Celecoxib does not protect against fibrosis and inflammation in a carbon tetrachloride-induced model of liver injury

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Abbreviations:
APAP: Acetaminophen; BDL: Bile duct ligation; CCl₄: Carbon Tetrachloride; COL1A1: Collagen 1A1; COX: Cyclooxygenase; CUDA: 12-(3-cyclohexyl-ureido)dodecanoic acid; CYP: Cytochrome P450; DHET: Dihydroxyicosatrienoic acids; EET: Epoxyeicosatrienoic acid; EpFA: Epoxy fatty acids; ER: Endoplasmic reticulum; HSC: Hepatic stellate cell; LOX: Lipoxygenase; LXA₄: Lipoxin A4; MMP: Matrix metalloprotease; NSAID: Non-steroidal anti-inflammatory drug; PGD₂: Prostaglandin D2; PGE₂: Prostaglandin E2; PGI₂: Prostaglandin I2; PGJ₂: Prostaglandin J2; PTUPB: 4-(5-phenyl-3-[3-(4-trifluoromethylphenyl)-ureido]-propyl)-pyrazol-1-yl)-benzenesulfonamide; sEH: Soluble epoxide hydrolase; sEHI: Soluble epoxide hydrolase inhibitor; SMA: Smooth muscle actin; TAA: Thioacetamide; TIMP: Tissue inhibitors of metalloproteases; TGF: Transforming growth factor; TPPU: 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea; TXA₂: Thromboxane A₂.
Abstract

The cyclooxygenase-2 (COX-2) selective inhibitor, celecoxib, is widely used in the treatment of pain and inflammation. Recently, celecoxib has been explored as a possible treatment for liver fibrosis with contradictory results depending on the model. The present study reports the effect of celecoxib in a five week carbon tetrachloride (CCl₄)-induced liver fibrosis mouse model. Celecoxib alone and a combination of celecoxib and inhibitors of the enzyme soluble epoxide hydrolase (sEH) as well as a dual inhibitor that targets both COX-2 and sEH were administered via osmotic minipump to mice receiving intraperitoneal injections of CCl₄. We found that there was elevated collagen deposition in the mice treated with both celecoxib and CCl₄ compared to the Control or CCl₄ only groups, as assessed by trichrome staining. Histopathology revealed more extensive fibrosis and cell death in the animals treated with both celecoxib and CCl₄ compared to all other experimental groups. While some markers of fibrosis, such as MMP-9, were unchanged or lowered in the animals treated with both celecoxib and CCl₄, overall, the hepatic fibrosis was more severe in this group. Co-treatment with celecoxib and an inhibitor of sEH or treatment with a dual inhibitor of COX-2 and sEH decreased the elevated levels of fibrotic markers observed in the group that received both celecoxib and CCl₄. Oxylipid analysis revealed that celecoxib reduced the level of PGE₂ relative to the CCl₄ only group. Overall, celecoxib treatment did not decrease liver fibrosis in CCl₄ treated mice.
Introduction

Celecoxib is a non-steroidal anti-inflammatory drug used by millions of patients to alleviate the pain and inflammation associated with diseases such as rheumatoid arthritis (Bessone et al., 2016). Reviews of controlled trials have found no significant difference in the incidence of liver damage between patients administered celecoxib and those receiving placebo (Bessone et al., 2016).

Liver fibrosis is the result of a normally beneficial wound healing process that can be initiated by toxicants such as ethanol or carbon tetrachloride (Liedtke et al., 2013). This inflammatory process involves the activation of resident macrophages called hepatic stellate cells (HSCs) and the recruitment of macrophages, both of which express pro-inflammatory signaling molecules, along with enzymes and structural proteins that remodel the extra-cellular matrix (Pellicoro et al., 2014). This remodeling includes the increased deposition of matrix proteins such as collagen, as well as changes in the populations of metalloproteases (Pellicoro et al., 2014). If damage due to exposure to the toxicant continues, liver fibrosis will alter the architecture of the organ and lead to liver failure (Liedtke et al., 2013).

Due to its anti-inflammatory effect, celecoxib has been explored as a possible therapy in several models of liver fibrosis, such as the thioacetamide (TAA)- and carbon tetrachloride (CCl₄)-induced rodent models and the surgical bile duct ligation (BDL) rodent model. CCl₄ acts primarily through an increase in hepatic lipid peroxidation and oxidative stress while TAA acts primarily through an increase in oxidative stress, processes which damage hepatocytes and trigger fibrosis (Martinez et al., 2014). BDL is a surgical model in which the bile duct is partially ligated, leading to cholestasis, liver damage and fibrosis (Martinez et al., 2014). In some models, treatment with celecoxib has resulted in a reduction in fibrosis and inflammation, while in others, including some...
rat CCl\textsubscript{4}-induced models, celecoxib has worsened the liver damage and fibrosis (Chavez et al., 2010; Hui et al., 2006; Paik et al., 2009).

