Biochemical Pharmacology 81 (2011) 996–1003

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Opioid-like compound exerts anti-fibrotic activity via decreased hepatic stellate cell activation and inflammation

Stephani A. Day [a,1], Ashley M. Lakner [a,b,1], Cathy C. Moore [c], Mao-Hsiung Yen [d], Mark G. Clemens [a], Edwin S. Wu [e], Laura W. Schrum [a,b,*]

[a] Department of Biology, University of North Carolina at Charlotte, Charlotte, NC 28223, USA
[b] Liver, Digestive and Metabolic Disorders Laboratory, Carolinas Medical Center, Charlotte, NC 28203, USA
[c] Department of General Surgery, Carolinas Medical Center, Charlotte, NC 28203, USA
[d] Department of Pharmacology, National Defense Medical Center, Taipei, Taiwan
[e] Jenken Biosciences, Inc., Research Triangle Park, NC 27709, USA

ARTICLE INFO

Article history:
Received 3 December 2010
Accepted 20 January 2011
Available online 1 February 2011

Keywords:
Hepatic stellate cell
Opioid
Inflammation
Fibrosis
Nalmefene

ABSTRACT

Hepatic fibrosis is characterized by excess type I collagen deposition and exacerbated inflammatory response. Naltrexone, an opioid receptor antagonist used for treating alcohol abuse, attenuates hepatocellular injury in fibrotic animal models, which can be accompanied by deleterious side effects. Additionally, opioid neurotransmission is upregulated in patients with inflammatory liver disease. Several derivatives of Naltrexone, Nalmefene (Nal) and JKB-119, exert immunomodulatory activity; however, unlike Nal, JKB-119 does not show significant opioid receptor antagonism. To delineate the potential hepatoprotective effects of these compounds, we investigated if JKB-119 and Nal could modulate activation of hepatic stellate cells (HSCs), primary effector cells that secrete type I collagen and inflammatory mediators during liver injury. Our results demonstrated that Nal or JKB-119 treatment decreased smooth muscle α-actin, a marker of HSC activation, mRNA and protein expression. Despite decreased collagen mRNA expression, both compounds increased intracellular collagen protein expression; however, inhibition of collagen secretion was observed. To address a possible mechanism for suppressed collagen secretion or retention of intracellular collagen, endoplasmic (ER) protein expression and matrix metalloproteinase (MMP) activity were examined. While no change in ER protein expression (Grp78, PDI, Hsp47) was observed, MMP13 mRNA expression was dramatically increased. In an acute LPS inflammatory injury animal model, JKB-119 treatment decreased liver injury (ALT), plasma TNFα and PMN liver infiltration. Overall, these results suggest that JKB-119 can directly inhibit HSC activation attributed to anti-inflammatory activity and may, therefore, attenuate inflammation associated with HSC activation and liver disease.

1. Introduction

Opiate drugs, through interactions with specific receptors, modulate antibody response, natural killer cell activity, T-cell development and expression of cytokines and chemokines both in vitro and in vivo. Opioid neurotransmission is well documented in patients with inflammatory liver disease, including billiary cirrhosis, steatohepatitis, and hepatic fibrosis [1]. Increased secretion and deposition of type I collagen by activated hepatic stellate cells (HSCs) is ultimately the major cause of organ dysfunction during fibrosis. As a result of hepatic injury, HSCs undergo an activation and transdifferentiation process wherein they acquire hyper-contractile, pro-inflammatory, and pro-fibrogenic properties [2,3]. Activated HSCs are characterized by loss of vitamin A stores, expression of cytoskeletal smooth muscle α-actin (αSMA), and increased proliferation [3]. Initiation of HSC activation is associated with increases in several inflammatory cytokines which modulate collagen expression, including TGFβ1, TNFα, IL-1β [2] and IL-6 [4–7]. Specifically in HSCs, TNFα and IL-6 induce cellular transdifferentiation and activation, ultimately resulting in secretion of pro-fibrotic and proliferative factors, culminating in fibrosis [8]. Stellate cell-mediated increases in TNFα results in enhanced proliferation, overexpression of TIMPs (tissue inhibitors of metalloproteinases) and increases in MMP2 and MMP13 expression, two matrix metalloproteinases required for normal ECM degradation [9–11]. Activated HSCs also secrete IL-6 [12] further exacerbating type I collagen production via autocrine

* Corresponding author at: Carolinas Medical Center, Liver, Digestive and Metabolic Disorders Laboratory, 1000 Blythe Blvd., Charlotte, NC 28203, USA. Tel.: +1 704 355 9670; fax: +1 704 355 7648.
E-mail address: Laura.Schrum@carolinashealthcare.org (L.W. Schrum).
[1] These authors contributed equally.

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.01.015

signaling [2]. Increased and sustained inflammation as a result of liver injury/disease states perpetuates activation of the stellate cell ultimately leading to increased hepatic injury, thus many studies have been performed to investigate the use of anti-inflammatory drugs to abrogate HSC-mediated hepatic injury.

All opioid receptor classes are expressed by HSCs at both mRNA and protein levels and are increased as a consequence of liver injury in both tissue and plasma [13]. Cholestatic models have demonstrated opioid transmission is increased post insult suggesting a linkage between hepatic injury and opioid receptor stimulation [14]. Accordingly, activation of receptors by endogenous opioids increases HSC proliferation and type I collagen secretion via PKC/ERK/PI3K pathways [13]. Furthermore, opioid antagonists Naloxone and Naltrexone decreased severity of fibrosis via modulation of HSC activation and collagen expression in bile duct-ligated (BDL)/dimethylnitrosamine (DMN) treated animals [13–15]. Naltrexone, with additive anti-inflammatory properties, attenuated damage in BDL rats with positive regulation of collagen synthesis by decreased MMP2 expression, which directs extracellular matrix (ECM) remodeling through degradation of collagen (type IV), consequently providing area for fibrotic type I collagen deposition by HSCs [16]. Likewise, Nalmefene, an analog of Naltrexone, is predicted to demonstrate similar efficacy. JKB-119, a proprietary morphinan analog, is an opioid-like compound with immunomodulatory activity in the absence of significant opiate receptor binding affinity possessed by Nalmefene. Previously published works demonstrate the efficacy of anti-inflammatory compounds for the treatment of hepatic fibrosis, such as S-adenosyl-L-methionine [17] and vitamin E [18–21]; however, translation of these data from rodent models to the human condition has been devoid of therapeutic benefit, further supporting the need for development of novel compounds. JKB-119 and Nalmefene both exhibit immunomodulatory properties and novel anti-inflammatory profiles with Nalmefene possessing opioid receptor antagonist capabilities. To delineate whether potential hepatoprotective effects ascribed to both compounds can be attributed to inhibition of opioid or inflammatory signaling pathways, we determined the effects of each compound on HSC activation, a key component to hepatic damage and disease. Additionally, JKB-119 was examined in vivo for protective effects. In vitro studies demonstrated HSCs treated with these compounds showed decreased activation and collagen secretion independent of canonical ERK and NFκB pathways. JKB-119 and Nalmefene also significantly reduced HSC proliferation and increased cell death, suggesting these compounds act directly on HSC activation and collagen production independent of opiate receptor interaction. Further analyses of novel compound JKB-119 in vivo demonstrated reduced inflammatory liver injury as indicated by decreased plasma TNFα, PMN infiltration and serum ALT.

