Targeting Liver Fibrosis with a Cell-penetrating Protease-activated Receptor-2 (PAR2) Pepducin

Andrew M. Shearer, Rajashree Rana, Karyn Austin, James D. Baleja, Nga Nguyen, Andrew Bohm, Lidija Covic, and Athan Kuliopulos

From the 1Center for Hemostasis and Thrombosis Research, Molecular Oncology Research Institute, Tufts Medical Center and 2Tufts University School of Graduate Biomedical Sciences, Biochemistry/Developmental, Molecular and Chemical Biology, Tufts University School of Medicine, Boston, Massachusetts 02111

Chronic liver inflammation and fibrosis in nonalcoholic steatohepatitis can lead to cirrhosis and liver failure for which there are currently no approved treatments. Protease-activated receptor-2 (PAR2) is an emerging new target expressed on liver stellate cells and hepatocytes that regulates the response to liver injury and inflammation. Here, we identified a pepducin to block the deleterious actions of PAR2 in promoting liver fibrosis. Non-alcoholic fatty liver disease and early fibrosis were induced by the methionine-choline-deficient diet in mice. Fibrotic liver disease was induced by administering carbon tetrachloride for 8 weeks. Mice were treated with the pepducin PZ-235 either from onset of the experiment or after fibrosis was established. Hepatic fibrosis, collagen content, inflammatory cytokines, steatosis, triglycerides, and NAFLD activity score were assessed as primary outcome parameters depending on the model. The activity of the PAR2 pepducin on cultured stellate cell activation and hepatocyte reactive oxygen species production was evaluated. PZ-235 significantly suppressed liver fibrosis, collagen deposition, inflammatory cytokines, NAFLD activity score, steatosis, triglycerides, aspartate transaminase, alanine transaminase, and stellate cell proliferation by up to 50–100%. The PAR2 inhibitor afforded significant protective effects against hepatocellular necrosis and attenuated PAR2-mediated reactive oxygen species production in hepatocytes. PZ-235 was distributed to liver and other mouse tissues and was mediated reactive oxygen species production in hepatocytes.

Nonalcoholic steatohepatitis (NASH) is a progressive fibrotic disease of the liver with a rising incidence in both obese adults and children (1–3). Typically, individuals are clinically asymptomatic until the disease has progressed over several years, which has led NASH to be considered a “silent” liver disease. NASH has become one of the most common causes of chronic liver disease in the industrialized world; however, the underlyng etiology is largely unknown. NASH is often associated with central obesity, metabolic syndrome, and type II diabetes mellitus, thus reflecting a relationship with disorders of systemic metabolism and inflammation (2). A number of experimental therapeutic strategies to potentially treat NASH are in phase 1–3 clinical trials, including antioxidants, farnesoid X or peroxisome proliferator–activated receptor ligands, and antifibrotic agents (4). Despite these efforts, there are no therapies yet approved for the treatment of NASH and related fibrotic liver diseases (4, 5).

A critical trigger of NASH fibrosis is the activation of hepatic stellate cells by any one of multiple pathways associated with recurrent tissue damage, inflammation, and remodeling (6, 7). Stellate cells normally exist as quiescent vitamin A–enriched cells located in the space of Disse between luminal endothelial cells and hepatocytes (8). Upon activation, stellate cells convert to a myofibroblastic phenotype that up-regulates matrix and collagen deposition pathways, leading to the development of liver fibrosis and eventually cirrhosis (9). Activated stellate cells also secrete a number of inflammatory cytokines to create a proinflammatory environment and stimulate recruitment and activation of white blood cells (10, 11). Activated stellate cells also secrete a number of inflammatory cytokines to create a proinflammatory environment and stimulate recruitment and activation of white blood cells (10, 11). Activated stellate cells can persist in an activated state until the cessation of insult or tissue damage whereupon they either undergo apoptosis or re-enter an inactive state that is similar to their initial state (12). In cases of recurrent liver injury, stellate cells remain in an activated form and prolong the exposure of the liver to heightened inflammatory signals and collagen matrix deposition, leading to the development of overt liver pathology. The central importance of stellate cells in the development of fibrotic liver disease has led to a concerted effort by many groups to target and inhibit their activation (4).

The abbreviations used are: NASH, non-alcoholic steatohepatitis; s.c., subcutaneous; NAFLD, non-alcoholic fatty liver disease; MCD, methionine-choline-deficient; NAS, NAFLD activity score; ROS, reactive oxygen species; AST, aspartate transaminase; ALT, alanine transaminase; PAR, protease-activated receptor; GPCR, G protein-coupled receptor; i3, third intracellular; MCP-1, monocyte chemoattractant protein-1; PI, propidium iodide.

