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The Synthetic Triterpenoid RTA 405 (CDDO-EA) Halts Progression of Liver Fibrosis and Reduces Hepatocellular Carcinoma Size Resulting in Increased Survival in an Experimental Model of Chronic Liver Injury

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ABSTRACT

Patients with cirrhosis have an increased risk of developing liver cancer and a higher rate of mortality. Cirrhosis currently has no known cure, and patients may benefit from new agents aimed at alleviating their complications and slowing down the rate of disease progression. Therefore, the effects of the orally bioavailable synthetic triterpenoid 2-cyano-3,12-dioxooleana- 1,9(11)-dien-28-oate-ethyl amide (CDDO-EA, RTA 405), which has potent antioxidative and antiinflammatory properties, was evaluated in a chronic carbon tetrachloride (CCl_4)-induced model of liver cirrhosis and hepatocellular carcinoma (HCC). Mice were injected with CCl_4 (to induce fibrosis and cirrhosis) or placebo biweekly for 12 weeks followed by CDDO-EA in the diet for 18 weeks with continued biweekly injections of CCl_4. Chronic CCl_4 administration resulted in cirrhosis, ascites, and HCC formation, associated with increased serum transforming growth factor-β1, hepatic hydroxyproline content, and increased serum bilirubin. CDDO-EA, whose administration commenced after establishment of liver fibrosis, decreased liver fibrosis progression, serum bilirubin, ascites, and HCC formation and markedly increased overall survival. CDDO-EA also attenuated -TNFα (tumor necrosis factor-α), α-SMA (alpha smooth muscle actin), augmented -IL-10 levels, and improved histologic and serologic markers of fibrosis. Conclusions: CDDO-EA mitigates the progression of liver fibrosis induced by chronic CCl_4 administration, which is associated with the induction of antifibrogenic genes and suppression of profibrogenic genes.

Key words: liver fibrosis; carbon tetrachloride; RTA 405; triterpenoid; antioxidant; antiinflammatory; hepatocellular carcinoma; cirrhosis; CDDO-EA; and end stage liver disease

Cirrhosis, a major clinical issue both in health care cost and direct patient care (annual death rate of 27 000), is an important cause of liver-related deaths in the United States (Minino et al., 2006), and its incidence is on the rise (Lefton et al., 2009; Mehta and Rothstein, 2009). Cirrhosis results from a variety of insults that include infections (Hepatitis C Virus), autoimmune

processes, metabolic changes (nonalcoholic steatohepatitis), drugs of abuse, and chronic alcohol consumption, which ultimately results in chronic liver injury (Arundel and Lewis, 2007; Lee and Seremba, 2008). To model human chronic liver injury, the prolonged administration of carbon tetrachloride (CCl_4) is a well-established animal model system commonly used for investigating mechanisms involved in the induction of liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (Kang et al., 2012; Sakata et al., 1987).

Liver fibrosis results from chronic injury and excessive accumulation of extracellular matrix (ECM) proteins. Advanced fibrotic scars, disrupting the hepatic architecture and the subsequent development of regenerating hepatocyte nodules, eventually lead to portal hypertension and liver failure. The pathogenesis of liver fibrosis is believed to depend on the activation of hepatic stellate cells (HSCs) that participate in the process of fibrotic scar formation through a complex interaction of matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitor of metalloproteinases (TIMPs). More importantly, HSCs participate in the production of collagen type I which is major component of fibrotic tissue formation (Friedman, 2008b). Besides HSC, other nonparenchymal and immune cells also contribute to this process by disrupting normal homeostasis between matrix production and degradation (Jarnagin et al., 1994). The relevance of the immune system to liver fibrosis has been demonstrated in several mouse models. One example shows that toxin induced liver injury activates natural killer T-cells (NK T-cells) leading to inhibition of liver regeneration via a tumor necrosis factor-α (TNFα) dependent mechanism (Wei et al., 2010). In addition, these activated NK T-cells target activated HSCs via retinoic acid early inducible 1/NKG2D-dependent and tumor necrosis factor–related apoptosis-inducing ligand–dependent mechanisms to limit the extent of fibrosis (Radaeva et al., 2006).

The search for novel antifibrotic drugs to combat this important clinical issue is ongoing, and the synthetic triterpenoid 2-cyano-3,12-dioxooleana- 1,9(11)-dien-28-oate-ethyl amide (CDDO-EA) is a potential candidate. CDDO-EA, CDDO-Me, and many related compounds, including CDDO-Im, activate the Keap1-Nrf2 pathway, which facilitates induction of an array of antioxidative enzymes and alleviation of inflammation (Reisman et al., 2009a). In addition to its antifibrotic-properties, its potential applicable efficacy in combating liver cancer may be related to its ability to enhance the expression of TGF-β-dependent genes (Sporn et al., 2011; Suh et al., 2003), induce apoptosis (Zou et al., 2007, 2008), and inhibit interleukins such as IL-6 through direct interaction with both STAT3 and IKK (Ahmad et al., 2008; Duan et al., 2009). Semisynthetic triterpenoids also inhibit constitutive STAT3 phosphorylation and block the degradation of IKB-α when challenged with TNFα (Shishodia et al., 2006). CDDO-Me, an analog of CDDO-EA, has been used in several different cancer models. In human leukemia cell lines, CDDO-Me inhibits both constitutive and inducible NF-κB (Shishodia et al., 2006), whereas in lung cancer models it suppresses IFN-γ action and induces heme oxygenase-1 (Liby et al., 2007).

Despite its multiple mechanisms of action, the potential preventive role of CDDO-EA in liver cirrhosis/fibrosis has not been evaluated. In this study, we sought to determine if the oral administration of CDDO-EA would alter the course of liver fibrosis. Mice were treated with CCl_4 for 12 weeks and then administered CDDO-EA for 18 weeks (whereas CCl_4 challenge continued), and end stage liver disease outcomes were assessed for reversibility of fibrosis sequelae. Our results indicate that CDDO-EA treatments ameliorate CCl_4-induced liver injury and fibrosis.

Moreover, early treatment with CDDO-EA in the course of this disease alters ascites and HCC formation and markedly reduces mortality.