Celecoxib targets cyclooxygenase 2 (COX-2), an enzyme that metabolizes arachidonic acid to a class of oxidized fatty acids called prostaglandins (Shi and Klotz, 2008). These oxylipids have diverse effects in the liver, but many COX-2 metabolites increase inflammation and portal hypertension (Sacerdoti et al., 2015). Arachidonic acid is also metabolized by cytochrome P450s to form the epoxideicosatrienoic acids (EETs) (Morisseau and Hammock, 2013). The EETs have been investigated in several disease models and have been found to be anti-inflammatory, organ protective, and anti-fibrotic in heart and kidney models of fibrosis (Morisseau and Hammock, 2013). The EETs are further metabolized by soluble epoxide hydrolase (sEH) to the dihydroxyicosatrienoic acids (DHETs) that are less lipophilic and more readily conjugated and excreted by the organism (Morisseau and Hammock, 2013).

We previously modulated the oxylipids in a CCl\textsubscript{4}-induced model of hepatic fibrosis through the use of dietary manipulation of lipid intake as well as inhibition of sEH, which blocks the major route of metabolism of the EETs and other epoxy fatty acids (Harris et al., 2015; Harris et al., 2016). In general, perturbation of the oxylipids with sEH inhibitors reduced collagen deposition in addition to the expression and activity of pro-fibrotic MMPs (Harris et al., 2015; Harris et al., 2016). These results raised the question of how celecoxib, another modulator of oxylipids, would impact fibrosis in the CCl\textsubscript{4} model and whether tools we developed for inhibiting both sEH and COX-2 would alter the observed effects of COX-2 inhibition.

In this study, we treated mice with CCl\textsubscript{4} over a five-week period to induce liver fibrosis. Interestingly, we found that markers of fibrosis were elevated in the mice that received both celecoxib and CCl\textsubscript{4} compared to those animals receiving CCl\textsubscript{4} alone. Modulation of the EETs by
either an sEH inhibitor or a dual inhibitor of COX-2 and sEH, blunt some aspects of the pro-

fibrotic effect of COX-2 inhibition in the CCl₄ background.
Materials and Methods

Animals

Male C57BL/6NCrl mice (~25g) were obtained from Charles River, Inc. (Wilmington, MA, USA) one week prior to the experiment and kept on a 12h/12h light/dark cycle. The animal protocol was approved by University of California Davis Institutional Animal Care and Use Committee and the study was performed in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

Chemicals

All commercial chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. TPPU, 1-(4-trifluoromethoxyphenyl)-3-(1-propionylpiperidin-4-yl)-urea, and PTUPB, 4-(5-phenyl-3-[3-(4-trifluoromethylphenyl)ureido]-propyl)-pyrazol-1-yl)-benzenesulfonamide, were synthesized according to the previous procedures (Hwang et al., 2011; Rose et al., 2010). Celecoxib was a gift from Pfizer (New York, NY, USA). Structures of TPPU, PTUPB and Celecoxib are given in Supplemental Data Figure S1.

Experimental Design

The mice were randomly divided into five experimental groups (6 animals/group): (1) Control, (2) CCl₄ only, (3) Celecoxib+CCl₄, (4) Celecoxib+TPPU+CCl₄, (5) PTUPB+CCl₄. CCl₄ was administered following a protocol described in detail in (Constandinou et al., 2005). Briefly, CCl₄ was mixed 1:1 (v/v) with Neobee M5 (Sigma, St. Louis MO, USA) and injected i.p. every five days for a total of seven injections of 80 mg/kg. Mice were euthanized three days after the final injection. Celecoxib, PTUPB, and a combination of celecoxib and TPPU were administered
subcutaneously via Alzet model 2006 osmotic minipumps (Cupertino, CA, USA). These pumps produce a 3.6 µL/day continuous flow rate for up to 45 days. The Control group received Neobee M5 only. The Control group and CCl₄ only group were implanted with minipumps filled with the 1:1 (v/v) PEG400:DMSO solution.

Plasma blood level analysis

Mouse plasma (10 µL) was mixed with 50 µL of water containing 0.1% formic acid. The liquid-liquid extraction and LC-MS/MS analysis were previously performed as described (Harris et al., 2016).