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An emerging new target for the potential treatment of fibrotic diseases of the liver and other tissues is protease-activated receptor-2 (PAR2), a G protein–coupled receptor that has been shown to play an important role in mesenchymal cell activation and inflammatory signaling (13–17). PAR family receptors are activated by proteolytic cleavage of their extracellular N-terminal domain by certain serine or metalloproteases, creating a tethered ligand that activates the receptor in an unusual intramolecular mode (18–20). PAR2 is activated by trypsin-like proteases such as trypptase (13) and matriptase (21) and by coagulation factors VIIa and Xa (22–25) when tissue factor expression levels are up-regulated as occurs in subjects with fibrotic liver disease (26). The confluence of these PAR2 proteases during tissue injury and remodeling leads to a microenvironment that may trigger prolonged pathological activation of PAR2 signaling pathways. These include activation of mitogen-activated protein kinases (ERK, p38, and JNK) involved in proliferation, inflammation, and differentiation of mesenchymal cells via TGF-β, TNF-α, IL-1β, and NF-κB pathways during tissue fibrosis (14–16, 27, 28).

Here, we determined whether pepducin technology could be exploited as a potential treatment of liver fibrosis by blocking the profibrotic actions of PAR2 in liver. Pepducins (29–31) comprise a short peptide derived from a GPCR intracellular loop tethered to a hydrophobic moiety such as palmitate. This structure allows the lipopeptide to anchor in the cell membrane lipid bilayer, rapidly flip across to the inner leaflet in a reversible manner (32–35), and target the GPCR/G protein interface via an intracellular allosteric mechanism (30, 36, 37). We describe a PAR2 pepducin that is able to effectively suppress PAR2-driven fibrosis, inflammation, steatosis, and hepatocellular necrosis, suggesting a novel multipronged approach to treat severe liver disease.

Results

The Cell-penetrating Pepducin PZ-235 Reduces Steatosis and Inflammation in the Methionine-Choline-deficient (MCD) Diet-induced Model of NAFLD in Mice—To identify an effective PAR2 antagonist that might be potentially useful as an agent to treat NASH, we screened several hundred pepducins with mutations of critical third intracellular (i3) loop pharmacophores (13). In a primary screen, the i3 loop pepducin, PZ-235 (P2pal-18S), was identified as a full antagonist of PAR2-G protein signaling (13, 38) or PAR2 calcium responses in either vehicle or 5 mg/kg PZ-235 for 21 days. There was a striking reduction in hepatocyte vesicular fat and inflammation in the PZ-235 treatment group (Fig. 1A). The NAFLD activity score (NAS) is a histological assessment used to grade and diagnose NASH in patients with a score of ≥5 as the defining threshold for NASH. The three histologic features include steatosis, lobular inflammation, and hepatocyte ballooning. The NAS values were lower in the PZ-235-treated livers (Fig. 1B) with the largest reductions in both steatosis and lobular inflammation. PZ-235 treatment significantly reduced the overall NAS by 50%. Liver triglycerides were also significantly reduced by 66% in the PZ-235-treated animals (Fig. 1C). Likewise, PZ-235 afforded significant protective effects on plasma alanine transaminase (ALT) and aspartate transaminase (AST) (Fig. 1, D and E). PAR2 expression in the liver increased by 7.4-fold in the MCD mice, and this was significantly suppressed by PZ-235 treatment. These data suggest that PAR2 antagonism with PZ-235 protects against liver steatosis, triglycerides, and inflammation in the MCD model.

To assess whether the pepducin could potentially impact early liver fibrosis, we extended the MCD model out to 8 weeks, a time point at which liver fibrosis becomes evident in this NASH model. Although not significant due to the variance in the percentage of fibrosis in the untreated group (0.1–1.4%), the PZ-235 treatment gave an apparent 30% mean protective effect on liver fibrosis at the 8-week end point in the MCD mice (supplemental Fig. 2, A and B).

Suppression of CCl4-induced Liver Fibrosis and Inflammatory Cytokines with Delayed PZ-235 Treatment—The efficacy of PZ-235 in suppressing more severe liver fibrosis was then tested using 8-week treatment with carbon tetrachloride (CCl4) to induce extensive liver injury. We delayed administration of PZ-235 until 4 weeks after initiation of CCl4-induced liver fibrosis. Liver fibrosis was quantified after an additional 4 weeks of daily injections of PZ-235 (5 mg/kg) or vehicle control at the 8-week end point (Fig. 2A). In vehicle animals, liver sections revealed a 6-fold increase in liver fibrosis from both sinusoidal collagen deposition and septal fibrosis that was significantly reduced by 75% by delayed PZ-235 treatment (Fig. 2, B and C). A negative control PAR2 pepducin, PZ-234, which has no observed inhibitory effects on PAR2-G protein signaling (P2pal-14GF (13, 38)) or PAR2 calcium responses in either SW620 cells or HUH7 hepatocytes (supplemental Fig. 1, B and C) gave no antiﬁbrotic effects in the 8-week liver fibrosis model (Fig. 2, B and C).