MATERIALS AND METHODS

Animals, reagents used, and experimental design. CCl_4 and all other chemicals used in these studies were from Sigma-Aldrich (St. Louis, Missouri), unless specified otherwise. Male mice between ages of 8 and 12 weeks were obtained from The Jackson Laboratory [(C57BL/6J mice) Bar Harbor, Maine]. All animal studies were carried out in compliance with accepted standards of humane animal care as described in the Guide for the Care and Use of Laboratory Animals (Council, 1996) and were approved by the UT Southwestern Institutional Animal Care and Use Committee. Mice were euthanized by CO_2 inhalation consistent with the AVMA Guidelines on Euthanasia. Mice were injected intraperitoneally with CCl_4 dissolved in corn oil in a 3:1 ratio (vol/vol) at a dose of 1–2 mg/kg body weight, or control animals received an equal volume of corn oil starting at week 0 and continued as defined in the different experimental protocols (Supplementary Figure 1). Briefly, CCl_4 was administered biweekly for 30 weeks. CDDO-EA (also referred to as RTA 405, Reata Pharmaceuticals, Inc., Irving, TX) was then introduced in the *ad libitum* diet of the mice (200 mg/kg) at week 12 for a period of 18 weeks with continued biweekly injections of CCl_4. The diet composition was as follows: 95% Purina diet 5002, 1.25% Ethanol, 3.75% Neobee Oil, 0.02% RTA 405, and 0.03% FD&C Red Dye#40. The placebo diet used in this study includes a vehicle without the dye (or RTA 405) with the difference made up with the Purina diet 5002. At the end of the experiment, animals were sacrificed at the indicated times after a 1-week washout to eliminate acute effects of CCl_4 and assessed for fibrosis, HCC, and ascites formation. For the acute CCl_4 toxicity experiments, animals were placed on CDDO-EA diet for 2 weeks prior to the administration of CCl_4 as described above. Animals were sacrificed 8 h post CCl_4 injection to assess the acute effects on CYP2E1 and alanine transaminase (ALT) levels.

Serum liver function assay. Following euthanasia, blood samples were collected for isolation of plasma for assessment of liver injury. Serum levels of ALT, aspartate transaminase, albumin, total bilirubin, creatinine, sodium, gamma-glutamyl transpeptidase (GGT), total protein, alkaline phosphatase, and blood urea nitrogen (BUN) were measured using Vitros-250 Chemistry Analyzer at the University of Texas-Southwestern (Dallas, Texas) core facility.

Immunoblotting. Western blotting was performed as previously described (Getachew et al., 2010). Immunodetection was performed using the Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, Illinois). Anti-α-SMA (alpha smooth muscle actin) mouse monoclonal and anti-CYP 2E1 polyclonal rabbit primary antibodies were obtained from Abcam, Inc. (Cambridge, Massachusetts). Beta-actin and GAPDH purified antibody, HRP-conjugated anti-rabbit and HRP-conjugated anti-rabbit Ig secondary antibodies were obtained from Pierce Biotechnology (Thermo Scientific, Rockford, Illinois). Survivin and Nrf2 antibodies were purchased from Fisher Scientific (Chicago, Illinois).

Quantitative RT-PCR of liver mRNA. RNA isolation and real time PCR analysis has been previously described (Getachew, et al., 2010). Primer sequences used are listed in Supplementary
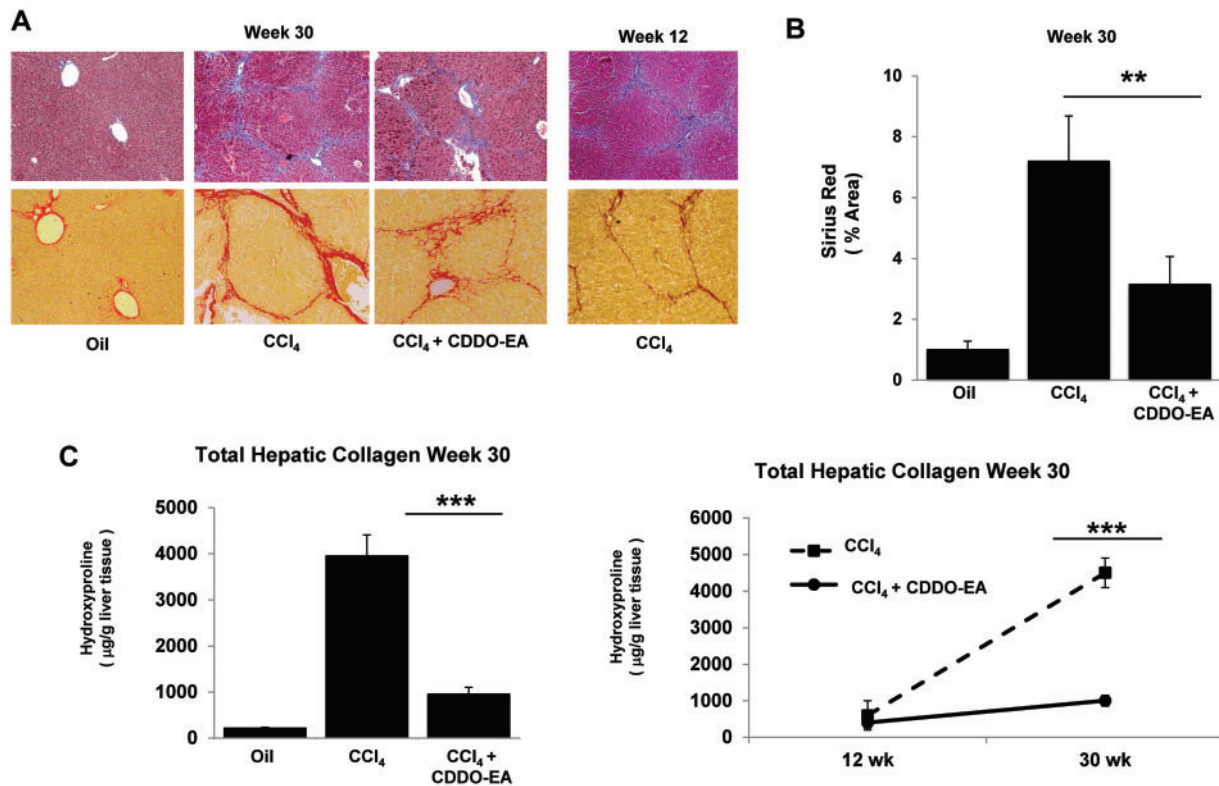
FIG. 1. CCl$_4$-treated mice exhibit decreased level of fibrosis after CDDO-EA rescue treatment. A, Liver histology was assessed with trichrome and Sirius red stain. Data show a representative slide in all groups (magnification at 20×). Slides depict time from week 30 (left 3 panels) as well as week 12 (right panel). The amount of Sirius red stain collagen is shown in (B) as a histogram. C, Total hepatic collagen content is shown as hydroxyproline content. Data represent the mean ± SE of all mice (N = 10 per group). *P < .05, **P < .01, ***P < .001.