Determination of liver tissue hydroxyproline levels

Hydroxyproline content was determined following a method in (Reddy and Enwemeka, 1996). Briefly, 10 µL of 10 N NaOH was added to 40 µL of 0.33 mg/µL liver tissue homogenate and the solution was autoclaved at 120 °C for 25 min. After the reaction cooled to room temperature, chloramine T reagent (0.84% chloramine-T, 42 mM sodium acetate, 2.6 mM citric acid, and 39.5% isopropanol) was added (450 µL) bringing the volume to 500 µL. After incubation at room temperature for 25 min, DMAB reagent, 15% 4-(dimethylamino)benzaldehyde in 2:1 (v/v) isopropanol/perchloric acid mixture, was added (500 µL) bringing the volume to 1 mL. The solution was then incubated at 65 °C for 20 min and the absorbances of the samples measured at 550 nm.

mRNA transcript analysis
Liver tissue was immediately stored in RNA later solution (Thermo Fisher Scientific, Waltham, MA USA) for 24 h at 4 °C before freezing at -80 °C. Tissue was homogenized using a roto stator grinder (IKA Works Inc., Wilmington, NC, USA) and passed through a QIAshredder column (Qiagen, Valencia, CA, USA). Total RNA was purified using RNeasy kit (Qiagen). cDNA synthesis from total RNA was performed by the QuantiTect Reverse Transcription Kit (Qiagen). RTPCR was performed using an Applied Biosciences Fast 7500 Real-Time PCR System (Foster City, CA, USA). The following Taqman probes were purchased from Life Technologies Corporation: GAPDH, Mm99999915_g1; TGFB1, Mm01178820_m1; MMP-2, Mm00439498_m1; MMP-9, Mm00442991_m1; COL1A1, Mm00801666_g1; TIMP-1, Mm00441818_m1 (Life Technologies Corp., Grand Island, NY, USA). GAPDH was used as the internal control.

Histopathology scoring

Liver samples were immersion-fixed in 10% neutral-buffered formalin for 48 hours. Samples were stored in 70% (v/v) ethanol in distilled deionized water prior to routine processing. Tissues were processed, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) by the Histopathology service in the UC Davis Veterinary Medical Teaching Hospital (Davis, CA, USA). H&E-stained sections of liver were scored for inflammation, fibrosis and cellular damage by a board-certified laboratory animal pathologist (DMI). The scoring system is reported in Supplemental Data Table S1.

Trichrome staining and Immunohistochemistry

αSMA and F4/80 immunohistochemistry and trichrome staining were performed by the Genomic
Pathology Laboratory at UC Davis and quantitated using imaging analysis software from Aperio (Sausalito, CA, USA) using the IHC and positive pixel count (PPC) algorithms.

**Oxylipid analysis**

Oxylipids in the COX and LOX branches of the arachidonic acid cascade were extracted and detected as described (Harris et al., 2016). Oxylipids in the P450 branch of the arachidonic acid cascade were detected as follows. Samples were analyzed on a Waters Acquity Ultra Performance Liquid Chromatograph coupled to a Waters Xevo TQ-S Mass Spectrometer in negative electrospray ionization mode. Samples were injected (5 µL) and separated using a Phenomenex Kinetex column (150 x 2.1 mm; 1.7 µm) at 40°C using the following mobile phase gradient, consisting of water (A) and acetonitrile (B) each containing 0.1% acetic acid: initial conditions of 65:35 A:B for 2.9 min \((t = 2.9 \text{ min})\), changing to 45:55 at 3 min \((t = 3 \text{ min})\) and decreasing to 35:65 over 5.5 min \((t = 8.5\) min), decreasing to 5:95 over 4 min \((t = 12.5\) min), holding at 5:95 for 1 min \((t = 13.5\) min), reverting to initial conditions of 65:35 in 0.1 min \((t = 13.6 \text{ min})\) and re-equilibrating for 1.9 min \((t = 15.5 \text{ min})\). Mass spectral analysis was accomplished using a capillary voltage of 3 kV, a desolvation temperature of 200°C, a desolvation gas flow of 800 L/hr, a cone gas flow of 150 L/hr, nebulizer pressure of 6 bar, and collision gas flow of 0.15 mL/min. Mass transitions for each EET were as follows: 14,15-EET \((319.0 > 219.0); 11,12\)-EET \((319.0 > 167.0)\); 8,9-EET \((319.0 > 155.0)\). Analyte concentration was quantified against an internal standard calibration curve, normalizing analyte response to 11,12-EET-d11 \((330.2 > 167.2)\), and 12-(3-cyclohexyl-ureido)dodecanoic acid (CUDA, 341.3 > 216.4) to correct extraction efficiency and instrument response, respectively. Dwell time for each analyte was 25 ms. The detailed information for all analytes are reported in Supplemental Data Table S2.
Statistics

Normality was assessed by the Shapiro-Wilk test. Significance was determined by one-way ANOVA followed by the Dunnett multiple comparison test. All statistical calculations were performed using SigmaPlot (Systat Software, Inc., San Jose, CA, USA).
Results

Subcutaneous osmotic minipumps produced high plasma concentrations of celecoxib

Male C57BL mice were implanted with osmotic minipumps two days before the start of CCl₄ exposure via i.p. injections as described in Materials and Methods. The five experimental groups were (1) Control, (2) CCl₄ only, (3) Celecoxib+CCl₄, (4) Celecoxib+TPPU+CCl₄, and (5) PTUPB+CCl₄. The drugs were loaded into minipumps so that the calculated dose of each compound would be 10 mg/kg/day.