As hepatocytes are very sensitive to proinflammatory stimuli, we examined the expression of markers associated with inflammation and stellate cell activation in the livers of mice treated with and without PZ-235. There were pronounced 3.5–6.5-fold elevations in gene transcripts associated with liver inflammation, including IL-8, IL-6, TNFα, and monocyte chemoattractant protein-1 (MCP-1) in vehicle control animals treated for 8 weeks with CCl4 as compared with normal mice (Fig. 3). There was also a significant increase in type IV collagen α1 (collagen IV) in the untreated CCl4 livers. Delayed PZ-235 treatment completely suppressed the increases in expression of IL-8, IL-6, and TNFα to baseline or below (Fig. 3). PZ-235 treatment also suppressed the increases in MCP-1 and collagen IV by 36–60%, which is notable as type IV collagen is considered to be the major collagen type involved in early stage septal fibrosis (39).

PZ-235 Inhibits PAR2-dependent Stellate Cell Activation—To directly determine the activity and specificity of the PAR2 antagonist on stellate cells, we showed that PZ-235 blocked PAR2 calcium signaling without inhibiting the thrombin
stimulation of the stellate cells with 10 nM trypsin significantly
control group. ****, stellate cell viability was inhibited by 75% with 1
increased cell viability over a 4-day period. This increase in
treatment that was blocked by PZ-235 (Fig. 4
in MCP-1 secretion in response to both SLIGRL and trypsin
induce secretion of MCP-1. LX-2 stellate cells gave an increase
esis in liver (40), we tested whether PAR2 activation could
ment of inflammatory cells via MCP-1 during active fibrogen-
production—
PAR2-driven Mitochondrial Reactive Oxygen Species (ROS)
(Fig. 4
staining of liver sections from PZ-235
phology when comparing the hematoxylin and eosin (H&E)
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The PAR2 pepducin PZ-235 reduces hepatic steatosis and inflammation in the experimental MCD model in mice. C57BL/6 male mice were fed normal chow or an MCD diet for 3 weeks and treated daily with PZ-235 (5 mg/kg/day) or vehicle (Veh). A and B, representative 200× photomicrographs of H&E-stained liver sections from vehicle- and PZ-235-treated MCD mice. Liver histology was scored according to the NASH Clinical Research Network criteria to determine the NAS. Liver triglycerides (C), plasma ALT (D), and plasma ALT (E) were measured at the 3-week end point in the MCD mice. F, quantitative PCR of SYBR Green (mean ± S.E., n = 4–6) was determined by the ΔΔCt method where PAR2 mRNA was normalized to GAPDH and the lowest adjusted value in the control group. ****, p < 0.0001; ***, p < 0.001; **, p < 0.01 using the Holm-Sidak correction for multiple comparisons. Error bars represent S.E. gm, gram.
response in the LX-2 stellate cell line (Fig. 4A). The pepducin
completely prevented activation of phospho-ERK induced by
the PAR2 agonists SLIGRL and trypsin (Fig. 4B). Accordingly,
stimulation of the stellate cells with 10 nM trypsin significantly
increased cell viability over a 4-day period. This increase in
stellate cell viability was inhibited by 75% with 1 μM PZ-235 (Fig. 4C). As stellate cells have been implicated in the recruit-
ment of inflammatory cells via MCP-1 during active fibrogen-
esis in liver (40), we tested whether PAR2 activation could
 induce secretion of MCP-1. LX-2 stellate cells gave an increase
in MCP-1 secretion in response to both SLIGRL and trypsin
treatment that was blocked by PZ-235 (Fig. 4D).