Table 1, and unless otherwise indicated, all primers are of mouse origin.

Evaluation of ascites and HCC. The volume of ascites was estimated at the time the mice were euthanized. After laparotomy, the abdominal fluid was drained by paper strips that were then weighed before and after placement in the abdominal cavity for absorbing the ascites fluid. Livers were removed and grossly visible lesions on the surface of the liver were counted and sized. Numbers of tumors and their size and histopathology were evaluated in a nonblinded fashion. The livers were then sectioned beginning at the medial hilar surface, and stained with hematoxylin & eosin (H&E) per standard histologic techniques.

Histopathological examination. H&E, Masson's trichrome and Sirius red staining protocols have been previously described (Dooley *et al.*, 2003). To evaluate the relative fibrosis area, the measured collagen area was divided by the net field area and then multiplied by 100. From each animal analyzed, the amount of fibrosis as a percentage was measured and the average value presented using the Sirius red method.

Hepatic hydroxyproline content. Hydroxyproline content was assessed as previously described (Kliment *et al.*, 2011). Following homogenization in 6 N hydrochloric acid (HCl), liver tissues underwent hydrolysis at 110°C for a period of 18 h. After cooling of the hydrolysate, it was treated with activated charcoal, filtered, evaporated, and resuspended in distilled water at which point chloramine T was added. Five minutes later, Ehrlich's reagent was added to the mixture, followed by incubation for a period of 30 min at 60°C. Using a reagent blank containing the complete system without added tissue, samples were read on a spectrophotometer at 560 nm. The hepatic hydroxyproline content was expressed as micrograms per gram of tissue (dry weight).

Cell line experimental culture conditions. Primary human HSCs (HHSCs, Cat# 5300) (ScienCell Research Laboratories) from a frozen stock were purchased and cultured in stellate cell medium (SteCM, Cat# 5301) at density of 5 × 10^5 per 1 ml volume according to manufacturer's instruction. CDDO-EA was dissolved in dimethyl sulfoxide at a concentration of 10 mM, and aliquots were stored at −80°C. The stock solution was diluted to the desired final concentrations with growth medium just prior to use. *Escherichia coli* LPS (100 ng/ml; Sigma-Aldrich Co., St. Louis, Missouri) was used 6 h daily to activate HHSC cells for 3 days followed by media change. RNA and protein extraction were performed at day 3 post induction. Plating cells under normal conditions for 5 days with daily medium change achieved culture activation without the addition of LPS. A rat hepatoma cell line (FAO) was cultured in Ham's F12 Media with 15%–18% fetal bovine serum, and supplemented with 1% penicillin/streptomycin (10 000 units/ml/10 000 μg/ml), 5% L-glutamate (29 mg/ml), and 5% sodium pyruvate (11 mg/ml) [Sigma-Aldrich] was used in the cell based experiments.

Isolation of mouse hepatic stellate cells. Mouse HSCs (MHSCs) were isolated using the collagenase-pronase perfusion protocol that has been previously described (Bataller *et al.*, 2003). Briefly, mice
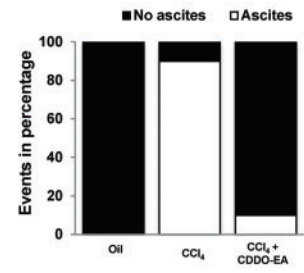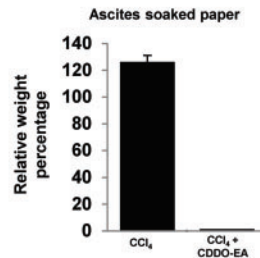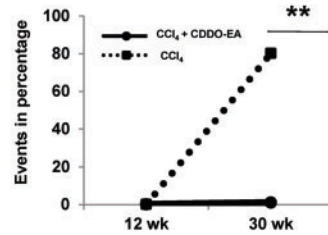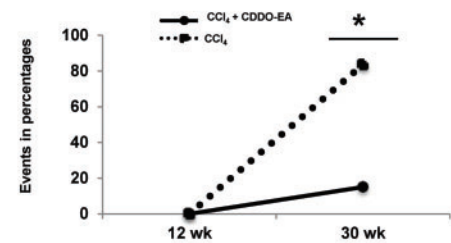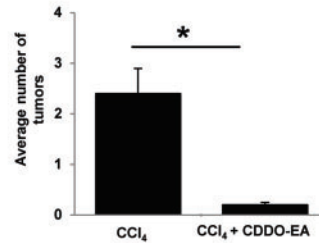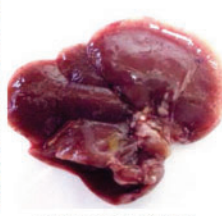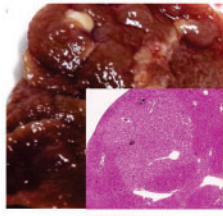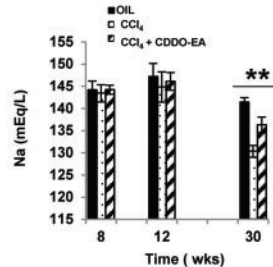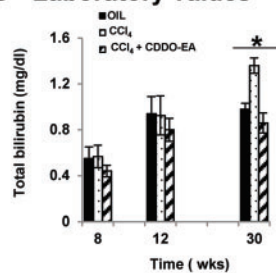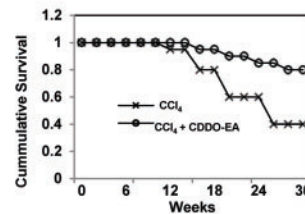
FIG. 2. CDDO-EA treatment reduces complication of end stage liver disease and improves mortality. A, Representative ascites photograph at week 30 in CCl_4 treated group. Longitudinal graph shows percentage of ascites formation. Histogram shows relative weight of soaked ascites paper (middle panel) whereas right panel depicts event noted per group for the formation of ascites. B. Representative photograph of gross livers from mice at week 30 post treatment of CCl_4 plus CDDO-EA. Histogram depicts average number of tumors identified per group. Data are presented as mean ± SE (n = 10 per group). Longitudinal graph shows percentage of HCC formation after CCl_4 treatment for 12 weeks followed by 18 more weeks of CDDO-EA. C. Liver function test over time in CDDO-EA treated versus nontreated. Data represent mean ± SE (n = 20 per time point). D, CCl_4 treated mice had > 70% mortality after 30 weeks while addition of CDDO-EA at week 12 had < 20% mortality during the same period of time. (N = 40 per group). *P < .05, **P < .01, ***P < .001.