When we examined plasma levels of the drugs, we found that the TPPU and celecoxib levels in the Celecoxib+TPPU+CCl₄ group were 1.11±0.018 µM and 1.32±0.039 µM, respectively. The plasma concentration of celecoxib in the Celecoxib+CCl₄ group was 1.58±0.47 µM and the PTUPB concentration in the PTUPB+CCl₄ group was 0.65±0.027 µM. These plasma concentrations are far above the IC₅₀ of for celecoxib and TPPU with their respective target mouse enzymes and approximately half the IC₅₀ for PTUPB with human COX-2 and far above the IC₅₀ for PTUPB with human sEH. Despite developing robust fibrosis after the five week CCl₄ treatment, the weight of the mice increased, with no statistical difference between the groups (p-value < 0.05), the CCl₄ toxicity being less severe than other models (Supplemental Data Figure S2).

Celecoxib increased collagen deposition and liver damage in CCl₄ treated mice

Trichrome staining revealed that the Celecoxib+CCl₄ had a higher amount of collagen deposition than the CCl₄ only group (Figure 1, A-F). The PTUPB+CCl₄ group had a reduced level of collagen deposition when compared to the Celecoxib+CCl₄ group, though still higher than the Control group (Figure 1F). Hydroxyproline levels in hepatic tissue from the Celecoxib+CCl₄ group was the same as CCl₄ only group (p-value > 0.05), and the levels from both the
Celecoxib+TPPU+CCl4 and PTUPB+CCl4 groups were lower than the CCl4 only group (Figure 1G).

We next performed a histopathological assessment of the extent of liver fibrosis and damage (Table 1). The categories were defined as follows: vacuolation: lacy or vacuolated cytoplasm that distends the hepatocyte; necrosis: single cell death of hepatocytes in centrilobular to random areas; lipofuscinosis: accumulation of lipid pigments, associated with nondegradable cellular breakdown products in macrophages; fibrosis: fibroblast proliferation with increased deposition of collagen around centrilobular regions occasionally connecting between centrilobular regions (bridges) or extending into the surrounding parenchyma (dissecting); inflammation: mixed neutrophilic and mononuclear inflammation around predominantly centrilobular regions. The slides were read in a blind fashion. The scoring system is reported in Supplemental Data Table S1.

The Celecoxib+CCl4 group had the highest composite lesion scores due to greater indices of hepatocellular damage (vacuolar degeneration, necrosis, fibrosis, regeneration). When the Celecoxib+TPPU+CCl4 group was examined, it was found that the addition of TPPU dampened the effect of celecoxib (p-value = 0.0117), predominantly by decreasing hepatic necrosis (p-value = 0.0256) and regeneration (p-value = 0.0134).

**Celecoxib did not significantly raise the level of pro-fibrotic markers in CCl4 treated mice**

To further gauge the extent of fibrosis, we measured the mRNA level of common markers by RTPCR (Figure 2). The expression levels of collagen 1A1 (COL 1A1) was increased in the CCl4 only group compared to the Control group (p-value < 0.05), and further increased in the Celecoxib+CCl4 group, but this difference did not reach statistical significance (p-value > 0.05) (Figure 2A). The mRNA expression of MMP-2 and MMP-9 were also elevated in the CCl4 only group (Figure 2B and C). For MMP-2, the three treatment groups were no different than the CCl4.
only group (Figure 2B). In the case of MMP-9, levels in the Celecoxib+CCl4, Celecoxib+TPPU+CCl4 and PTUPB+CCl4 groups, while not different than CCl4 only group, were also not different than the Control group (p-value > 0.05) (Figure 2C). TIMP-1 was elevated in the CCl4 only group, but the further increase observed in the Celecoxib+CCl4, Celecoxib+TPPU+CCl4 and PTUPB+CCl4 groups did not reach significance (Figure 2D). For TGF-β, the levels of the Celecoxib+CCl4 and Celecoxib+TPPU+CCl4 groups were lower than the CCl4 only group, but this difference did not reach statistical significance (Figure 2E).