PZ-235 Protects against Hepatocellular Necrosis and
PAR2-driven Mitochondrial Reactive Oxygen Species (ROS)
Production—We noted a pronounced difference in liver mor-
phology when comparing the hematoxylin and eosin (H&E)
staining of liver sections from PZ-235 versus vehicle-treated
animals after 8 weeks of CCl4 (Fig. 2B). In particular, the hepa-
tocytes of animals not receiving PZ-235 showed enlarged cellular
volumes and clear cytosols, consistent with hepatocellular
necrosis. These results raised the possibility that the PAR2
inhibitor PZ-235 may not only protect against the activation of
stellate cells and fibrosis but may also promote hepatocellular
viability. To address the possibility that the PAR2 pepducin
could meaningfully impact the development of liver necrosis,
we repeated our study with concurrent treatment with PZ-235
and CCl4 over the entire 8-week period of the experiment (Fig.
5A). Concurrent PZ-235 treatment afforded significant sup-
pression of liver fibrosis compared with vehicle animals (Fig. 5,
B and C). In addition to improvements in overall tissue mor-
phology, there was a striking 66% protection of the number of
viable hepatocytes retained per field in the PZ-235-treated ani-
mals (Fig. 5, B and D). As expected, there was a very minor
increase in sporadic TUNEL staining in the livers of CCl4-
treated animals versus normal controls, consistent with CCl4
causing liver necrosis independently of a caspase-driven mech-
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FIGURE 2. Suppression of CCl4-induced liver fibrosis with delayed PZ-235 treatment. A, timeline of treatment. C57BL/6 mice were treated twice weekly for 8 weeks with 1 μl of CCl4/g of body weight and PZ-235 (5 mg/kg/day), vehicle (Veh), or inactive control PAR2 pepducin PZ-234 (5 mg/kg/day) started at week 4. B, photomicrographs (200 ×) of Sirius red and H&E staining of liver sections from mice in the different treatment groups at the 8-week end point. C, quantification of percentage of Sirius red staining per 10 fields (mean ± S.E.). Vehicle (no CCl4), n = 5; CCl4 + vehicle, n = 8; CCl4 + PZ-235, n = 8; CCl4 + PZ-234, n = 3. **, p < 0.01. Error bars represent S.E.

FIGURE 3. PZ-235 suppresses liver expression of inflammatory cytokines in CCl4-treated mice receiving delayed pepducin treatment initiated at week 4. Livers were isolated from mice in Fig. 2 at week 8, and quantitative PCR by SYBR Green (mean ± S.E., n = 4–6) was determined by the ΔΔCt method where mRNA expression was normalized to GAPDH and the lowest adjusted value in the control group. #, p = 0.06; *, p < 0.05 using the Holm-Sidak correction for multiple comparisons. Error bars represent S.E. Veh, vehicle.

FIGURE 4. PZ-235 blocks PAR2-calcium signaling and activation of LX-2 stellate cells. A, stellate cell (LX-2) calcium mobilization in response to the PAR2 agonist SLIGRL (100 μM) is inhibited by PZ-235 (30 μM) but does not inhibit the PAR1 agonist thrombin (Thromb) (10 nM), B, ERK phosphorylation (pERK) induced by 30 μM SLIGRL or 1 nM trypsin in LX-2 cells is inhibited by 3 μM PZ-235. C, PZ-235 (1 μM) inhibits LX-2 cell viability induced by 10 nM trypsin (Tryp). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolum (MTT) activity was determined after 96-h stimulation with 10 nM trypsin. D, secretion of MCP-1 into LX-2 medium in response to PAR2 stimulation by the agonists SLIGRL or 1 nM trypsin. Error bars represent S.E. RFU, relative fluorescence units.

stimulated with PAR2 agonists and the kinetics of mitochondrial ROS production were measured. As shown in Fig. 6, A and B, there was a significant increase in the ROS production following PAR2 stimulation by either trypsin or the PAR2-activating peptide LIGRLO as compared with vehicle control. Mitochondrial ROS production induced by either PAR2 stimulus was completely inhibited by PZ-235 to slightly below baseline in hepatocytes and in fibroblasts (Fig. 6C). These data indicate that PAR2 contributes to both ROS production and decreases in hepatocellular viability; therefore, PAR2 signaling may be generally increased in the livers of the mice treated with CCl4. Indeed, as shown in Fig. 6D, treatment of the HepG2 cells with CCl4 gave significant increases in pERK staining that was significantly protected by treatment with PZ-235 (Fig. 6C). These data indicate that PAR2 contributes to enhanced hepatocellular necrosis by stimulating excessive ROS production in fibrosis models in mice and that the PAR2 pepducin PZ-235 can afford significant protective effects both in hepatocytes and in vivo.

Biodistribution of PZ-235 to Liver and Other Tissues—To quantify the relative propensity of the PAR2 pepducin to be distributed to liver and other organs in vivo, PZ-235 was radioactively labeled at the bridging C1 carbon joining the palmitate

anism (supplemental Fig. 2, C and D). These data indicate that PZ-235 may prevent hepatocellular death by protecting cells against a necroptotic rather than an apoptotic mechanism in the fibrotic mouse livers. To determine whether PAR2 could induce mitochondrial ROS production in hepatocytes that can trigger necroptotic processes, liver-derived THLE2 cells were
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PZ-235 was fairly evenly distributed to most tissues (ages to other organs. On a per gram of tissue basis, however, distributed 20% to kidney and 5–10% to lung and in lesser percent-
h in the panel of whole organs examined. PZ-235 was also dis-

erved twice weekly with 1 µl of CCl4/q of body weight and with PZ-235 (10 mg/kg/day) or vehicle (Veh) for the entire 8 weeks of the study. B, representative 200× photomicrographs of H&E and Picosirius red staining from the livers of the different treatment groups. C, quantification of nuclei number per 200× field (mean ± S.D. of 10 fields). D, quantification of percentage of Picosirius staining per 200× field (mean ± S.D. of 10 fields). **, p < 0.01; *, p < 0.05 using the Holm-Sidak correction for multiple comparisons. Error bars represent S.E.