2. Materials and methods

2.1. Animal model of liver injury and damage assessment

Wistar-Kyoto rats were randomly assigned to the following groups: (I) control group: treated with vehicle (saline; i.v.); (II) JKB-119 group: treated with JKB-119 (4 mg/kg, i.v.); (III) LPS group: treated with *E. coli* lipopolysaccharide serotype O127:B8 [LPS, (Sigma–Aldrich, St. Louis, MO) 10 mg/kg, i.v.]; (IV) JKB-119 post-treatment group (LPS/JKB-119): treated with JKB-119 (4 mg/kg, i.v.) at 30 min after LPS (10 mg/kg; i.v.). Blood was collected at indicated time points and tested for presence of alanine aminotransferase (ALT), a bio-marker for liver damage, and TNFα. Livers were harvested 8 h post treatment for histopathological studies, which was quantitatively analyzed as an index of injury severity. Index was determined by counting number of polymorphonuclear neutrophils (PMN) in 10 randomly selected high-power fields and by histological changes.

2.2. Cell culture and treatments

Primary hepatic stellate cells (HSCs) were isolated from male Sprague–Dawley rats (>500 g) as previously described [22]. Freshly isolated HSCs were cultured in DMEM (Sigma–Aldrich) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 1× Antibiotic/Antimycotic (Cellgro, Kansas City, MO), and 200 mM glutamine (Gibco, Carlsbad, California) and grown in a 5% CO_2–95% air atmosphere at 37 °C. Growth medium was exchanged every other day. Culturing HSCs on plastic is routinely used to mimic the in vivo activation process. All animal procedures were performed under the guidelines set by the University of North Carolina at Charlotte Institutional Animal Care and Use Committee and are in accordance with those set by the National Institutes of Health. In vivo studies were approved by the internationally accepted principles in the care and the use of experimental animals of the National Defense Medical Center. JKB-119 and Nalmefene (Nal), were provided by Jenken Biosciences, Inc. (Research Triangle Park, NC) JKB-119 and Nal were solubilized in sterile water at 2 mg/mL, and 10 mg/mL respectively. Dose responses with JKB-119 (0.2 μg/mL, 2 μg/mL, and 20 μg/mL) and Nal (1 μg/mL, 10 μg/mL, and 100 μg/mL) were performed and most effective doses were chosen. No treatment was used as a negative control for all experiments. Prior to treatment, culture-activated HSCs were serum starved for 24 h. After 24 h of serum starvation, day 5 culture-activated HSCs were treated with either opioid compound JKB-119 (20 μg/mL) or Nal (100 μg/mL) in 0% FBS DMEM. For p-ERK experiments, day 5 culture-activated HSCs were treated with or without serum in combination with either compound.

2.3. Isolation of RNA and reverse transcription PCR (RT-PCR)

Total RNA was isolated from HSCs after 48 h of JKB-119 or Nal treatment using TRIzol (Invitrogen Corporation, Baltimore, MD), followed by integrity gels and quantitation as previously described [22]. RNA was reverse transcribed with Superscript II (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR amplifications were performed as previously described for 18–22 cycles. Densitometric quantitation of band intensity was performed using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA).

2.4. Cell lysate preparation and immunoblotting

Whole cell extracts were harvested from HSCs after 30 min, 48 h or 72 h of JKB-119 or Nal treatment with protein lysis buffer (50 mM Tris–HCl, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2% SDS, EDTA 1 mM, aprotinin, and protease inhibitor cocktail) at 4 °C. Lysates were centrifuged at 16,000 × *g* for 10 min at 4 °C and supernatants collected. For secreted protein analysis, after 48 or 72 h of JKB-119 or Nal treatment, cell culture media was centrifuged at 14,000 × *g* for 10 min at 4 °C in Nanosep filtration tubes (Pall Corporation; Ann Arbor, MI). Concentrated protein was washed with 1× PBS, and equal amounts of protein lysis buffer with protease inhibitors were added. Protein concentrations were measured by Bradford assay (Bio-Rad Laboratories, Inc.). Membranes were washed, and bound enzymes were detected with enhanced chemiluminescent (ECL) solution (Pierce Biotechnology, Inc., Rockford, IL). Densitometric quantification of Western blot signal intensity was performed using a densitometric analysis program (Quantity One, Bio-Rad Laboratories, Inc.). Western blot analysis was performed as previously described [23]. p-ERK, Hsp47, Grp78, PDI and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GAPDH and αSMA

antibodies were purchased from Millipore (Billerica, MA) and Dako Corporation (Carpinteria, CA) respectively.

2.5. Proliferation assays

Cellular viability and proliferation were assessed by direct cell count and BrdU incorporation assays. HSCs were seeded at 1 million cells per 9.5 cm^2 well for 4 days in standard culture medium (as described in Section 2.1). After 24 h of serum starvation, day 5 culture-activated HSCs were treated with either JKB-119 (20 μg/mL) or Nal (100 μg/mL) in 0% FBS DMEM for 48 or 72 h. Cells were then trypsinized using 1× 0.5% Trypsin–EDTA (Gibco, Invitrogen Corporation, Baltimore, MD), centrifuged at 500 × *g* for 5 min to pellet, and counted on a hemocytometer (Hausser Scientific, Horsham, PA). Trypan blue analyses were performed to determine cellular viability following treatment. For BrdU incorporation assays, HSCs were seeded on a 96-well plate (0.32 cm^2/well) at a density of 60,000 cells per well. For cell cycle synchronization, day 4 culture-activated HSCs were serum starved for 24 h. Proliferation of day 5 HSCs was induced by 10% FBS exposure in culture medium containing JKB-119 or Nal, and BrdU added for the last 24 h of treatment. BrdU proliferation assay was performed according to the manufacturer's instructions (Exalpha Biological, Inc., Watertown, MA) 48 or 72 h post treatment with JKB-119 or Nal.