**Celecoxib increased αSMA but not F4/80 staining in CCl4 treated mice**

F4/80 is a mouse cell surface protein expressed in many populations of macrophages. CCl4 increased the F4/80 immunohistochemical staining relative to the Control group (Figure 3). While the amount of positive staining in the Celecoxib+CCl4, PTUPB+CCl4, and Celecoxib+TPPU+CCl4 groups were not significantly reduced (p-value > 0.05) when compared with the CCl4 only group (Figure 3F), these groups were also not different than the Control group.

To assess hepatic stellate cell (HSC) activation, we performed IHC on liver tissue with an anti-αSMA (smooth muscle actin) antibody (Figure 4). We found that HSC activation was greatest in the Celecoxib+CCl4 group, where it was significantly increased relative to both the Control and CCl4 only groups (p-value < 0.05). Co-treatment with celecoxib and TPPU or treatment with PTUPB in the CCl4 background decreased the level of HSC activation compared to the animals receiving celecoxib and CCl4. In the case of the Celecoxib+TPPU+CCl4 group, the HSC activation was significantly less than the Celecoxib+CCl4 group (p-value < 0.05). In the PTUPB+CCl4 group, the level of HSC activation was not statistically different than the CCl4 only group (p-value < 0.05), unlike the Celecoxib+CCl4 group.

**Celecoxib lowered the tissue level of COX metabolites of arachidonic acid in CCl4 treated**

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We next examined the hepatic oxylipids by LC-MS/MS after solid phase extraction of lipids from tissue lysates. A simplified diagram of these pathways is given in Supplemental Data Figure S1. Although the differences did not reach statistical significance for every analyte, CCl₄ treatment displayed the same trend across several COX metabolites, raising their concentration relative to the Control group (Figure 5 and Supplemental Data Table S4). Three metabolites in the COX branch of the arachidonic acid cascade were significantly altered in the Celecoxib+CCl₄ group relative to either the Control or the CCl₄ only group (Figures 5A-D). The PGE₂ and PGD₂ levels were significantly higher in the CCl₄ only group compared to the Celecoxib+CCl₄ group. Although they did not reach the level of statistical significance, PGJ₂ and 15-deoxy-Δ12,14-PGJ₂ show similar trends, the Celecoxib+CCl₄ group being lower than the CCl₄ only group. Interestingly, the LOX metabolite of arachidonic acid, Lipoxin A₄ (LXA₄) was significantly elevated by celecoxib (Figure 5E). The complete oxylipid profile is summarized in Supplemental Data Table S4.

Finally, we determined the effect of the treatments on hepatic EET levels (Supplemental Data Figure S3 and Table S4). Although the differences in the levels of these analytes did not achieve statistical significance between any of the groups, the means for 8,9-, 11,12-, and 14,15-EET were elevated in CCl₄ only group relative to the Control group (Figures S3A-C). The Celecoxib+CCl₄ group had lowered 11,12- and 14,15- levels relative to the Control group (Figures S3B-C). In the Celecoxib+TPPU+CCl₄ and PTUPB+CCl₄ groups, the EET levels were increased relative to the Celecoxib+CCl₄ group (Figures S3A-C).
Discussion

There is a current disagreement in the literature regarding the use of celecoxib as a treatment for liver damage and fibrosis (Hui et al., 2004; Paik et al., 2009; Wen et al., 2014; Yu et al., 2009). Relevant to this study, opposing results have been reported in CCl₄ models of liver fibrosis. In a short-term model, celecoxib reduced liver inflammation and damage during the early phase of CCl₄ toxicity (Washino et al., 2010). A similar short-term study found that celecoxib reduced markers of lipid peroxidation, but had no effect on hepatic toxicity or necrosis (Ekor et al., 2013). Chavez et al reported that celecoxib reduced fibrosis as judged by collagen deposition and other biochemical markers (Chavez et al., 2010). However, Hui et al reported that treatment with celecoxib increased hepatic fibrosis, gauged by collagen deposition and MMP production (Hui et al., 2006). In their discussion of these CCl₄ studies, Chavez et al suggested that differences in CCl₄ model parameters might explain the conflicting results, in particular, the dose and length of time of toxicant exposure (Chavez et al., 2010).