Discussion

NASH is a growing worldwide epidemic, rising in incidence with obesity, diabetes, and metabolic syndrome, and is becoming the most prevalent cause of liver failure (1, 2). There are no effective therapies yet available to treat the potentially life-threatening complications resulting from liver fibrosis (5), pointing to an urgent need to more deeply understand the pathophysiology of NASH and identify new points of therapeutic intervention. Emerging evidence points to a novel mecha-
nism linking the development of NASH fibrosis with PAR2, a cell surface protease-activated receptor highly expressed in liver stellate cells, hepatocytes, inflammatory cells, and other mesenchymal cells that regulates the response to tissue injury (13, 14, 42, 43). Here, we demonstrate that the PAR2 pepducin PZ-235 suppresses collagen production, fibrosis, steatosis, triglycerides, and lobular inflammation in mouse models of NASH. PZ-235 blocked PAR2-driven activation, proliferation, and viability of stellate cells and reduced the deposition of collagen and production of inflammatory cytokines as depicted schemati-
cally in Fig. 8. The ability of PAR2 to promote progression/ differentiation of the phenotype of mesenchymal cells has been observed in multiple organ systems used in different models of
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Mitochondrial ROS Production

![Graph A: Mitochondrial ROS Production](image)

**A**

Mitochondrial ROS Production

![Graph B: Mitochondrial ROS Production](image)

**B**

Hepatocellular ROS Sensitivity

![Graph C: Hepatocellular ROS Sensitivity](image)

**C**

Hepatocellular CCI4 Sensitivity

![Graph D: Hepatocellular CCI4 Sensitivity](image)

**D**

The hepatocellular necrosis observed in the fibrotic livers may also be caused by the release of inflammatory cytokines from activated stellate cells. TNFα generally acts to reduce hepatocellular viability when cells are under stress (47–49) and aids in the promotion of IL-8, IL-6, and MCP-1 expression (11). IL-8 and MCP-1 are critically important in the recruitment of inflammatory cells to the nidus of activated stellate cells, which can propagate local fibrosis and cause additional liver damage. The role of stellate cells in controlling the development of fibrosis is complicated by the fact that they appear to operate directly to drive cell death and tissue remodeling (6) as well as being active in liver regeneration (50) and repairing tissue functionality. Hepatocytes are very sensitive to low TNFα levels with signaling in certain contexts promoting hepatocellular cell survival, proliferation, and regeneration (51, 52), whereas chronic exposure to TNFα promotes hepatocellular cell death and necrosis (49, 53). A similar pattern is also seen with the cytokine IL-6, which in acute doses can cause anti-inflammatory responses (54) but with long term exposure promotes inflammation and lowered rates of hepatocellular proliferation and tissue regeneration (55, 56). The chronic suppression of both of these cytokines by the PZ-235 pepducin is likely to be an additional explanation for our observed net increase in hepatocellular viability and reduced fibrosis and may help to maintain organ functionality in the setting of chronic disease.

We show for the first time that pharmacologic inhibition of PAR2 in the 3-week MCD model in mice significantly reduces the NAS. We then extended the MCD diet-induced NASH model out to 8 weeks to induce early fibrosis, which was also apparently suppressed by the PAR2 pepducin. Thus, in addition to reducing the liver histological features of fibrosis, lobular inflammation, and hepatocyte ballooning, the PAR2 pepducin also significantly reduced steatosis as validated biochemically by the >50% drop in liver triglycerides. The mechanistic reason(s) for the PAR2-promoting effects on liver steatosis in the MCD model is not known at this point but is likely quite complex and could reflect changes in lipid and/or sugar metabolism or other biochemical pathways. Unlike obese mouse models of NASH in which mice accumulate abundant visceral fat, mice in the MCD model are lean and actually lose weight. Thus, the effects of the PAR2 pepducin on suppressing liver steatosis reported here are likely due to direct effects on liver metabolism rather than indirect effects on PAR2 in inflammatory cells located in visceral adipose tissue (24).