were anesthetized and the portal vein was accessed with an 18G catheter. Perfusion was performed as described by Bataller *et al.* (2003) with perfusate containing pronase E, collagenase D, and DNase in sequential manner for total of 45 min. The liver was then removed and incubated for 15 min in a solution containing DNase and collagenase D and afterwards filtered through a sterile nylon 120 μm mesh. Subsequent cell washes and suspension was performed in a solution containing Accudenz solution to achieve a gradient white layer isolate that contains HSCs. Cell sorting and purification were performed using a CyAnTM LX flow cytometer (Dako Cytomation, Carpintaria, California) and FlowJo software (TreeStar, Ashland, Oregon). Cell gating was performed on singlets, excluding doublets, after depletion of Kupffer cells using anti-F4/80 (BD Biosciences) and anti-CD11b (BD Biosciences). MHSC activation was achieved as described above using chronic CCl_4 administration over 30 weeks. Cells that were isolated were stained for collagen-1α1 and DAPI.

Statistical evaluation. Data are presented as mean ± standard error (SE). To detect differences among groups, statistical significance was evaluated by *t* test (for comparison of 2 groups) or 1-way ANOVA (followed by a posttest using Bonferroni for comparison of multiple groups; Graph Pad Prism Version 5 [Graph Pad Software, San Diego, California]); differences at the level of *P < .05, **P < .01, and ***P < .001 are noted separately.

RESULTS

CDDO-EA Reduces Liver Fibrosis Progression

To characterize the importance of CDDO-EA in reducing liver fibrosis in the course of CCl_4-induced liver injury, wild type (C57BL/6J) mice were treated with 2 mg/kg of CCl_4 over a course of 30 weeks. Administration of CCl_4 resulted in marked fibrosis as determined by Masson's trichrome and Sirius red stains as depicted in Figure 1A. All control (oil treated) mice revealed a normal distribution of collagen, while extensive collagen deposition and nodule formation was evident in liver tissue from CCl_4 only treated groups. Sirius red staining demonstrated that CDDO-EA administration was associated with a marked decrease in the amount of fibrotic tissue compared with control
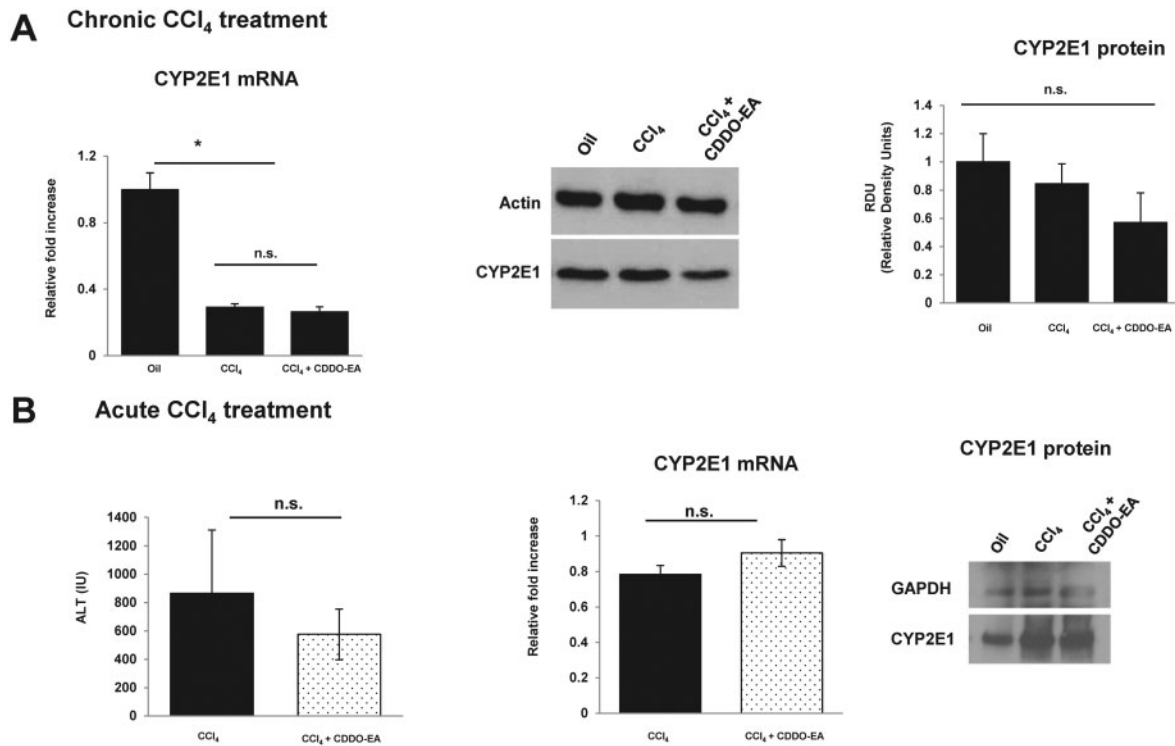
FIG. 3. CDDO-EA reduces CCl₄-induced fibrosis without altering CYP2E1 levels compared with CCl₄ treatment. A, mRNA and protein expression expression of CYP2E1 was assessed at 30 weeks post CCl₄ treatment versus CCl₄ + CDDO-EA. Histogram depicts relative density units of protein expression. B, Acute effect of CCl₄ was assessed with measurement of ALT, mRNA and protein expression expression of CYP2E1. Protein expression was assessed via Western blot analysis. Data are presented as mean ± SE (n = 10 per group). Each experiment was performed in duplicate. *P < .05, **P < .01, ***P < .001.

groups with CCl₄-induced liver injury (Figure 1A), which was significant upon quantification of the area of the injury (Figure 1B). Further, analysis of hydroxyproline content was consistent with the histological findings and demonstrated that CDDO-EA almost completely inhibited accumulation of hepatic collagen induced by chronic CCl₄ administration (Figure 1C).