In the current study we obtained results similar to those reported by Hui et al after treatment with celecoxib in their CCl₄ rat model of liver fibrosis, as well as those obtained by Reilly et al using COX-2 knockout mice in an APAP model of acute liver damage (Hui et al., 2006; Reilly et al., 2001). Like Hui et al, we found that celecoxib treatment increased the amount of collagen deposition by approximately 25% compared to the CCl₄ only group (Hui et al., 2006). We also observed an increase in αSMA staining, a marker for activated HSCs, in the Celecoxib+CCl₄ group compared to the CCl₄ only group, though our model produced an almost twofold increase compared to their reported 40% increase (Hui et al., 2006). However, we did not see an increase in MMP-2 or MMP-9 mRNA expression in the Celecoxib+CCl₄ group compared to the CCl₄ only group, unlike Hui et al. It should be kept in mind that Hui et al treated with a much higher dose of
CCl₄, 3200 mg/kg delivered twice weekly compared to our 80 mg/kg delivered twice weekly. Our study also parallels results using COX-2 transgenic mice in a liver damage model. When COX-2 deficient mice were given a single bolus of APAP in an acute liver damage model (Reilly et al., 2001), the COX⁻²⁻ and COX⁻²⁺ animals displayed a greater degree of bridging perivenous hepatocyte necrosis than wild type (Reilly et al., 2001). Similarly, we observed an increase in hepatic necrosis in the Celecoxib+CCl₄ group compared to the CCl₄ only group. Although the mechanism of toxicity of APAP and CCl₄ are different, this may indicate that celecoxib accelerates hepatic necrosis in models that involve severe oxidative stress.

We used two pharmacological tools in addition to celecoxib to perturb the oxylipid homeostasis, TPPU and PTUPB. TPPU is a potent inhibitor of soluble epoxide hydrolase, an enzyme that metabolizes epoxy fatty acids (Rose et al., 2010). In previous studies we have reported that TPPU treatment reduces hepatic fibrosis (Harris et al., 2015; Harris et al., 2016). PTUPB contains the pharmacophores from both celecoxib and TPPU and inhibits both enzymes (Hwang et al., 2011). We found that treatment with PTUPB or co-treatment with TPPU and celecoxib decreased the elevated collagen deposition observed in the Celecoxib+CCl₄ group. The most striking result obtained with these inhibitors concerns the elevated αSMA in the Celecoxib+CCl₄ group. While not returning αSMA levels to that of the Control group, co-treatment with the sEH inhibitor attenuated the elevated αSMA expression observed in the Celecoxib+CCl₄ group. Changes in associated oxylipid mediators will be discussed below. Interestingly, we saw a slight reduction in F4/80 expression, a marker of macrophages, in the Celecoxib+CCl₄ group compared to the CCl₄ only group, indicating that the increase in fibrosis in the Celecoxib+CCl₄ group is not due to increased macrophage migration.

The downstream COX-2 metabolites PGE₂ and 15-deoxy-Δ¹²,¹⁴-PGJ₂ have been proposed
to mediate both the pro- and anti-fibrotic effects of COX-2 inhibition or gene deletion, depending on the model. In general, prostaglandins have been shown to have organ protective effects in the liver (Reilly et al., 2001), and PGE\(_2\) reduced collagen production in a human cell culture model of HSCs (Hui et al., 2004). Results that support a pro-fibrotic role for COX-2 downstream metabolites include the observation that plasma PGE\(_2\) levels were elevated after treatment with TAA (Wen et al., 2014). In a separate study using the BDL and TAA models, PGE\(_2\) levels were increased in the fibrotic animals and reduced after treatment with celecoxib, with an accompanying attenuation of fibrosis (Paik et al., 2009). 15-deoxy-\(\Delta^{12,14}\)PGJ\(_2\) is another potential pro-fibrotic downstream COX-2 metabolite elevated in the serum of fibrotic rats treated with CCl\(_4\) (Planaguma et al., 2005). However, 15-deoxy-\(\Delta^{12,14}\)PGJ\(_2\) has also been shown to induce apoptosis of human myofibroblasts, a process that contributes to the resolution of fibrosis (Li et al., 2001).

We performed an analysis of oxylipids of the arachidonic acid cascade to determine the effect of COX-2 and sEH inhibition. Overall, we found that COX metabolites were elevated in the CCl\(_4\) only group, though the levels did not reach statistical significance in most cases. PGE\(_2\) levels were elevated in the CCl\(_4\) only group and substantially lowered in the Celecoxib+CCl\(_4\) group as expected for a COX inhibitor. As outlined above, there was extensive fibrosis in both of these groups, the greatest degree of fibrosis found in the Celecoxib+CCl\(_4\) group where the lowest PGE\(_2\) level was detected. Consistent with an anti-inflammatory effect of celecoxib in the liver, LXA\(_4\) was greatly elevated by celecoxib. Interestingly, treatment with TPPU and celecoxib or the dual COX-2/sEH inhibitor in the CCl\(_4\) background raised PGE\(_2\) levels and lowered LXA\(_4\) levels compared to the Celecoxib+CCl\(_4\) group, in both cases bringing the levels of these lipid mediators closer to those observed in the Control group. It is possible that a drastic reduction in inflammatory processes in the CCl\(_4\) background results in even greater damage to the liver and that, depending
on the inflammatory state of the liver, an elevation in PGE$_2$ levels will either increase or decrease liver damage and fibrosis. This might explain some of the contradicting conclusions regarding the role of PGE$_2$ in different liver fibrosis models.