We found that the PAR2 pepducin is rapidly distributed to liver and other tissues with prolonged kinetics, which may account for its high efficacy in the 8-week liver fibrosis models. PZ-235 has a C16 palmitate tail, which may confer preferred

Reduced antioxidant defense capacity and increased inflammatory response. These data suggest adaptation of the liver at early stages of obesity-related insulin resistance and NAFLD that is subsequently lost during NASH fibrosis/progression. Our study made the surprising observation that activation of PAR2 significantly stimulated ROS production in hepatocytes, which may contribute to the development of liver necrosis, and that pharmacological inactivation of PAR2 is a potential means to mitigate the necrotic damage associated with enhanced ROS in fibrotic liver disease.

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We found that the PAR2 pepducin is rapidly distributed to liver and other tissues with prolonged kinetics, which may account for its high efficacy in the 8-week liver fibrosis models. PZ-235 has a C16 palmitate tail, which may confer preferred
residency in tissues with higher fat content such as liver and/or in highly vascularized tissues such as kidney and lung. The peptidic portion of the PZ-235 pepducin formed a remarkably well structured $\alpha$-helix that closely resembled the juxtamembrane helical region of the analogous TM6 and i3 loop of PAR2 and squid rhodopsin (57). Notably, squid rhodopsin has a hyperextended TM6-i3 helix as compared with higher eukaryotic GPCRs in the rhodopsin family such as the $\beta_2$-adrenergic receptor. Unlike other opsin GPCRs that signal through a Go(i) signaling pathway, squid rhodopsin instead signals through a Gq pathway (58). PAR2 efficiently couples to both Gq and Gi pathways (59). Likewise, we found that PZ-235 inhibits both PAR2-Gq (calcium mobilization) and PAR2-Gi (ERK) signaling. As PZ-235 does not have agonist activity, we speculate that PZ-235 replaces/inserts into this most mobile portion (analogous cytoplasmic TM6-i3 loop segment) of its cognate receptor to block the ability of PAR2 to directly couple with intracellular G proteins or induces a PAR2 conformation that is not conducive for PAR2-G protein signaling. The ability of PZ-235 to provide substantial effects in suppressing liver fibrosis and necrosis, in addition to blocking PAR2-ROS production and induction of inflammatory cytokines, indicates the potential for PAR2 inhibitors to be efficacious in the treatment of liver fibrosis.

**Experimental Procedures**

**NMR Structural Determination of PZ-235—PZ-235 (P2pal-18S; palmitate-RSSAMDEEKLKrKAIK-NH$_2$) antagonist and negative control pepducin PZ-234 (P2pal-14GF; palmitate-GDENSEKKSAIF-NH$_2$) were synthesized as before (13) by standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) solid-phase methods and purified to 99.1% purity by reverse phase high performance liquid chromatography (HPLC). The methionine residue in PZ-235 was replaced with an isoleucine to reduce oxidation during NMR data acquisition. The lyophilized pepducin was dissolved in a buffer comprising 270 mM dodecylphosphocholine-$d_{31}$, 10 mM acetate-$d_4$, 0.02% NaN$_3$, and 0.016 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid at pH 5.66. The final concentration of the pepducin was 1 mM. NMR spectra included total correlation spectroscopy with a mixing time of 40 ms and nuclear Overhauser effect spectroscopy (NOESY).
TABLE 1
NMR structural parameters of the PZ-235 PAR2 i3 loop pepducin

| Parameter                                      | Value       |
|------------------------------------------------|-------------|
| NMR spectra of PZ-235 | recorded using total correlation spectroscopy with a mixing time of 40 ms, NOESY with a mixing time of 200 ms, and natural abundance 13C heteronuclear single quantum coherence spectroscopy. Data were collected at 600 MHz at 20°C. r.m.s., root mean square. |
| Experimental restraints                        |             |
| Distance restraints from NOEs                   | 294         |
| Dihedral angle restraints                       | 49          |
| Hydrogen bond restraints                        | 0           |
| Total no. of experimental restraints            | 343         |
| r.m.s. deviations from experimental data        |             |
| Average distance restraint violation (Å)        | 0.011 ± 0.037|
| Dihedral restraint violations > 5°              | 0           |
| r.m.s. deviations from ideal stereochemistry    |             |
| Bonds (Å)                                       | 0.0008 ± 0.0001|
| Angles (°)                                      | 0.240 ± 0.008|
| Impropers (°)                                   | 0.092 ± 0.008|
| Ramachandran analysis of the structures (%)     |             |
| Residues in favored regions                     | 82.4        |
| Residues in additionally allowed regions        | 11.8        |
| Residues in generously allowed regions          | 5.9         |
| Residues in disallowed regions                  | 0           |
| Lennard-Jones potential energies                |             |
| Ensemble average (kcal/mol)                     | 8.8 ± 1.5   |
| Coordinate precision of peptide (Å)             |             |
| Backbone                                        | 0.78 ± 0.23 |
| All heavy atoms                                 | 1.81 ± 0.29 |

with a mixing time of 200 ms. Data were collected on a Bruker Avance 600-MHz spectrometer at 20 °C. Resonances were assigned and distance restraints were obtained by standard methods. Proton chemical shifts, dihedral restraints obtained using PREDITOR, and distance restraints were used as input to generate 29 structures through simulated annealing, energy minimization, and refinement using CNSolve version 1.3.