2.6. Zymography

MMP enzymatic activity was determined using media harvested from HSCs 72 h after treatment with or without JKB-119 or Nal. MMP2 and MMP13 enzymatic activity was determined using 10% pre-cast gels with gelatin or 12% pre-cast gels with casein (Bio-Rad Laboratories, Inc.) respectively. Zymogram assays were performed according to the manufacturers instructions (Bio-Rad Laboratories, Inc.) following 72 h of treatment with JKB-119 or Nal.

2.7. Statistical analysis

Results of a minimum of three independent experiments per condition are presented as mean ± standard error of mean. Analyses performed include paired *t*-tests, repeated measures one-way ANOVA or Mann–Whitney Rank Sum test where appropriate to determine overall significance. A *p*-value of <0.05 was considered significant.

3. Results

3.1. JKB-119 and Nalmefene alter pro-fibrotic collagen and αSMA expression

OR antagonists have been shown to be hepatoprotective in liver injury animal models [13,14]; therefore, we wanted to determine if two such compounds, JKB-119 and Nalmefene (Nal), could abrogate pro-fibrotic activities of HSCs and which property, either anti-inflammatory or opioid antagonism provides maximal benefit. To verify HSCs have the ability to respond to opioids and OR antagonists, we examined OR mRNA expression. Total RNA was harvested from day 5 culture-activated HSCs, and RT-PCR determined mRNA expression of all three opioid receptor classes (δOR, κOR, μOR) (data not shown). Additionally, DeMinicis et al. showed HSCs express these three OR classes at the protein level [24].

We next examined mRNA expression of type I collagen, the hallmark of liver fibrosis, and αSMA, a marker of activated HSCs. RT-PCR analyses determined treatment with JKB-119 for 48 h caused a 44% decrease in α1(I) collagen mRNA expression, a 32% decrease in α2(I) collagen and a 53% decrease in αSMA mRNA compared to control (Fig. 1A). Treatment with Nal for 48 h caused a 49% decrease in α1(I) collagen mRNA expression, a 40% decrease in α2(I) collagen mRNA expression, and a 48% decrease αSMA mRNA expression compared to the control (Fig. 1A). Additionally, Western blot analyses showed that both JKB-119 and Nal significantly decreased αSMA protein expression by 47% and 44%, respectively, after 48 h compared to control (Fig. 1B). Surprisingly, a significant increase was seen in intracellular type I collagen following 48 h of JKB-119 treatment with no significant changes observed as a result of Nal exposure. Constitutively expressed β-actin was used to verify equal protein loading in Western blot analyses. Although protein concentrations were measured by Bradford assay it appeared JKB-119 was modulating

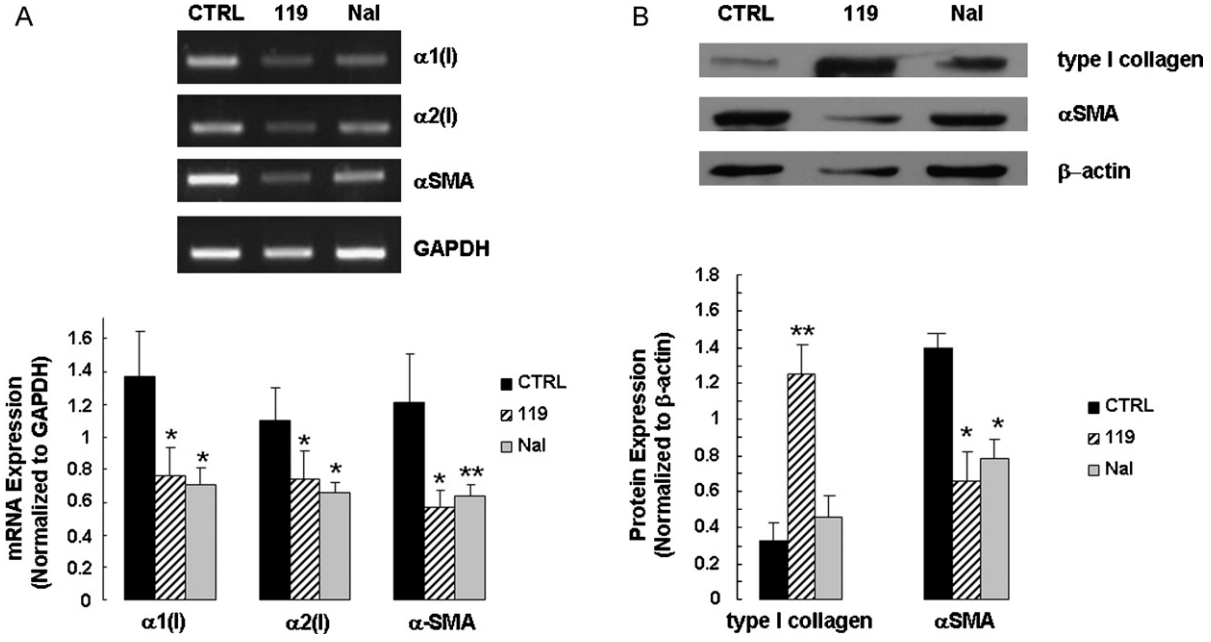

Fig. 1. Effects of JKB-119 and Nalmefene (Nal) on type I collagen and αSMA mRNA and protein expression. Day 5 culture-activated HSCs were treated with or without JKB-119 or Nal and total RNA and protein were harvested after 48 h. (A) mRNA expression of procollagens, α1(I) and α2(I), and αSMA as detected by RT-PCR. (B) Western blot analyses of type I collagen and αSMA. Representative gels/blots, top panel; densitometric analysis, bottom panel. Data expressed as mean ± SEM. $^*p < 0.05$ vs control (CTRL), $^{**}p < 0.001$ vs CTRL.

expression of β-actin; therefore, blots were re-probed with GAPDH, another invariant control, and we observed that changes in αSMA and type I collagen expression would only be further altered if normalized to GAPDH (data not shown).

3.2. JKB-119 and Nalmefene decrease secreted type I collagen protein expression

Activated HSCs are the main source of fibrillar collagens in hepatic fibrosis. Specifically, type I collagen is considered the primary collagen, followed by type III, which alters basic hepatic architecture post liver injury [25]; therefore, secretion of this protein is critical in development and progression of the disease state. Treatment of activated HSCs with JKB-119 for 48 h resulted in significantly decreased collagen secretion, whereas Nal treatment decreased secretion of the protein but not significantly compared to control (Fig. 2).