There was high variation in the PGJ$_2$ and 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ levels, but the trend of metabolites was similar. While CCl$_4$ elevated their mean levels compared to the Control group, the levels were slightly decreased in other groups. Based on these data, the 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ levels do not explain the increased fibrosis in the Celecoxib+CCl$_4$ group, or the attenuation in fibrosis after TPPU co-treatment.

sEH inhibitors and presumably the underlying elevation in epoxy fatty acid chemical mediators have been shown to reduce pathological fibrosis in several models (Hye Khan et al., 2016; Kim et al., 2014; Liao et al., 2016; Zhou et al., 2016) including ischemia driven heart failure (Sirish et al., 2013). Earlier we demonstrated that sEHI reduced hepatic fibrosis in the same CCl$_4$ model used in this study (Harris et al., 2015; Harris et al., 2016). However, unlike some other pathological changes reversed by sEHI, the effects were not enhanced by a diet depleted in $\omega$-6 relative to $\omega$-3 fatty acids.

sEH inhibitors synergize with NSAIDs in many biological systems (Hwang et al., 2011; Hye Khan et al., 2016; Schmelzer et al., 2006; Zhang et al., 2014). Surprisingly, sEH inhibitors prevent and reverse gastrointestinal erosion caused by the NSAID diclofenac (Goswami et al., 2017; Goswami et al., 2016), and indomethacin (Goswami, forthcoming), and in mice they block some of the cardiovascular effects caused by COX-2 inhibitors possibly by returning the enhanced blood levels of TXA$_2$ to PGI$_2$ to more normal levels (Schmelzer et al., 2006). Since celecoxib is a selective COX-2 inhibitor and PTUPB is a highly selective COX-2 inhibitor, we expected a strong positive synergistic effect between the sEHI TPPU and celecoxib and an dramatic reduction in
fibrosis with PTUPB. Surprisingly, the profibrotic effect of celecoxib in our CCl₄ model overpowered possible positive synergistic interaction with sEHI giving only moderate efficacy in reducing fibrosis and fibrosis markers. This result suggests that celecoxib is not an attractive drug to treat hepatic in a system driven by pathological peroxidation. However, both sEHI alone and dual COX/sEH inhibitors may be worth examining in hepatic fibrosis models driven by agents other than CCl₄. In patients with hepatic fibrosis who are on celecoxib or other NSAIDs for a difference disease, sEHI may ameliorate the exacerbation of the hepatic fibrosis by celecoxib.

Finally, although the differences observed in the EETs did not achieve statistical significance, the trends suggest that the sEH inhibitors were able to elevate hepatic EET levels, although CCl₄ treatment also raised the level of these oxylipids. Given the variation in the data one must use caution in interpretation, but it is possible that the slight increase in EET levels after CCl₄ treatment is insufficient to counteract pro-fibrotic environment and that the further increase in EETs caused by sEH inhibition tips the balance, partially countering the pro-fibrotic effects of celecoxib in the CCl₄ background. In the oxylipin, as well as the mRNA expression and histological analyses, small n sizes might be responsible for trends not reaching significance in this study. Given the variability of fibrotic and inflammatory responses in chronic studies, small group sizes can limit the ability to detect differences between groups.

Due to the differences in animal models and the dose of celecoxib used, the effectiveness of COX-2 inhibition as a treatment of liver fibrosis is questionable. In the current study, we have an indication that oxidized fatty acids other than COX-2 metabolites of arachidonic acid may be involved in the pro-fibrotic properties of celecoxib in a CCl₄ mouse model of liver fibrosis. Given their effectiveness in modulating the EETs as well as their anti-fibrotic effects in the liver and other tissues, sEHI are promising tools for the study of the role of these oxylipids in hepatic fibrosis.
and damage.
Author Contributions

Participated in research design: Harris, Kodani, Hammock.

Conducted experiments: Harris and Rand.

Contributed new reagents or analytic tools: Rand, Yang, and Hwang.

Performed data analysis: Harris, Imai.

Wrote or contributed to the writing of the manuscript: Harris, Imai and Hammock.
References


7. Harris TR, Bettaieb A, Kodani S, Dong H, Myers R, Chiamvimonvat N, Haj FG and Hammock BD (2015) Inhibition of soluble epoxide hydrolase attenuates hepatic fibrosis and...