Mouse Experiments—C57BL/6 male mice (10–12 weeks) were purchased from Charles River Laboratories and maintained in climate-controlled rooms in the certified and accredited Division of Laboratory Animal Medicine at Tufts University. All experiments were performed in accordance with the guidelines of the National Institutes of Health and approved by the Tufts Medical Center Institutional Animal Care and Use Committee. At the end of each experiment, animals were weighed, and blood and liver samples were collected. Animals were euthanized with CO2 following anesthesia.

Pharmacokinetics and Biodistribution of PZ-235—C57BL/6 mice were injected either intravenously or subcutaneously with various doses of PZ-235. Blood was collected at 5-min–48-h time points, and pepducin concentrations in plasma were measured by quantitative LC-MS/MS methods (Novabioassays, Woburn MA). To determine the biodistribution of PZ-235, 14C-labeled and unlabeled pepducins were mixed at a ratio of 1:2 and injected at 5000 cpm/animal; selected animal tissues were harvested and dissolved in 4 M NaOH with 1% Triton X-100 and 1% SDS overnight. Subsequently, 7.5 ml of scintillation fluid (PerkinElmer Life Sciences) was added, and radioactivity was measured using a scintillation counter.

MCD-induced Mouse NAFLD Model—C57BL/6 male mice were fed an MCD diet or a normal chow diet for 3- (experimental model for hepatic steatosis and inflammation) and 8-week (experimental model for early hepatic fibrosis) time periods. MCD mice were treated with subcutaneous injections of the cell-penetrating PAR2 inhibitory pepducin PZ-235 (5 mg/kg/day) or vehicle. All mice were permitted food and water consumption ad libitum. Body weight and food consumption were monitored weekly. At completion of the experiment, whole blood and livers were collected from each animal. Liver tissue was snap frozen at −80°C for subsequent biochemical analyses. Plasma ALT and AST and hepatic triglycerides were measured using commercially available kits (Cayman Chemical and Sigma).

CCl4-induced Mouse Fibrosis Model and Treatment Protocols—Intraperitoneal injections of CCl4 (Sigma-Aldrich) at 1 μl/g of body weight (dissolved 1:10 in olive oil) twice a week for a period of 8 weeks were used to generate liver fibrosis in the C57BL/6 mice. PZ-235 or PZ-234 was dissolved in 5% dimethyl sulfoxide (DMSO). Treatment groups received s.c. injections of 5 mg/kg PZ-235 or PZ-234 six times a week either starting at week 4 (delayed treatment) or at week 0 (concurrent treatment), whereas control groups received vehicle (5% DMSO). Animals were sacrificed 3 days following the last CCl4 injection, and blood samples were collected in tubes containing heparin. Plasma was then separated by centrifugation at 3000 rpm for 10 min. Liver samples were harvested and weighed. A segment of each harvested liver was fixed with 10% neutral buffered formalin for histological analysis, and the remaining tissue was snap frozen and stored at −80°C for subsequent biochemical and gene expression analyses.

Histopathological Analysis—Liver tissues were sectioned (5 μm) and stained with either Sirius red or H&E and observed with a Nikon Eclipse 80i microscope connected to a Spot 7.4 Slider camera. Slides were assigned a number, and 10 randomly chosen 200 × fields were visualized. The assigned fields were analyzed using ImageJ (National Institutes of Health, Bethesda, MD). Sirius red staining values were obtained by adjusting the threshold setting of the green channel from the original image; values are displayed as a percentage of the total pixels. Hepatocellular nuclei were identified by morphology and tabulated using the counter function.

mRNA Isolation and Quantitative RT-PCR—Total mRNA was extracted from the harvested liver samples using the RNeasy Mini kit (Qiagen). mRNA was reversed transcribed, and real time quantitative PCR was conducted using SYBR Green Master Mix (Roche Applied Science) and a 40-cycle thermocycling protocol. The data are represented as relative fold changes in mRNA normalized to GAPDH. The following primers were used: IL-8 forward primer, ATCTTCGTCGCTTTCCGGTAG; IL-8 reverse primer, CTGGTTGACATAATGGTCTCGAA; IL-6 forward primer, ACAAACCGGGCGCTCTACTT; IL-6 reverse primer, GTGTTTGCAGAGCTGACACT; TNFa forward primer, CTTGATCGTGGTATGCTAAAT; TNFa reverse primer, GGTTTCACGAGCAGTTGA; MCP-1 forward primer, AGTTCTGGTTGTCGACT; MCP-1 reverse primer, CTCAATTCGAGCTCTTTGG; collagen IVa1 forward primer, GATGGTCAAGAACCCACGG; collagen IVa1 reverse primer, GCAGAGGCGAGGGTCACTAGT; PAR2 forward primer, CTCTGACCAGGAGCCA; PAR2 reverse primer, AAGAGCTGGTTTCTACCGGAAC.