CDDO-EA Reduces End Stage Liver Disease Complications and Improves Overall Survival

CDDO-EA treatment resulted in an almost complete reduction to naïve control levels in complications of end stage liver disease including overall mortality. Ascites, spleen size, and HCC formation were evaluated. The amount of ascites formation was noted to be higher in CCl₄-treated mice compared to CCl₄ plus CDDO-EA-treated groups (Figure 2A). Spleen sizes were similar in both groups of mice (data not shown). As shown in the Supplementary Table 2, other liver tests did not have statistically different values. To investigate whether CDDO-EA reduces HCC formation, livers of mice were visually inspected at experimental termination for the formation of tumor nodules. As depicted in Figure 2B, higher numbers of HCCs were noted in CCl₄-treated mice, and CDDO-EA treatment resulted in a marked reduction in the absolute numbers of HCC formed. CCl₄ administration produced tumors that were 3 mm to 1.5 cm in size and were either single or multiple nodules with well-differentiated HCC histological characteristics but remarkably, only a single 5 mm tumor was noted in the CCl₄ + CDDO-EA treated cohort. Assessment of liver function tests showed CDDO-EA treated groups had near normal levels of total bilirubin and serum sodium, whereas the CCl₄ treated only group had decreased serum sodium and elevated total bilirubin

(Figure 2C). Further, assessment of overall mortality demonstrated CDDO-EA-treated mice had greater than 80% survival, while the CCl₄ only treated group had only a 30% survival rate after 30 weeks (Figure 2D).

CDDO-EA Reduces CCl₄-Induced Fibrosis Without Altering CYP2E1 Levels

CYP2E1 mRNA and protein expression were evaluated by real time PCR and Western blot analysis, respectively. As depicted in Figure 3A, mRNA expression of CYP2E1 was decreased in the CCl₄ group and CDDO-EA + CCl₄ groups. The addition of CDDO-EA did not alter either mRNA or protein expression of CYP2E1. FAO cell lines were used to test the ability of CDDO-EA to induce CYP2E1 but no statistically significant elevation of either mRNA and protein expression was observed (Data provided in Supplementary section and Supplementary Figure 2). In addition, to assess the impact of CCl₄ on expression of CYP2E1, CCl₄ was administered in acute setting and mRNA and Western blot was assessed. As depicted in Figure 3B no difference was noted but ALT measurements showed a trend toward reduction in CDDO-EA group.

CDDO-EA Attenuates Fibrosis Correlating with Altered Levels of Proinflammatory and Antiinflammatory Cytokines

Synthetic triterpenoids amplify expression of antiinflammatory cytokines and attenuate expression of proinflammatory cytokines (Deeb et al., 2010; Duan et al. 2009; Sporn et al. 2011; Suh et al. 2003). The livers of CCl₄-treated and CDDO-EA-treated mice were assessed for levels of various proinflammatory and antiinflammatory cytokines. Proinflammatory cytokine mRNA encoding for TNFα were up regulated in CCl₄-treated groups,
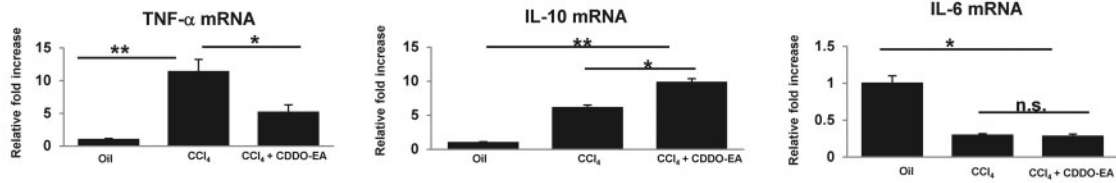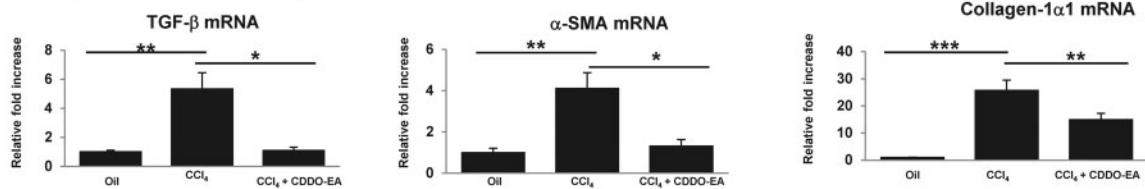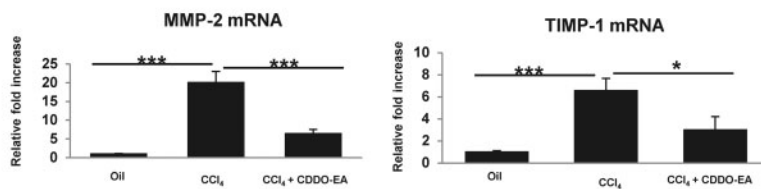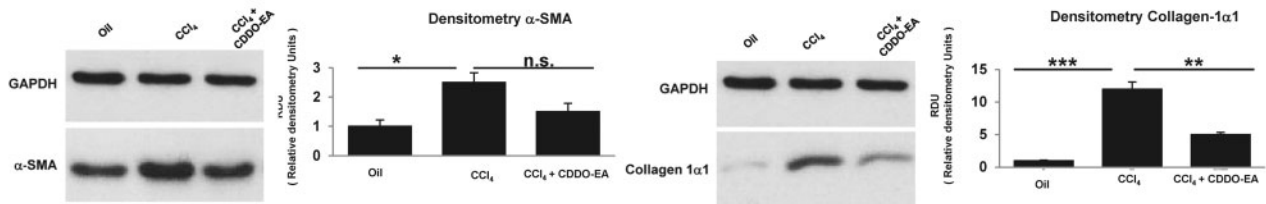
FIG. 4. CDDO-EA attenuates fibrosis by altering expressions of proinflammatory and antiinflammatory mediators. A, mRNA expression of proinflammatory and antiinflammatory cytokines. B, mRNA expression of profibrogenesis cytokines was assessed which includes TGF-β, collagen-1α1, and α-SMA. C, mRNA expression of tissue remodeling cytokines was assessed at baseline and week 30 post-CCl₄ treatment in the presence of CDDO-EA versus control. Data are presented as mean ± SE (n = 10 per group). D, A representative Western blot showing α-SMA and collagen-1α1 with relative amount of protein expression determined by densitometry were measured at week 30 after CCl₄ versus CCl₄ for 12 week followed by 18 weeks of CDDO-EA + CCl₄. Data are presented as mean ± SE of all mice (N = 10 per group). Each figure legend depicts an independently performed 2 experiments. *P < .05, **P < .01, ***P < .001.