3.3. JKB-119 and Nalmefene alter MMP2 and MMP13 expression and activity

Since JKB-119 and Nal were shown to inhibit type I collagen mRNA expression and decrease secretion, effects of these compounds on MMP/TIMP expression and activity were determined. Day 5 culture-activated HSCs were treated with or without JKB-119 or Nal for 48 h. Total RNA was harvested and analyzed by RT-PCR. Culture media was harvested at 72 h and analyzed by zymogram staining for enzymatic activity. RT-PCR analyses determined these compounds had no significant effect on MMP2 mRNA expression; however, JKB-119 treatment significantly increased MMP13 mRNA expression (Fig. 3A). Additionally, RT-PCR determined Nal significantly reduced TIMP1 mRNA expression, and while both compounds decreased TIMP2 mRNA expression, these changes were not found to be statistically significant (data not shown). However, zymogram analyses determined JKB-119 significantly increased enzymatic activity of MMP2, while Nal had a minimal effect. Additionally, both JKB-119 and Nal decreased MMP13 activity (Fig. 3B).

3.4. JKB-119 and Nalmefene treatment do not alter ER protein expression

JKB-119 and Nal treatments decreased secreted type I collagen protein expression, suggesting these compounds may interfere with intricate processing and/or folding of type I collagen, thus inhibiting secretion. Since type I collagen is processed in the ER prior to secretion, we examined the effects of JKB-119 and Nal on several regulatory ER proteins, including heat shock protein 47 (Hsp47), 78-kDa glucose regulated protein (Grp78), and protein disulfide isomerase (PDI). No change in resident ER protein expression was observed upon treatment with JKB-119 or Nal at 48 or 72 h (Fig. 4).

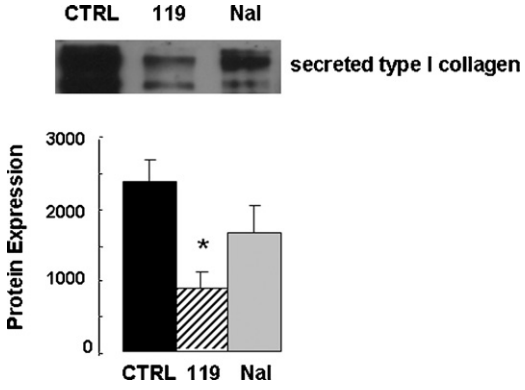

Fig. 2. JKB-119 and Nal decrease secreted type I collagen. Day 5 culture-activated HSCs were treated with or without JKB-119 or Nal and culture media harvested. Secreted proteins were concentrated and type I collagen detected using Western blot analysis. Representative blot, top panel; densitometric analysis, bottom panel. Data expressed as mean ± SEM. $^*p < 0.05$ vs control (CTRL).

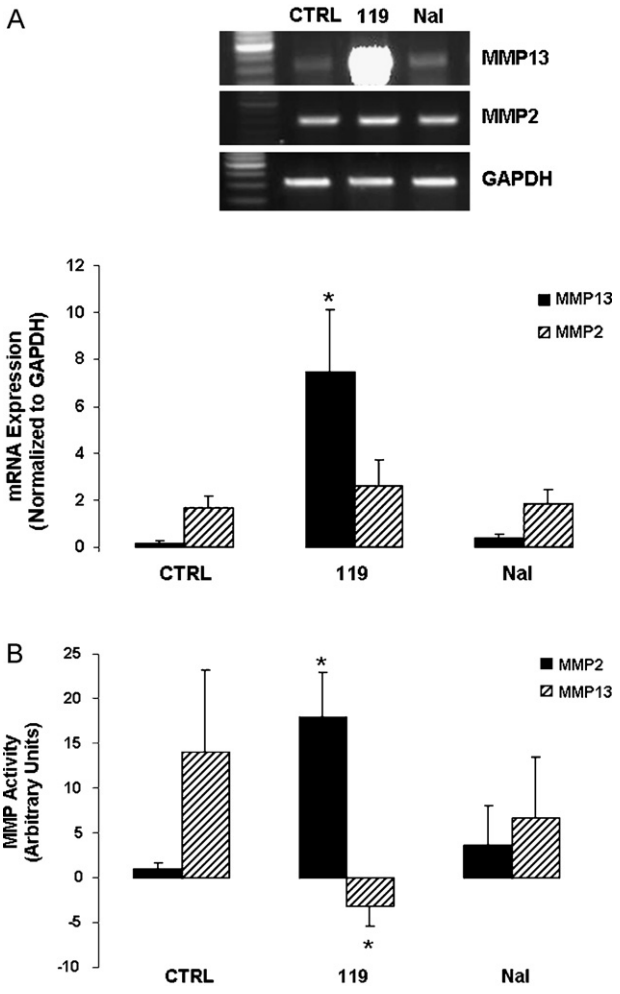

Fig. 3. Effects of JKB-119 and Nal on MMP13 and MMP2 mRNA expression and activity. Day 5 culture-activated HSCs were treated with or without JKB-119 or Nal and total RNA and culture media harvested at 48 h. (A) RT-PCR analyses of MMP13 and MMP2. (B) Zymography analyses of MMP enzymatic activity. Data are presented as mean ± SEM. $^*p < 0.05$ vs control (CTRL).

3.5. JKB-119 and Nalmefene decrease HSC proliferation independently of p-ERK

Cellular viability after drug treatment was determined by performing trypan blue exclusion assay. JKB-119 decreased cellular viability at 48 and 72 h, whereas Nal had no significant affect (Fig. 5A). Proliferation was significantly decreased after both 48 and 72 h of JKB-119 treatment and after 72 h with Nal. (Fig. 5B). As treatment resulted in altered cellular viability and proliferation, we further explored effects of these compounds on canonical proliferation pathways. We observed the effects of JKB-119 or Nal on p-ERK, a key signaling component in sustained HSC activation and subsequent accumulation of type I collagen. Day 5 HSCs were treated with or without 10% serum to induce proliferation. Additionally, cells were treated with or without JKB-119 or Nal in 10% serum. Treatment with either derivative for 15 or 30 min did not significantly alter p-ERK expression (Fig. 5C).

