genes that showed stable expression in all samples (SDHA, HMBS, HPRT). The PCR-efficiency of each primer pair was calculated using a standard curve of reference cDNA. Amplification efficiency was determined using the formula $10^{-1/slope-1}$. The primer set sequences are listed below.

### Primer pairs used for qRT-PCR

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$E = \text{amplification efficiency}$

2.8 | Primer pairs used for qRT-PCR

2.9 | ELISA

Quantification of NE levels in hepatic tissue lysates and serum was performed using commercial high sensitivity ELISAs (R&D Systems, Abingdon, United Kingdom) according to the manufacturer’s instructions. Data from tissue samples were normalized to the total protein content.

2.10 | Myeloperoxidase (MPO) assay

MPO assay was performed as previously described. Briefly, liver samples were suspended in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich, Bornem, Belgium) followed by sonication on ice for 15 s. The supernatant was separated from the solid phase by centrifugation at 16,000 xg for 20 min. A total of 10 µl of the supernatant was mixed with 140 µl of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml-dianisidine dihydrochloride (Sigma-Aldrich) and 0.005% H2O2 (Sigma-Aldrich). MPO activity was derived from an observed change in absorbance measured by spectrophotometry at 450 nm (BioRad) and normalized to the total protein content of the supernatant.

2.11 | Histology

Liver samples were fixed in 4% phosphate buffered formaldehyde solution (Klinipath, Olen, Belgium), dehydrated, embedded in paraffin, and sectioned. Liver sections were stained with H&E. Bright field images were captured using an Olympus (BX41, Antwerp, Belgium) light microscope (objective 10x). Necrotic areas were quantified in at least 10 fields per mouse using Cell-ID software (Olympus). Results are expressed as the mean necrotic area per field (% ± SE). The final score is represented as the mean of the scores determined by two independent researchers, who were blinded to the study samples.

2.12 | Boyden chamber chemotaxis assay

Cell culture medium (HBSS, Invitrogen) with and without NE (native human NE protein, Abcam) was subjected to 0.5 x 10⁶ human peripheral blood mononuclear cells (PBMCs), isolated from a healthy volunteer using gradient centrifugation (SepMate, STEMCELL Technologies, Grenoble, France) in a Boyden transwell assay (ThinCertTM 3 µm pore size, TC Insert system, Greiner bio-one, Vilvoorde, Belgium). Leukocytes migration was counted in a coulter chamber after 4 h.

2.13 | Statistical analysis

Data analysis was performed using Graphpad (GraphPad Software, Inc., San Diego, CA, USA) and SPSS 25 (SPSS, Inc., Chicago, IL, USA). Data distribution was evaluated with the Shapiro-Wilk test. Normally distributed data were analyzed with the Student’s t-test or ANOVA with posthoc correction. For nonparametric data, data were analyzed using the Mann-Whitney U-test or Kruskal-Wallis test with Dunn’s posttest. Measurements were expressed as means ± SE. Two-tailed probabilities were calculated and P-values <0.05 were considered significant.
RESULTS

3.1 Experimental AILI is characterized by hepatic neutrophil and monocyte accumulation and production of NE

We first analyzed biochemical and histologic features of APAP intoxication. Mice were i.p. injected with 300 mg/kg APAP or PBS control, and the severity of liver damage was assessed 24 h postinjection. APAP induced significant liver injury, as indicated by a 138-fold increase in serum alanine transaminase (ALT: 6506 ± 3102 vs. 47 ± 10, \( P < 0.0001 \)) and a 24-fold increase in aspartate transaminase (AST: 4208 ± 2835 vs. 173 ± 51, \( P < 0.0001 \)) compared to control mice (Fig. 1A). Histologic analysis showed extensive necrotic regions in mice that received APAP overdose (20% ± 5% vs. 0% in control mice, \( P < 0.0001 \)) (Fig. 1B).

Inflammatory mediators are increased during the initial phase of hepatocellular injury induced by APAP\(^4\). Chemokines Cxcl2, responsible for neutrophil attraction, and Ccl2 and its receptor Ccr2, which guide monocyte recruitment, were up-regulated in livers of APAP-injected mice compared to control mice (both \( P < 0.0001 \)) (Fig. 1C). Elevated hepatic levels of Tnf\(a \) (\( P < 0.0001 \)), Ifn1\(b \) (\( P = 0.0029 \)), and Tlr4 (\( P = 0.013 \)) were observed in APAP-overdosed mice as well (Fig. 1C).