Cell Culture—The LX-2 human hepatic stellate cell line was a generous gift from Dr. Scott Friedman (Mt. Sinai School of Medicine, New York, NY). The LX-2 cells and SW620 aden-
carcinoma cells (ATCC) were grown in DMEM (Corning CellGro) supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). The THLE-2 cells (ATCC) were propagated following the manufacturer’s recommendations using the BEGM Bullet kit (Lonza) with the addition of phosphoethanolamine (70 ng/ml) and 10% FBS but without gentamycin and epinephrine.

**Ca**²⁺ **Flux Assays**—The SW620 and LX-2 cells were resuspended in Krebs-Ringer bicarbonate buffer and labeled with 2.5 μM Fura-2/AM (Molecular Probes) by incubating for 30 min at 37 °C with gentle shaking. The fluorescence emission was recorded at 510 nm with dual excitation at 340 and 380 nm at 25 °C using a PerkinElmer Life Sciences LS 50B spectrofluorometer.

**Immunoblotting**—LX-2 cells were propagated till confluent and treated with either the agonist SLIGRL (30 μM) or trypsin (1 nM). The PAR2 antagonist pepducin PZ-235 (3 μM) was added to the cells 15 min prior to agonist treatment. Cells were lysed with 300 μl of Tissue Protein Extraction Reagent (T-PER; Fisher Scientific) at 4 °C, and protein was quantified using a Bradford assay. Equal amounts of proteins were loaded for SDS-PAGE, and gel bands were transferred to a PVDF membrane (Millipore, Billerica, MA) as described (60) with horseradish peroxidase (HRP)-conjugated secondary antibodies used at a dilution of 1:5000. The immunoblots were probed for phospho-ERK1/2 (Cell Signaling Technology catalog number 4370S; 1:1000), total ERK antibody (Cell Signaling Technology catalog number 2146S; 1:5000) and β-tubulin (Cell Signaling Technology catalog number 2146S; 1:5000) and visualized using ECL reagent (Pierce).

**Enzyme-linked Immunosorbent Assay (ELISA) for MCP-1**—LX-2 cells were treated with either the agonist SLIGRL (100 μM) or trypsin (1 nM) with and without PZ-235 (1 μM) treatment for 24 h. The conditioned medium was collected and assayed for MCP-1 by sandwich ELISA using a commercially available kit (Quantikine, Minneapolis, MN) according to the manufacturer’s instructions.

**ROS Production Assay**—THLE2 cells were harvested using 5 mM EDTA, PBS and replated in 96-well plates. Cells were allowed to settle overnight, and medium was removed and replaced with Hanks’ balanced salt solution (with calcium and magnesium) and 1% FBS. Cells were preincubated with either PZ-235 (3 μM) or vehicle (0.2% DMSO) for 45 min and pre-treated with MitoSOX (Life Technologies) for 10 min according to the manufacturer’s instructions. Following incubation, cells were washed with PBS and fresh Hanks’ balanced salt solution with 1% FBS with additional PZ-235 in addition to 1 μM LIGRLO or 10 nM trypsin. Fluorescence was measured (excitation, 510 nm; emission, 580 nm) using a FlexStation 3 multimode microplate reader at times 0, 2, 4, and 8 h post-treatment. Baseline signal at time 0 was subtracted from all readings and compared with the average increase in signal recorded in untreated condition.

**Statistical Analyses**—All of the values in the figures are expressed as means ± S.E. Comparisons between experimental and control cohorts were performed by one-way analysis of variance, and the means of the different groups were compared using the Holm-Sidak post hoc correction. Analysis was performed using GraphPad Prism 6.0 (San Diego, CA). Statistical significance was defined as *p* < 0.05 (*), *p* < 0.01 (**), or *p* < 0.001 (***)

**Author Contributions**—A. M. S., R. R., K. A., N. N., A. B., and J. D. B. performed experiments, analyzed the data, and wrote the manuscript. L. C. contributed to writing the manuscript and experimental design. A. K. conceived the study, analyzed the data, and wrote the manuscript.

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