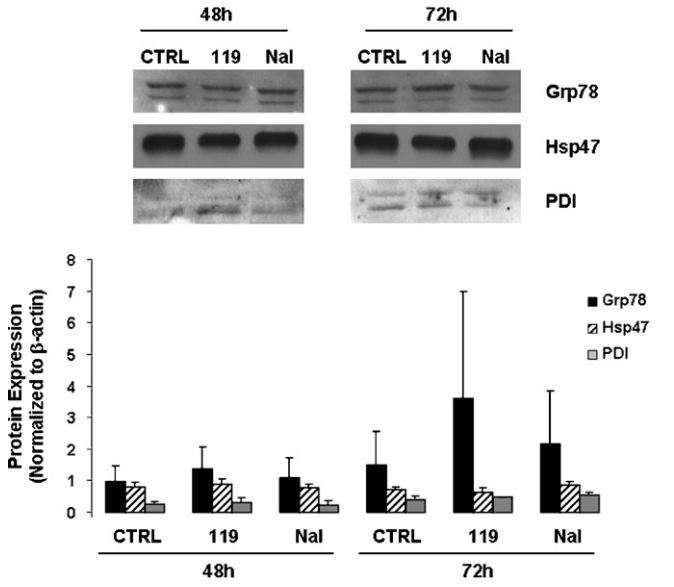

Fig. 4. JKB-119 and Nal do not alter ER protein expression. Day 5 culture-activated HSCs were treated with or without JKB-119 or Nal and total protein harvested at 48 and 72 h. Representative blots, top panel; densitometric analysis, bottom panel. Data expressed as mean ± SEM.

3.6. JKB-119 inhibits inflammatory liver injury

Increased inflammation and cytokine signaling are critical components in progression of liver disease. Bacterial endotoxin (lipopolysaccharide, LPS), provides a reproducible and well controlled model of inflammatory stress that can be observed in a variety of hepatic injury/disease states [17]. LPS administration allows for proof-of-concept that use of JKB-119 is able to prevent an exacerbated inflammatory response observed in liver disease (specifically, steatosis and fibrosis). Furthermore, JKB-119 was a more significant inhibitor of HSC activation which is the major contributor to development of fibrosis and thus an important therapeutic target. As indicated in Fig. 6A, LPS treatment (1–2 h) resulted in significant increases in serum TNFα levels. However, when LPS was administered in combination with JKB-119 this effect was abrogated. Additionally, similar results were observed with liver PMN infiltration (Fig. 6B), where JKB-119 treatment significantly blunted the acute deleterious inflammatory response. Not only did JKB-119 reduce inflammation, but reduced overall hepatic injury as serum ALT levels were significantly decreased compared to control (Fig. 6C).

4. Discussion

Hepatic fibrosis is correlated with increased opioid expression with numerous models of liver injury demonstrating increased tissue and plasma opioid peptide levels [15,26–30] indicative of neurotransmission [14]. Additional roles for opioids include regulation of cytokine and cytokine receptor expression, such as TNFα, IL-1 and IL-6, all of which are both pro-infammatory and pro-fibrotic. Inhibition of endogenous opioid signaling via OR antagonists was previously proposed as a therapeutic strategy for treatment of liver injury. Antagonist anti-inflammatory compounds Naltrexone and Naloxone were shown to decrease liver injury in several animal models [13,15,31]. Despite effective anti-fibrotic activity of Naltrexone, the FDA's black box label warning of hepatotoxicity discourages its use. Moreover, Naloxone is associated with a greater variety of risks and complications, including drug dependence resulting in restricted access in the United States. Therefore, new opioid compounds devoid of deleterious side-effects are being developed for anti-fibrotic therapies. JKB-119 is a morphinan analog of Naltrexone and is predicted to possess immunomodulatory properties without significant opioid receptor binding as opposed to Nalmefene (Nal, Naltrexone derivative) which displays opioid receptor antagonism and immunomodula-

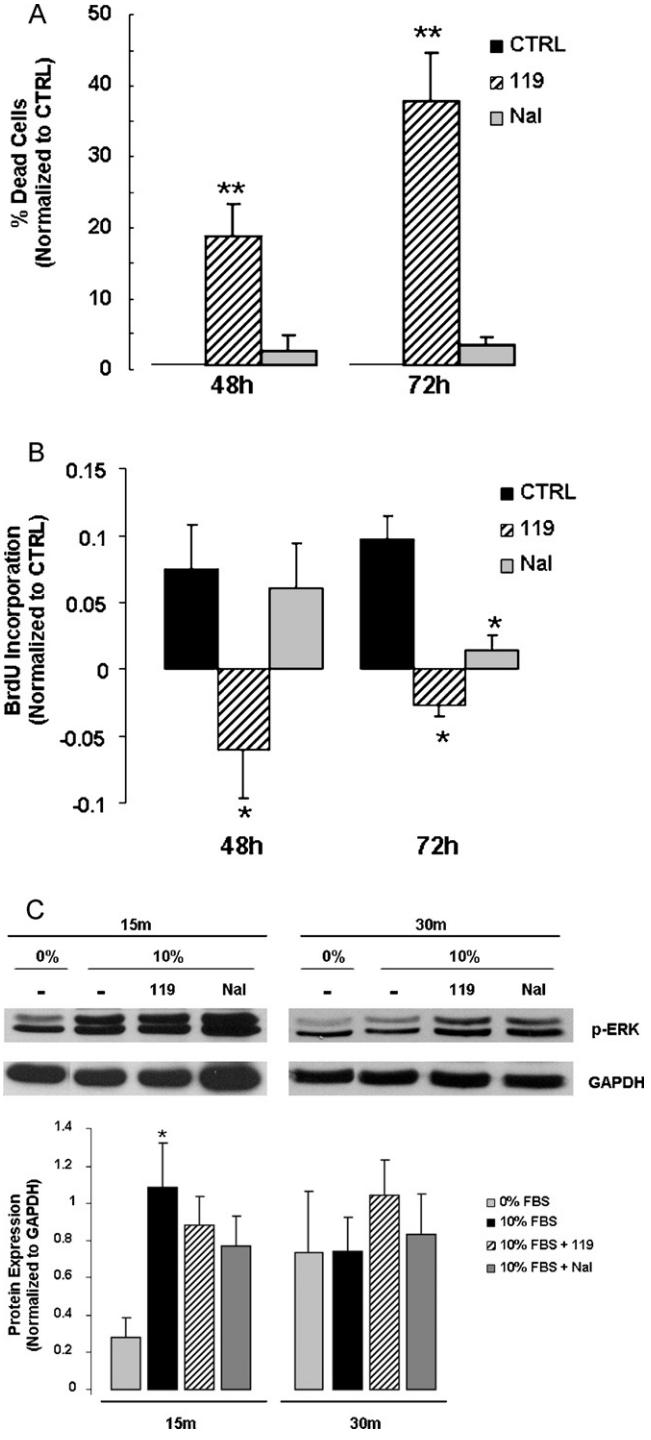

Fig. 5. Effects of JKB-119 and Nal on HSC viability and proliferation. Day 5 culture-activated HSCs were treated with or without JKB-119 or Nal for 48 and 72 h in 10% serum. (A) Cell viability was assessed by trypan blue exclusion assay. Positive cells were counted and results expressed as percent dead cells normalized to control (CTRL). $^{**}p < 0.001$ vs CTRL. (B) HSC proliferation was assessed by BrdU incorporation assay and normalized to CTRL. $^{*}p < 0.05$ vs CTRL. (C) Cells were treated with or without JKB-119 for 15 or 30 min (m) and phopsphorylated-ERK (p-ERK) protein expression was detected by Western blot. Representative blot from p-ERK, top panel; densitometric analysis, bottom panel. Data expressed as mean ± SEM, $^{*}p < 0.05$ vs 0% FBS.