Footnote

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Legends for Figures

Figure 1. Celecoxib increases the collagen deposition caused by CCl₄ treatment. Mice were injected (i.p.) with CCl₄ for 5 weeks. The inhibitors were administered subcutaneously via osmotic minipumps that delivered the compounds at a calculated dose of 10 mg/kg/day for a 30 gram mouse. A-E) Representative slides of liver sections stained with trichrome (40x). F) Quantification of staining as determined by the Aperio positive pixel count algorithm. The negative pixel ratio represents the % area of collagen deposition, which is stained blue by the Trichrome stain. G) Quantification of hepatic levels of hydroxyproline. Error bars represent standard deviation. *p-value vs. Control group < 0.05. †p-value vs. CCl₄ only group < 0.05. ‡p-value vs. Celecoxib+CCl₄ group < 0.05. N = 6 for Celecoxib+CCl₄ group, N = 5 for all other groups. Statistical tests are described in Materials and Methods. The raw data used for this figure is reported in Supplemental Data Table S3.

Figure 2. Celecoxib modulates markers of hepatic fibrosis that are increased after CCl₄ treatment. Total RNA from hepatic tissue and cDNA were prepared as described. Quantitative PCR was performed using Taqman probes on an Applied Biosystems Fast PCR instrument as described. Mice were injected (i.p.) with CCl₄ for 5 weeks. The inhibitors were administered subcutaneously via osmotic minipumps that delivered the compounds at a calculated dose of 10 mg/kg/day for a 30 gram mouse. Error bars represent standard deviation. *p-value vs. Control group < 0.05. N = 3 for Celecoxib+CCl₄ group, N = 4 for all other groups. Statistical tests are described in Materials and Methods. The raw data used for this figure is reported in Supplemental Data Table S3.
Figure 3. Celecoxib attenuates the increased recruitment of macrophages caused by CCl₄ treatment. (A-E) IHC on liver tissue with an anti-F4/80 antibody (40x). (F) Quantification of IHC stain using the Aperio positive pixel count algorithm. Mice were injected (i.p.) with CCl₄ for 5 weeks. The inhibitors were administered subcutaneously via osmotic minipumps that delivered the compounds at a calculated dose of 10 mg/kg/day for a 30 gram mouse. Error bars represent standard deviation. *p-value vs. Control group < 0.05. N = 5. Statistical tests are described in Materials and Methods. The raw data used for this figure is reported in Supplemental Data Table S3.

Figure 4. Celecoxib further increases the hepatic stellate cell activation triggered by CCl₄ treatment. (A-E) IHC on liver tissue with an anti-αSMA antibody (40x). (F) Quantification of stain using the Aperio positive pixel count algorithm. Mice were injected (i.p.) with CCl₄ for 5 weeks. The inhibitors were administered subcutaneously via osmotic minipumps that delivered the compounds at a calculated dose of 10 mg/kg/day for a 30 gram mouse. Error bars represent standard deviation. *p-value vs. Control group < 0.05. ‡p-value vs. CCl₄ only group < 0.05. #p-value vs. Celecoxib+CCl₄ group < 0.05. N = 5. Statistical tests are described in Materials and Methods. The raw data used for this figure is reported in Supplemental Data Table S3.

Figure 5. Celecoxib modulates the increase in metabolites in the COX and LOX branches of the arachidonic acid cascade caused by CCl₄ treatment. (A-D) Hepatic tissue levels of metabolites in the COX branch. (E) Hepatic tissue levels of Lipoxin A₄, a metabolite in the LOX branch. Tissue levels of compounds were analyzed by LC-MS/MS after solid phase extraction as described in Materials and Methods. Mice were injected (i.p.) with CCl₄ for 5 weeks. The inhibitors were
administered subcutaneously via osmotic minipumps that delivered the compounds at a calculated dose of 10 mg/kg/day for a 30 gram mouse. For a simplified diagram of these pathways see Supplemental Data Figure S1. Error bars represent standard deviation. *p-value vs. Control group < 0.05. ‡p-value vs. CCl₄ only group < 0.05. #p-value vs. Celecoxib+CCl₄ group < 0.05. N = 6 for Celecoxib+CCl₄ group, N = 5 for all other groups. Statistical tests are described in Materials and Methods. The raw data used for this figure is reported in Supplemental Data Table S3.
1 **Tables**

2 Table 1. Histological scoring of H&E-stained sections for inflammation, fibrosis and cellular damage on a 0-3 scale as described in Supplemental Data Table S1.

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Figure 1
Figure 2

A. COL 1A1

B. MMP-2

C. MMP-9

D. TIMP-1

E. TGF-β

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Figure 3
Figure 4

A Control

B CCl₄ only

C Celecoxib+CCl₄

D Celecoxib+TPPU+CCl₄

E PTUPB+CCl₄

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Figure 5