and decreased with CDDO-EA treatment. IL-6 mRNA levels were reduced in all groups of CCl₄-treated mice. IL-10, in contrast, was markedly elevated in all groups of CCl₄-treated mice and was further upregulated by the administration of CDDO-EA (Figure 4A). No differences were noted in other cytokines, including IL-1β, IL-18 and IL-4 (data not shown). As a main positive regulator of fibrosis, TGF-β was significantly reduced in CDDO-EA-treated mice. In parallel, production of collagen-1α1 and α-SMA was noted to be reduced in CDDO-EA treated group (Figs. 4B and D). Also depicted in Figure 4C, analysis of MMP2 and TIMP1, which are implicated in tissue remodeling, showed down-regulation associated with CDDO-EA treatment.

CDDO-EA Attenuates In Vivo MHSC Profibrogenic Cytokines
To assess the role of CDDO-EA on MHSC, MHSC cells were isolated and analyzed by quantitative real-time PCR, Western blot, flow cytometry, and cell staining. The purity of MHSC was achieved by depleting macrophages and B-cells as shown in Figure 5A. CDDO-EA treatment resulted in attenuation of MHSC activation as evidenced by immunohistochemistry stain in Figure 5B and mRNA cytokine expression of inflammatory and

fibrotic mediators (Figs. 5C–E). Further analysis of these groups showed that downstream targets of Nrf2 gene were up regulated in CDDO-EA treated mice (Figure 5F).

CDDO-EA Treatment Attenuates LPS Activation of HHSC Cells In Vitro
To assess the role of CDDO-EA on HHSC activation, HHSC were treated in vitro as detailed in the methods section. Cells that were treated with CDDO-EA showed lower level of markers of stellate cell activation (Figure 6A) and a reduction in markers of proinflammatory cytokines (Figs. 6B and C). In addition, HHSCs that were treated with CDDO-EA showed a significant elevation of Nrf2 protein levels (Figure 6D) when compared with the LPS treated group and controls. However, CDDO-EA treatment did not result in an alteration of levels of survivin, another known regulator of antiapoptotic pathway (Data shown in Supplementary Figure 3).

DISCUSSION

The role of ECM in advancing liver fibrosis in response to chronic injury is already documented (Friedman, 2008b). During
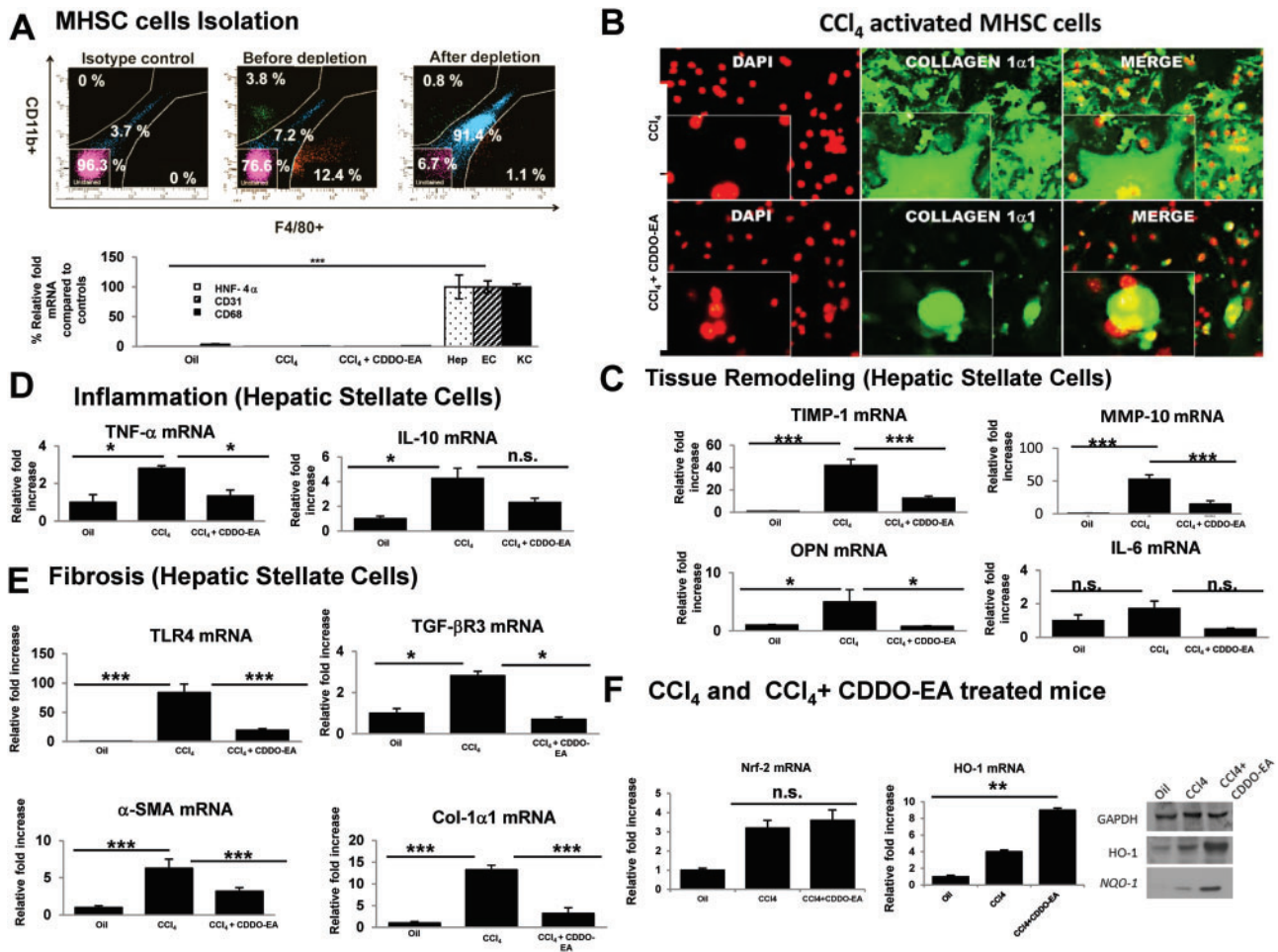
FIG. 5. Mouse hepatic stellate cells (MHSC) expresses profibrogenic genes with CCl$_4$ administration that is attenuated by CDDO-EA. A, MHSC cells were isolated and purified. Flow as well as mRNA data show isolation purity of MHSC cells after depletion. B, MHSC cells isolated were stained with collagen-1a1 to assess degree of activation. Pictogram shows a representative figure. C, mRNA expression in MHSCs of tissue remodeling cytokines. D, mRNA expression in MHSCs of inflammatory cytokines. E, mRNA expression of fibrogenetic cytokines were measured at week 30 after CCl$_4$ versus CCl$_4$ for 12 week followed by 18 weeks of CDDO-EA+ CCl$_4$. (F) mRNA expression in MHSCs for Nrf-2 and HO-1 as well as protein levels of HO-1 and Nqo-1 were measured. Data are presented as mean ± SE of all mice (N = 10 per group). Each figure legend depicts an independently performed 2 experiments. *P < .05, **P < .01, ***P < .001.