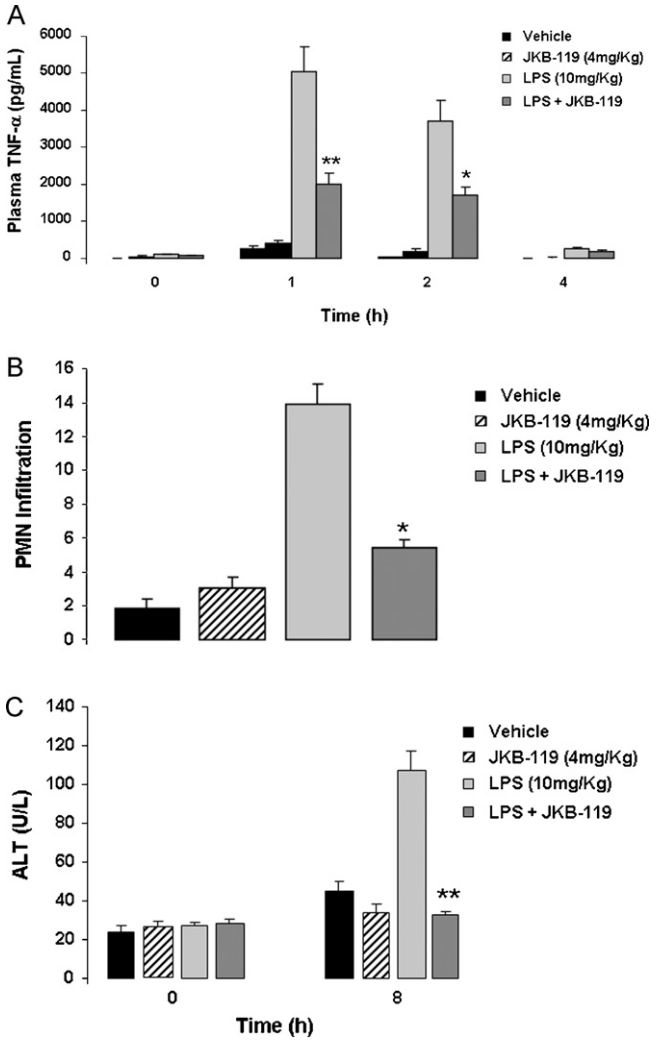

Fig. 6. In vivo effects of JKB-119 in a model of hepatic inflammation. Animals were randomly assigned to experimental groups. After indicated time points, blood and tissue were harvested for analysis of plasma TNFα (A), histopathological examination or tissue stained for polymorphonuclear neutrophils (PMN) at 8 h (B) and serum ALT (C). Data expressed as mean ± SEM, $^{*}p < 0.05$ vs LPS, $^{**}p < 0.001$ vs LPS.

tory properties. In vitro studies enabled us to compare which mechanism, immunomodulatory or opioid antagonism, was most effective in suppressing pro-fibrotic activation of HSCs. Additionally, using an inflammatory LPS injury animal model, we were able to test the novel compound JKB-119 for therapeutic benefit in vivo as opposed to previously investigated compound Nal [32–34].

To discern whether compounds possessing immunomodulatory or OR antagonist properties were more effective at reducing injurious HSC activation, both JKB-119 and Nal were tested on primary rat HSCs. Treatment with JKB-119 or Nal significantly reduced collagen α1(I), collagen α2(I) and αSMA mRNAs with both compounds significantly reducing αSMA protein expression. Interestingly, intracellular type I collagen protein was increased as a result of JKB-119 administration, but significantly decreased by Nal, an unexpected result due to decreased mRNA expression observed following treatment with both derivatives. Although increased intracellular type I collagen is a consequence of HSC activation, it is the secretion of this protein that is the foundation of hepatic fibrosis. Following 48 h of JKB-119 treatment, type I collagen secretion by activated HSCs was significantly reduced as compared to control and Nal. These data suggest that JKB-119 may interfere with the processing and/or folding of type I collagen, thereby inhibiting secretion, which would be important in prevention of fibrotic disease. Type I collagen, like other secretory proteins, must be extensively and properly processed in the endoplasmic reticulum prior to secretion. Several regulatory proteins, most notably Hsp47, Grp78 and PDI, are involved with the intricate processing of collagen. However; expression of all aforementioned regulatory proteins appeared unaltered as a result of either JKB-119 or Nal treatment, suggesting that JKB-119 may specifically block secretion of type I collagen through an alternate mechanism. The exact mechanism by which JKB-119 interferes with type I collagen secretion remains unknown and it would be interesting to determine whether this particular compound affects other secretory proteins. Additionally, reduced changes in αSMA and type I collagen expression may be due to reduced oxidative stress and inhibition of TGFβ signaling. Studies from our group have shown that S-adenosyl-L-methionine (SAMe), a precursor to glutathione, can inhibit oxidative stress and TGFβ signaling thus decreasing collagen and SMA expression in activated HSCs [17]. Furthermore, Naltrexone, an opioid compound similar to JKB-119, also demonstrated reduced collagen and SMA expression in a fibrotic liver animal model, while levels of GSH were increased suggesting reduced oxidative stress as a possible mechanism [14].