Next, flow cytometric analysis was performed to study the hepatic immune cell populations during APAP hepatotoxicity. The gating strategy is presented in Figure S1. AILI was accompanied by significantly increased percentage of hepatic CD11b\(^{+}\)Ly6C\(^{hi}\)Ly6G\(^{+}\) neutrophils (\( P < 0.0001 \)), CD11b\(^{+}\)Ly6G\(^{−}\)Ly6C\(^{+}\)Tim4\(^{−}\) monocytes (\( P < 0.0001 \)), and CD11b\(^{+}\)Ly6G\(^{−}\)Ly6C\(^{−}\)Tim4\(^{−}\) macrophages (\( P = 0.029 \)), and a decrease in F4/80\(^{−}\)CD11b\(^{+}\)Ly6G\(^{−}\)Ly6C\(^{−}\)Tim4\(^{−}\) Kupffer cells (\( P < 0.0001 \)) compared to control mice (Fig. 1D), consistent with previous studies.\(^{23-27}\) MPO activity in liver tissue, used as a surrogate marker of neutrophil infiltration and activation, confirmed flow cytometric data (\( P = 0.006 \); Fig. 1D). Collectively, these data confirmed the concept of sterile inflammation as a second phase of liver injury following direct APAP-induced hepatotoxicity in AILI.

Next, we were interested whether the inflammatory response in AILI is associated with local production and circulation of NE, a product of neutrophils with both destructive and proinflammatory functions. The expression of NE was significantly increased in liver tissue and serum of mice that received APAP injection compared to control mice (\( P = 0.0006, P = 0.017 \), respectively) (Fig. 2).

3.2 Combination therapy of the NE inhibitor sivelestat and NAC attenuates liver damage and exceeds current standard treatment for AILI

Pharmacologic inhibition of NE has been shown to successfully reduce the inflammatory response and disease severity in different pathologies mediated by acute inflammation.\(^{9-17}\) As we and others demonstrated a pronounced acute inflammatory response in AILI, and given the increased production of NE, we hypothesized that NE inhibition may be protective against AILI. A first group of APAP-intoxicated mice were treated with the NE inhibitor sivelestat. NAC monotherapy, currently the gold standard for the medical treatment of AILI, was included as a comparator group. Given the limitations of NAC treatment in clinical practice, we were specifically interested whether targeting NE by adding sivelestat to standard therapy with NAC surpasses monotherapy with NAC. Treatment with either NAC or sivelestat led to a similar reduction in serum AST (NAC: \( −42\% \), \( P = 0.02 \); sivelestat: \( −45\% \), \( P = 0.01 \)) and ALT levels (NAC: \( −32\% \), \( P = 0.028 \); sivelestat: \( −19\% \), \( P = 0.067 \)) compared to mice with AILI that received vehicle (Fig. 3A). Hepatic necrosis was less severe in actively treated APAP-intoxicated mice compared to untreated mice (\( −38\% \) in NAC; \( −30\% \) in sivelestat; both \( P < 0.0001 \)) (Fig. 3B). The combination of NAC and sivelestat resulted in significantly lower serum transaminase levels (AST: \( 1305 \pm 506 \) vs. \( 4208 \pm 2835 \), \( P = 0.0001 \); ALT: \( 2674 \pm 1037 \) vs. \( 6506 \pm 3102 \), \( P = 0.0002 \)) and in less liver necrosis (\( 12\% ± 4\% \) vs. \( 20\% ± 5\% \), \( P < 0.0001 \)) compared to untreated mice with AILI (Fig. 3A and B).

Moreover, this combination strategy resulted in less APAP-induced cell death, indicated by both lower serum AST (\( −47\% \), \( P = 0.0003 \)) and ALT (\( −37\% \), \( P = 0.003 \)) in comparison with NAC monotherapy (Fig. 3A).

Animal weights can be found in Supplementary Table S1.

3.3 Cotreatment with NAC and sivelestat suppresses liver inflammation in AILI

Further analysis of whole liver tissue demonstrated lower expression of the chemokines Cxcl2 (\( P = 0.02 \)), Ccl2 (\( P = 0.033 \)), and of the chemokine receptor Ccr2 (\( P = 0.0004 \)) in APAP-challenged mice that received cotreatment with NAC and sivelestat, compared to mice that were administered vehicle (Fig. 4A). In line, hepatic levels of proinflammatory mediators Tnf\(a \) (\( P = 0.019 \)), Ifn1\(b \) (\( P = 0.019 \)), and Tlr4 (\( P = 0.05 \)) were lower in mice that received both NAC and sivelestat, compared to untreated mice with AILI (Fig. 4A). Hepatic gene expression of proinflammatory markers was not significantly decreased in mice that received NAC or sivelestat monotherapy (Fig. 4A).