liver injury, quiescent HSCs become activated and acquire a myofibroblast-like phenotype (Friedman, 2008a) with the excess production of collagen-1α1 and α-SMA and reduced ECM degradation. The perpetuation of this proinflammatory and profibrogenic background, mediated by the interplay of paracrine and autocrine molecules, ultimately replaces the normal liver architecture with fibrotic tissue and regenerative parenchymal nodules (Friedman, 2008b).

This study demonstrates that CDDO-EA, an orally available antioxidant/antiinflammatory synthetic small molecule, treatment leads to decreased liver fibrosis. CDDO-EA attenuates the chronic liver fibrosis and continual remodeling and correlates with decreased levels of expression of α-SMA protein, suggesting CDDO-EA suppresses the activation of HSCs. This is further supported by the significant amount of fibrosis reduction depicted with Sirius red staining and the halting of fibrosis progression when CDDO-EA was given to the mice starting after week 12 of CCl$_4$ treatment (Figure 1C). We also demonstrated that the administration of CDDO-EA reduces the incidence and accumulation of ascites (Figure 2A). In mice that received CDDO-EA at an early fibrosis stage, ascites formation was abrogated.

We also evaluated the potential effects of CDDO-EA on HCC incidence and formation. In agreement with previous observations (Fujii et al., 2010), administration of CCl$_4$ resulted in increased HCC formation. In this study, we were able to demonstrate that treatment of CDDO-EA at the early stage of advanced fibrosis resulted in a marked reduction in the formation of HCC (Figure 3C). The antitumorigenic properties of CDDO-EA and its analogs have been evaluated and validated in a variety of cancer models including prostate, pancreatic cancer, and colorectal cancer cells. In these cancer models, synthetic triterpenoid compounds attenuate ROS generation and inhibit Akt, NF-κB and mTOR signaling proteins (Deeb et al., 2010; Gao et al., 2010), as well as directly interact with STAT3 and IKK to decrease constitutive IL-6 secretions (Liby et al., 2010). Suppression of IL-6 (Figure 5A) in MHSCs, STAT3, and NF-κB levels (data not shown) following CDDO-EA administration was also observed and consistent with a previous finding.(Shishodia et al., 2006) Also Inami et al reported that HCC formation is enhanced via Nrf2 pathway through p62 activation (Inami et al., 2011). CDDO-EA has multiple mechanisms that include activation of Nrf2, which in a controlled manner plays an important antiinflammatory role. Our findings suggest that the smaller number of HCCs
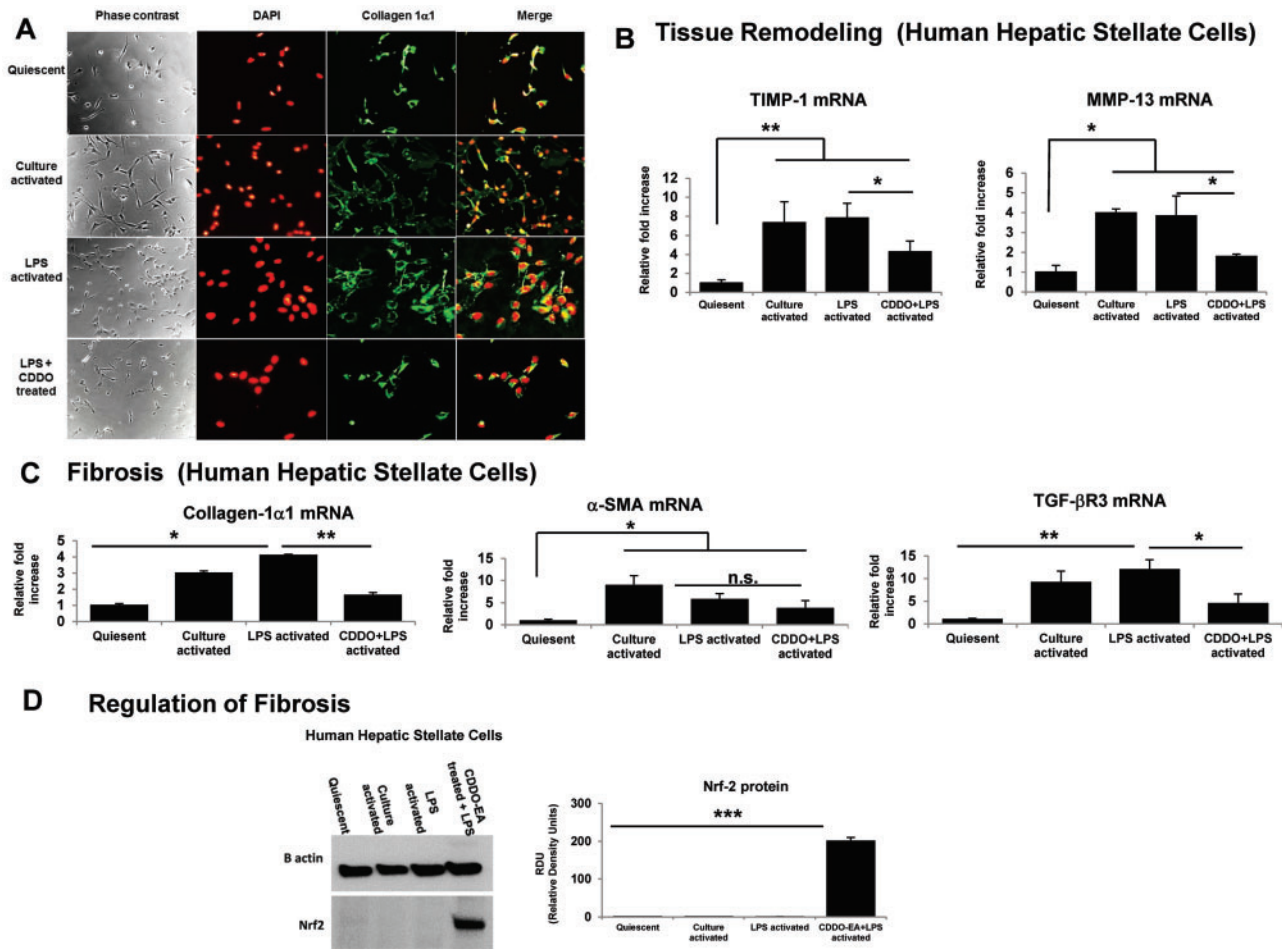
FIG. 6. CDDO-EA treatment attenuates LPS induced HHSC cells activation *in vitro*. A, A representative immunohistochemistry pictogram showing culture activation, LPS activation and CDDO-EA + LPS activation. B, mRNA expression of HHSC tissue remodeling cytokine (C) mRNA expression of HHSC fibrogenic cytokine (D) protein expression of potential effectors of CDDO-EA mechanism of action. Data are presented as mean ± SE of the 4 different groups tested. (N = 3 per group). Each figure legend depicts 2 independently performed experiments. *P < .05, **P < .01, ***P < .001.