HSC activation is characterized by significant increases in cellular proliferation which ultimately contribute to increased deposition of fibrotic scar matrix. Treatment with JKB-119 significantly reduced proliferation of activated HSCs after 48 h of administration, whereas Nal only reached significant reduction after 72 h of treatment. However, canonical ERK phosphorylation was not significantly altered despite the decreased proliferation detected by BrdU, suggesting these compounds act through alternate pathways including p38 MAPK and JNK. In addition to reduced DNA synthesis, total cell number was also significantly reduced (data not shown). Upon further analysis, trypan blue exclusion assays revealed a detrimental effect on HSC viability after 48 and 72 h of JKB-119 treatment. While non-specific cytotoxicity is alarming, specific killing of activated HSCs would be extremely beneficial in a therapeutic setting if concentrations needed did not affect neighboring cell types. Preliminary studies in our lab using parenchymal hepatocytes showed JKB-119 does not cause similar decreases in cell viability; however, further experimentation is necessary not only in hepatocytes but also in other non-parenchymal cells and in HSC co-culture systems. Since JKB-119 and Nal inhibited type I collagen expression and decreased collagen secretion, in the case of JKB-119, MMP and TIMP expression/activity were also determined. In rats, MMP13 degrades type I collagen, whereas MMP2 directs ECM remodeling via degradation of type IV collagen. If either treatment decreased collagen levels by modulating MMP enzymatic activity, we would expect to see decreased MMP2 reducing normal matrix degradation and increased MMP13 activity, promoting type I collagen degradation. Treatment with JKB-119 or Nal had no significant effect on MMP2 expression; however, JKB-119 markedly increased MMP13 mRNA. In contrast to expression data, JKB-119 significantly decreased MMP13 activity and significantly increased MMP2, with Nal having no significant effects. Future in vitro studies should examine the discrepancy between MMP13 mRNA and activity. As a putative mechanism miRNAs 27a and 27b are predicted (by TargetScan) to bind to MMP13 mRNA suggesting that these miRNAs could inhibit mRNA translation and thus decrease protein expression and activity even in the presence of increased message. Additionally, these miRNAs have already been shown to influence HSC activation [35], making this an interesting avenue to explore in future studies.

In this study we observed that JKB-119 was capable of protecting against hepatic inflammation in vivo. Administration of the compound significantly decreased levels of TNFα and hepatic PMN infiltration signifying decreased inflammation and

liver damage compared to LPS-treated animals. Finally, in vivo studies showed that JKB-119 administration significantly reduced serum ALT levels, showing that not only is inflammation reduced but overall liver damage is decreased by JKB-119. A primary role for TNFα in inflammatory liver injury/disease has been proposed in initiation of HSC activation. Studies utilizing TNFα knockout mice in a fibrotic model demonstrated these mice possessed lower hepatic collagen expression and decreased fibrosis. Additionally, levels of αSMA, a marker for activated HSCs, were markedly reduced compared to wild-type mice [36]. Additionally, although large contributors, HSCs are not the primary source of TNFα in the liver, future studies should examine the effect of JKB-119 on resident macrophages (Kupffer cells, KCs). It is likely that JKB-119 will impede TNFα secretion from KCs, since unpublished studies from our group demonstrated that Nal inhibits LPS-induced TNFα secretion from KCs.

Overall our studies showed that the morphinan derivative JKB-119 is anti-inflammatory in vivo, which holds clinical promise. Additionally, both JKB-119 and Nal appear to exert anti-fibrotic effects through decreased HSC activation. As similar results were obtained using JKB-119 and Nal in vitro, our data suggest the effects seen on HSC activation are independent of opiate receptor antagonism. Although we and others have demonstrated opioid receptor expression in activated HSCs, our system did not employ agonists, further suggesting that both compounds act primarily through modulation of inflammatory responses.

Currently, there are no FDA-approved drug regimes for the treatment of liver fibrosis. The use of anti-oxidants to diminish deleterious inflammation associated with pathology of the disease is common; however, limited clinical benefit in humans has been observed. It is apparent that JKB-119 possesses promise for therapeutic use by inhibiting HSC activation and decreasing liver injury in an inflammatory animal model. Interestingly, this compound did not modulate canonical signaling pathways and/or molecules associated with HSC proliferation and secretion of fibrotic type I collagen. Future studies should focus on elucidating the exact mechanism by which JKB-119 is able to suppress HSC activation. During the process of HSC activation, numerous changes in gene expression profile and morphology are observed [22], in particular toll-like receptor 4 (TLR4) signaling is increased and perpetuates the fibrotic response through downstream activation of pro-fibrogenic cytokine TGFβ [37]. Recent studies have shown that opioid-antagonists, Naloxone and Naltrexone, are capable of suppressing glial cell activation, which display similar HSC markers of origin, via TLR4 antagonism [38], making this an attractive potential mechanism to investigate. HSCs are involved in the hepatic immune response through TLR4 signaling and antigen presentation [39]. TLR4-mediated upregulation of NFκB and JNK leads to overexpression of adhesion molecules and chemokines promoting the inflammatory response [40]. Thus, a treatment that could modulate HSC activation and inflammation could be an effective therapeutic for treating liver disease. Therefore, future studies will be aimed at examining the anti-inflammatory effects of JKB-119 in a fibrotic animal model.

Acknowledgements

The authors thank Cathy Culberson for her technical assistance and insightful comments during the preparation of this manuscript. This work was supported by SRL of North Carolina Biotechnology Center.