3.4 Treatment with NAC and sivelestat reduces hepatic monocyte infiltration after APAP overdose but does not affect its proinflammatory phenotype

We next questioned whether add-on therapy with sivelestat influences hepatic immune cell accumulation during AILI. Flow cytometry revealed significantly less CD11b\(^{+}\)Ly6G\(^{−}\)Ly6C\(^{+}\)Tim4\(^{−}\) monocyte influx in livers from mice treated with the combination therapy of NAC and sivelestat compared to APAP mice (\( P = 0.0003 \)) (Fig. 4B), which is compatible with the observation of lower hepatic expression of inflammatory markers. In agreement with serum transaminase and histology data, monocyte infiltration was significantly lower in mice that were administered both therapeutic compounds, compared to animals that received NAC only (\( P = 0.0001 \)) (Fig. 4B). Moreover, the APAP-induced reduction in F4/80\(^{−}\)CD11b\(^{hi}\)Ly6G\(^{−}\)Ly6C\(^{−}\)Tim4\(^{−}\) Kupffer cells was less pronounced in the combination treatment group compared to mice that received vehicle (\( P = 0.001 \)) (Fig. 4B). Hepatic neutrophil and...
FIGURE 1  Experimental acetaminophen (APAP) overdose results in liver damage and hepatic influx of immune cells. Mice were i.p. injected with 300 mg/kg APAP or PBS and were euthanized 24 h later. (A) Serum AST and ALT levels. (B) Left: quantification of necrotic area (mean necrotic area per field ± se) on H&E stained liver sections. Right: representative images of liver sections, showing hepatic necrosis after APAP overdose. Original magnification, ×40. (C) Hepatic mRNA expression of proinflammatory markers Cxcl2, Ccl2, Ccr2, Tnfa, Il1b, and Tlr4 as measured by RT-qPCR, and hepatic MPO levels. (D) Quantification of hepatic neutrophils, monocytes, macrophages, and Kupffer cells, expressed as % of CD 45+ cells, by flow cytometry. Data are represented as means ± se or means ± se relative to the mean of the PBS control group as fold change. *P < 0.05, **P < 0.01, and ***P < 0.001.
Pharmacologic inhibition of NE reduces liver injury without impairing the repair process

As newly recruited immune cells, especially neutrophils, are known to be involved in the removal of necrotic cell debris in preparation for the resolution phase, we questioned whether interfering with NE delays the repair process in AILI. Mice were administered APAP, treated with the different compounds 2 h later, and euthanized at either 24 or 48 h. Importantly, sivelestat does not influence liver repair processes, as demonstrated by significant reductions in serum transaminase levels and necrotic areas at both 24 and 48 h post-APAP injection in actively treated animals (Fig. 6). Transaminase levels decreased in all treatments groups with increasing time after APAP overdose (Fig. 6A). Hepatic necrosis was reduced at 48 h compared to time point 24 h in sivelestat-treated mice (Fig. 6B), indicating ongoing repair, and further underscores the beneficial effect of this compound in experimental AILI.

4 | DISCUSSION

Accidental or intentional APAP overdose remains an important cause of DILI, overdose-related ALF and death. The only treatment options are NAC, which targets the earliest phase of AILI, or LT as a rescue measure. Given the limitations of both strategies, the development of novel therapies focuses on interfering with the second phase of AILI, the inflammatory response. In this study, we evaluated the therapeutic potential of NE inhibition to mitigate APAP-induced liver damage. We demonstrated that pharmacologic inhibition of NE with sivelestat and NAC additively (i) reduces liver damage and (ii) decreases hepatic influx of monocytes in experimental AILI.