observed in CDDO-EA-treated mice may be due to mechanisms that either inhibits *de novo* tumor formation and/or regression of tumors that were formed. In contrast, in a constitutive activation state Nrf2 can participate in the development of HCC. Our current findings suggest that other factors such as the degree of fibrosis and MMP-2 interaction with the ECM may also play a pivotal role in altering the fate of HCC formation in the liver.

This study also demonstrates that administration of CDDO-EA to mice with CCl_4-induce liver fibrosis is associated with the attenuation of several profibrogenic and proinflammatory mediators, as well as increases in some antiinflammatory mediators in chronic liver injury. CDDO-EA decreased the expression of proinflammatory cytokines including TNF-α and Osteopontin (OPN). IL-10, a major antiinflammatory cytokine, which reduces expression of TGF-β, TNFα, and TIMP-1 (Zhang *et al.*, 2006), was upregulated by CDDO-EA (Figure 4A), which is consistent with previous finding (Kang *et al.*, 2012).

The hepatoprotection related to CDDO-EA is also associated with changes in the levels of MMP-2 and TIMP-1 mRNA expression (Figure 4C), suggesting enhanced fibrolytic activity. Additionally, MMP-2 enhanced activity has been associated with cancer progression and metastatic invasion via type IV collagen degradation (Mook *et al.*, 2004) and by proteolytically activating TGF-β (Gialeli *et al.*, 2011). The levels of TGF-β, a

known potent mitogen that activates quiescent HSC to myofibroblast-like cells (Friedman, 2008a), is suppressed in the CDDO-EA-treated groups (Figure 4B). Potential mechanisms of action for the effect of CDDO-EA on TGF-β expression include downregulation of SOCS-3 (data not shown), a known activator of TGF-β transcription (Niwa *et al.*, 2005; Ogata *et al.*, 2006), and also attenuation of TLR-4 levels (Figure 5E). TLR-4 has been shown to be a major regulator of TGF-β expression (Seki *et al.*, 2007).

Chronic CCl_4 treatment resulted in reduction of CYP2E1 mRNA levels but further reduction by the administration of CDDO-EA post 12 weeks of CCl_4 treatment was not observed in these experiments (Figure 3A). In addition, treatment of FAO cell line treatment with CDDO-EA did not result in the reduction of CYP2E1 mRNA expression (Supplementary Figure 2). Thus, the protection that is observed by the treatment of CDDO-EA is unlikely to be related to differences in the formation of trichloromethyl free radicals (•CCl3), the electrophilic metabolite of CCl_4 that is implicated in the mechanism of CCl_4-induced liver injury. This was further confirmed by the treatment of mice that were on CDDO-EA diet 2 week prior to treatment of CCl_4 administration. Results as shown in Figure 3B show no alteration of CYP2E1 mRNA and protein level. ALT level analysis show a trend toward a reduced level in CDDO-EA group but that could

also be related to the antiinflammatory activity of CDDO-EA as seen in other agents such as acetaminophen (APAP) toxicity (Reisman *et al.,* 2009).

In addition, CDDO-EA resulted in the decreased level of activation of HSC as noted in Figures 5B and D. This was further supported by *in vitro* model of HHSC cells where CDDO-EA treatment resulted in decreased activation of these cells (Figure 6A). The potential mechanism for the decreased activation may have been related to the up regulation of Nrf2 (Figure 6B). The ability of CDDO-EA and related analogs to activate Nrf2 and induce its antioxidant target genes is a well-established mode of action for synthetic triterpenoids (Reisman *et al.,* 2009a). Nrf2 plays a major role in dampening inflammatory pathways likely contributing to the down regulation of proinflammatory cytokines. Others have shown that survivin, a known antiapoptotic gene plays a major role in protection against CCl_4 injury (Li *et al.,* 2010). However, analysis in our study showed no difference in the level of this mediator (Supplementary Figure 3), suggesting its minimal role in the protection that CDDO-EA affords in this model.

In summary, this study demonstrates an important role for CDDO-EA in the early stages of advanced fibrosis in a chronic CCl_4-induced liver injury mouse model. Moreover, CDDO-EA improved other sequelae of end stage liver cirrhosis and mortality. The multiple modes of action and diverse pharmacology of CDDO-EA may achieve the results in these studies by attenuation of proinflammatory mechanisms, as well as augmenting antioxidative, antiinflammatory, and anticarcinogenic pathways.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci. oxfordjournals.org/.

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