References

[1] Mani AR, Moore KP. New insights into the role of endogenous opioids in the pathogenesis of gastrointestinal and liver disease. Gut 2009;58:893–5.
[2] Armendariz-Borunda J, Katayama K, Seyer JM. Transcriptional mechanisms of type I collagen gene expression are differentially regulated by interleukin-1 beta, tumor necrosis factor alpha, and transforming growth factor beta in Ito cells. J Biol Chem 1992;267:14316–21.
[3] Bataller R, Brenner DA, Liver Fibrosis. J Clin Invest 2005;115:209–18.
[4] Choi I, Kang HS, Yang Y, Pyun KH. IL-6 induces hepatic inflammation and collagen synthesis in vivo. Clin Exp immunol 1994;95:530–5.
[5] Greenwel P, Rubin J, Schwartz M, Hertzberg EL, Rojkind M. Liver fat-storing cell clones obtained from a CCl4-cirrhotic rat are heterogeneous with regard to proliferation, expression of extracellular matrix components, interleukin-6, and connexin 43. Lab Invest J Tech Methods Pathol 1993;69:210–6.
[6] Natsume M, Tsuji H, Harada A, Akiyama M, Yano T, Ishikura H, et al. Attenuated liver fibrosis and depressed serum albumin levels in carbon tetrachloride-treated IL-6-deficient mice. J Leukoc biol 1999;66:601–8.
[7] Tsukamoto H. Cytokine regulation of hepatic stellate cells in liver fibrosis alcoholism. Clin Exp Res 1999;23:911–6.
[8] Elsharkawy AM, Mann DA. Nuclear factor-kappaB and the hepatic inflammation-fibrosis-cancer axis. Hepatology (Baltimore MD) 2007;46:590–7.
[9] Iredale JP, Goddard S, Murphy G, Benyon RC, Arthur MJ. Tissue inhibitor of metalloproteinase-I and interstitial collagenase expression in autoimmune chronic active hepatitis and activated human hepatic lipocytes. Clin Sci (Lond) 1995;89:75–81.
[10] Matsuoka M, Pham NT, Tsukamoto H. Differential effects of interleukin-1 alpha, tumor necrosis factor alpha, and transforming growth factor beta 1 on cell proliferation and collagen formation by cultured fat-storing cells. Liver 1989;9:71–8.
[11] Pinzani M, Gesualdo L, Sabbah GM, Abboud HE. Effects of platelet-derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat-storing cells. J Clin Invest 1989;84:1786–93.
[12] Greenwel P, Schwartz M, Rosas M, Peyrol S, Grimaud JA, Rojkind M. Characterization of fat-storing cell lines derived from normal and CCl4-cirrhotic livers Differences in the production of interleukin-6. Lab Invest J Tech Methods Pathol 1991;65:644–53.
[13] De Minicis S, Candelaresi C, Marzioni M, Saccomanno S, Roskams T, Casini A, et al. Role of endogenous opioids in modulating HSC activity in vitro and liver fibrosis in vivo. Gut 2007.
[14] Ebrahimkhani MR, Kiani S, Oakley F, Kendall T, Shariftabrizi A, Tavangar SM, et al. Naltrexone, an opioid receptor antagonist, attenuates liver fibrosis in bile duct ligated rats. Gut 2006;55:1606–16.
[15] Marzioni M, Alpini G, Saccomanno S, de Minicis S, Glaser S, Francis H, et al. Endogenous opioids modulate the growth of the biliary tree in the course of cholestasis. Gastroenterology 2006;130:1831–47.
[16] Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev 2008;88:125–72.
[17] Karaa A, Thompson KJ, McKillop IH, Clemens MG, Schrum LW. S-adenosyl-L-methionine attenuates oxidative stress and hepatic stellate cell activation in an ethanol-lps-induced fibrotic rat model. Shock 2007.
[18] Iida C, Fujii K, Koga E, Washino Y, Kitamura Y, Ichi I, et al. Effect of alpha-tocopherol on carbon tetrachloride intoxication in the rat liver. Arch Toxicol 2009;83:477–83.
[19] Sanyal AJ, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. N Engl J Med 2010;362:1675–85.
[20] Shingu C, Hagiwara S, Iwasaka H, Matsumoto S, Koga H, Yokoi I, et al. EPCK1, a vitamin C and E analogue, reduces endotoxin-induced systemic inflammation in mice. J Surg Res 2010.
[21] Yachi R, Igarashi O, Kiyose C. Protective effects of vitamin E analogs against carbon tetrachloride-induced fatty liver in rats. J Clin Biochem Nutr 2010;47:148–54.
[22] Lakner AM, Moore CC, Gulledge AA, Schrum LW. Daily genetic profiling indicates JAK/STAT signaling promotes early hepatic stellate cell transdifferentiation. World J Gastroenterol 2010;16:5047–56.
[23] Lakner AM, Walling TL, McKillop IH, Schrum LW. Altered aquaporin expression and role in apoptosis during hepatic stellate cell activation. Liver Int 2010.
[24] De Minicis S, Candelaresi C, Marzioni M, Saccomano S, Roskams T, Casini A, et al. Role of endogenous opioids in modulating HSC activity in vitro and liver fibrosis in vivo. Gut 2008;57:352–64.
[25] Iredale JP. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. J Clin Invest 2007;117:539–48.
[26] Bergasa NV, Liau S, Homel P, Ghali V. Hepatic Met-enkephalin immunoreactivity is enhanced in primary biliary cirrhosis. Liver 2002;22:107–13.
[27] Bergasa NV, Rothman RB, Vergalla J, Xu H, Swain MG, Jones EA. Central mu-opioid receptors are down-regulated in a rat model of cholestasis. J Hepatol 1992;15:220–4.
[28] Bergasa NV, Vergalla J, Swain MG, Jones EA. Hepatic concentrations of proenkephalin-derived opioids are increased in a rat model of cholestasis. Liver 1996;16:298–302.
[29] Jones EA, Bergasa NV. The pruritus of cholestasis. Hepatology 1999;29:1003–6.
[30] Swain MG, Rothman RB, Xu H, Vergalla J, Bergasa NV, Jones EA. Endogenous opioids accumulate in plasma in a rat model of acute cholestasis. Gastroenterology 1992;103:630–5.
[31] Kiani S, Ebrahimkhani MR, Shariftabrizi A, Doratotaj B, Payabvash S, Riazi K, et al. Opioid system blockade decreases collagenase activity and improves liver injury in a rat model of cholestasis. J Gastroenterol Hepatol 2007;22:406–13.
[32] Mason BJ, Ritvo EC, Morgan RO, Salvato FR, Goldberg G, Welch B, et al. A double-blind, placebo-controlled pilot study to evaluate the efficacy and

safety of oral nalmefene HCl for alcohol dependence. Alcohol Clin Exp Res 1994;18:1162–7.
[33] Mason BJ, Salvato FR, Williams LD, Ritvo EC, Cutler RB. A double-blind, placebo-controlled study of oral nalmefene for alcohol dependence. Arch Gen Psychiatry 1999;56:719–24.
[34] Wang HL, Yu KJ, Liu HT. Clinical observation of the effect of nalmefene in treatment of septic shock. Zhongguo Wei Zhong Bing Ji Jiu Yi Xue 2010;22:351–3.
[35] Ji J, Zhang J, Huang G, Qian J, Wang X, Mei S. Over-expressed microRNA-27a and 27b influence fat accumulation and cell proliferation during rat hepatic stellate cell activation. FEBS Letters 2009;583:759–66.
[36] Gabele E, Froh M, Arteel GE, Uesugi T, Hellerbrand C, Scholmerich J, et al. TNFalpha is required for cholestasis-induced liver fibrosis in the mouse. Biochem Biophys Res Commun 2009;378:348–53.
[37] Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nat Med 2007;13:1324–32.
[38] Hutchinson MR, Zhang Y, Brown K, Coats BD, Shridhar M, Sholar PW, et al. Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). Eur J Neurosci 2008;28:20–9.
[39] Winau F, Hegasy G, Weiskirchen R, Weber S, Cassan C, Sieling PA, et al. Ito cells are liver-resident antigen-presenting cells for activating T cell responses. Immunity 2007;26:117–29.
[40] Paik YH, Schwabe RF, Bataller R, Russo MP, Jobin C, Brenner DA. Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. Hepatology 2003;37:1043–55.