The hepatotoxic response to APAP is initiated by the generation of NAPQI, which induces hepatocyte death and the release of DAMPs, and is followed by production of proinflammatory mediators by Kupffer cells. We observed significant changes in the hepatic immune cell pool, compatible with previous studies, along with increased levels of proinflammatory mediators CXCL2, CCL2, CCR2, TNFα, IL1β, and TLR4 during the development of experimental AILI. Neutrophils are amongst the first immune cells that are recruited to the hepatic sinusoids and then extravasate into the parenchyma guided by chemokines in AILI. Whereas Kupffer cells are depleted during AILI, infiltrating monocytes accumulate and migrate to the sites of hepatic necrosis. Although finally these infiltrating immune cells will orchestrate removal of necrotic cell debris in preparation for repair and resolution, they exhibit a proinflammatory profile when recruited, which is responsible for amplification and maintenance of liver injury. Also, specifically neutrophils can produce a variety of proteases, and their excessive activation can cause significant collateral tissue damage, as shown in other models of acute liver disease, for example, alcoholic hepatitis, and obstructive cholestasis. NE is such a neutrophil protease that has destructive capacities, but can also potentiate the inflammatory response by stimulating the secretion of cytokines and chemokines. Therefore, we were interested in NE as a potential target to break the circle of sterile inflammation in AILI. We found abundant hepatic presence of NE, as well as circulating NE, in mice with AILI. Next, we evaluated the effects of the pharmacologic NE inhibitor sivelestat in mice with AILI. We could show that treatment with sivelestat leads to less liver injury after APAP overdose, with an effectiveness comparable to that of NAC monotherapy. More important, our experiments consistently demonstrated that the combination...
FIGURE 3  Sivelestat reduces liver damage in mice with acetaminophen-induced liver injury (AILI). Mice were i.p. injected with PBS or 300 mg/kg acetaminophen (APAP) and were treated with 200 mg/kg NAC, 150 mg/kg sivelestat, the combination of sivelestat and NAC, or PBS vehicle 2 h later. Liver and serum were collected 24 h after APAP injection. (A) Serum AST and ALT levels. (B) Quantification of necrotic area (mean necrotic area per field ± SE) on H&E stained liver sections and representative images of liver sections. Original magnification, ×100. Data are represented as means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001
FIGURE 4 Sivelestat dampens the inflammatory response and reduces hepatic monocyte infiltration in experimental acetaminophen-induced liver injury (AILI). Mice were i.p. injected with PBS or 300 mg/kg acetaminophen (APAP) and were treated with 200 mg/kg NAC, 150 mg/kg sivelestat, the combination of sivelestat and NAC, or PBS vehicle 2 h later. Liver samples were collected 24 h after APAP injection. (A) mRNA expression of proinflammatory markers Ccl2, Ccr2, Cxcl2, Tnfa, Il1b, and Tlr4, measured by RT-qPCR on hepatic tissue. Data are represented as means ± SE relative to the mean of the PBS control group as fold change. (B) Results of flow cytometric analyses of hepatic neutrophils, monocytes, macrophages, and Kupffer cells as percentage of CD45+ cells in different treatment groups. (C) Attraction of PBMC’s towards medium with and without neutrophil elastase (NE) was tested in a Boyden chamber assay. Data are represented as means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001
FIGURE 5  Sivelestat does not reverse the proinflammatory phenotype of hepatic immune cells in mice with acetaminophen-induced liver injury (AILI). Mice were i.p. injected with PBS or 300 mg/kg acetaminophen (APAP) and were treated with 200 mg/kg NAC, 150 mg/kg sivelestat, the combination of sivelestat and NAC, or PBS vehicle 2 h later. Liver samples were collected 24 h after APAP injection. (A) mRNA expression of proinflammatory markers in FACS-isolated (A) neutrophils, (B) monocytes, (Continues)
FIGURE 5  (Continued) (C) macrophages, and (D) Kupffer cells, measured by RT-qPCR. Data are represented as means ± SE relative to the mean of the PBS control group as fold change. *P < 0.05, **P < 0.01, and ***P < 0.001
strategy of sivelestat and NAC results in less liver damage, that even surpasses the effect of gold standard therapy with NAC.

These results suggest a pathogenic role for infiltrating neutrophils in mediating liver damage in AILI, which however has been subject of debate. Previous studies demonstrated attenuation of the neutrophil response and AILI in anti-HMGB1 antibody-treated mice,34,35 and reduced liver damage in TLR4-deficient mice,36 suggesting neutrophil influx and activation downstream of DAMPs sensing by TLR4 on Kupffer cells. Others investigated the effects of neutrophil depletion,37,38 knockout of intercellular cellular adhesion molecule 137 or TLR9,28,39 which resulted in similar conclusions, and strongly suggested a critical role for neutrophils in aggravating liver damage in AILI. In contrast, others believe that neutrophils are only implicated in the resolution phase of AILI, but do not contribute to liver injury.40 Our data further support the involvement of neutrophils and NE in the progression of acute liver injury during the early phase of AILI. Add-on therapy with sivelestat to NAC profoundly attenuated hepatic expression of proinflammatory markers compared to untreated animals, and resulted

FIGURE 6 Sivelestat reduces liver damage without impairing repair processes in experimental acetaminophen-induced liver injury (AILI). Mice were i.p. injected with PBS or 300 mg/kg acetaminophen (APAP) and were treated with 200 mg/kg NAC, 150 mg/kg sivelestat, the combination of sivelestat and NAC, or PBS vehicle 2 h later. Liver and serum were collected 24 and 48 h after APAP injection. (A) Serum AST and ALT levels. Original magnification, ×100. Data are represented as means ± SE.*P < 0.05, **P < 0.01, and ***P < 0.001, indicating comparisons between groups at the same time point. §§P < 0.05, §§P < 0.01, and §§§P < 0.001, indicating a comparison with the corresponding treatment group at the other time point.

(Continues)
FIGURE 6 (Continued) (B) Upper section: quantification of necrotic area (mean necrotic area per field ± SE) on H&E stained liver sections. Lower section: representative images of liver sections at 48 h post-APAP injection.

in significantly less hepatic influx of proinflammatory monocytes. Our in vitro chemotaxis assay showed the potential of NE to induce leukocyte migration, which could be reduced upon sivelestat treatment and, at least in part, contribute to our results. This mechanism of action shows parallels with observations in models of ventilator-induced lung injury,\(^1\) lipopolysaccharide-induced acute lung injury,\(^1\) and arthritis,\(^4\) where administration of sivelestat resulted in less leukocyte influx and lower cytokine expression in broncho-alveolar lavage or the affected joints, respectively. In this study, the reduction of liver damage is mainly due to a reduced number of immune
cells that are recruited and infiltrate the liver, and not by affecting their expression profile, as shown by gene expression analysis on FACS-isolated cells.

Until recently, the role of NE in hepatic disease had only been explored specifically in ischemia-reperfusion-induced liver injury. Experimental studies showed that NE, by various mechanisms, contributes to liver damage in this specific setting and that inhibition with sivelestat ameliorates hepatic injury. Very recently, Hilscher et al. also demonstrated for the first time a role for NE in the pathogenesis of portal hypertension. Genetic deletion of NE resulted in decreased portal hypertension in two experimental models, partial inferior vena cava ligation and common bile duct ligation. Pharmacologic inhibition with sivelestat confirmed the results, showing significantly lower portal pressure in inferior vena cava ligated mice. Together with our experiments in AILI, these observations further support the concept of neutrophils as contributors to liver disease and point to these cells and their products as potential targets for therapy.

A potential concern with immunomodulation therapies may be interfering not only with injury but also with resolution mechanisms. Importantly, our results showed that treatment with sivelestat does not hinder repair processes during resolution from AILI in experimental APAP overdose.

A pronounced inflammatory response post-APAP intoxication has been associated with poor clinical outcome in patients with AILI. In these patients, as well as in those who present late, treatment with NAC is generally unsuccessful as its mechanism of action is specifically directed at the very early phase of metabolic liver injury. Here, immune-modulatory therapies could potentially prolong the therapeutic window, and also may benefit patients with AILI who do not respond sufficiently to NAC. Sivelestat has been administered to thousands of patients, so clinical experience is substantial. Several studies reported improved survival and a shortened ventilator-assisted period for patients with acute lung injury and acute respiratory distress syndrome, although one multicenter trial could not confirm this effect. This however does not preclude clinical benefit, given the heterogeneous study population and differences in trial design. Furthermore, sivelestat was demonstrated to improve clinical outcome when preventively administered in patients undergoing cardiopulmonary bypass, thoracoscopic esophagectomy, or living-donor liver transplantation. Safety analyses demonstrate a favorable safety profile. No difference in adverse events between placebo- and sivelestat-treated patient groups was observed and no adverse effects were considered related to sivelestat. Given the role of neutrophils in host defense, the question arises whether inhibition of NE activity results in an increased infection risk. Experimental studies demonstrate preserved host defense and bactericidal capacity of neutrophils upon sivelestat treatment, and importantly, no difference in incidence of adverse events related to infection was noted in clinical trials.

In conclusion, our results indicate a role for NE in the pathophysiology of AILI, and suggest that add-on therapy with sivelestat to current standard treatment with NAC might represent a new therapeutic option to treat AILI.

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AUTHORSHIP


DISCLOSURES

The authors declare no conflicts of interest.

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REFERENCES


SUPPORTING INFORMATION

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