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A Retinoic Acid Receptor β 2 Agonist Reduces Hepatic Stellate Cell Activation in Nonalcoholic Fatty Liver Disease

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Abstract

Hepatic stellate cells (HSCs) are an important cellular target for the development of novel pharmacological therapies to prevent and treat non-alcoholic fatty liver diseases (NAFLD). Using a high fat diet (HFD) model of NAFLD, we sought to determine if synthetic selective agonists for retinoic acid receptor β 2 (RAR β 2) and RAR γ can mitigate HSC activation and HSC relevant signaling pathways during early stages of NAFLD, before the onset of liver injury. We demonstrate that the highly selective RAR β 2 agonist, AC261066, can reduce the activation of HSCs, marked by decreased HSC expression of α -smooth muscle actin (α -SMA), in mice with HFD-induced NAFLD. Livers of HFD-fed mice treated with AC261066 exhibited reduced steatosis, oxidative stress, and expression of pro-inflammatory mediators, such as tumor necrosis factor- α (TNF α), interleukin 1 β (IL-1 β), and monocyte chemotactic protein-1 (MCP-1). Kupffer cell (macrophage) expression of transforming growth factor- β 1 (TGF- β 1), which plays a critical role in early HSC activation, was markedly reduced in AC261066 treated, HFD-fed mice. In contrast, HFD-fed mice treated with an RAR γ agonist (CD1530) showed no decreases in steatosis, HSC activation, or Kupffer cell TGF- β 1 levels. In conclusion, our data demonstrate that RAR β 2 is an attractive target for development of NAFLD therapies.

Keywords

Retinoic Acid Receptor β 2; Retinoic Acid; Stellate Cells; Steatosis

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Conflict of Interest: Weill Cornell has filed a patent application on some of the intellectual property (IP) in this manuscript and this IP was licensed to Sveikatal, Inc. LJG and XHT are founders and have financial interests in Sveikatal, Inc.

Author contributions. S.E.T. performed experiments, analyzed data, wrote the manuscript, reviewed/edited manuscript. X.H.T. performed experiments, wrote manuscript, reviewed/edited manuscript. J.J. researched data, reviewed/edited manuscript. L.J.G. performed experiments, wrote manuscript, reviewed/edited manuscript. Dr. Lorraine Gudas is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analyses.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of liver diseases ranging from simple steatosis (fatty liver disease) to nonalcoholic steatohepatitis (NASH) and cirrhosis [1]. NAFLD is the most common liver disease in adults [2], and is emerging as a new health crisis worldwide [3]. No pharmacological therapies are FDA approved for prevention or treatment of NAFLD (1). Thus, the identification of new drugs for NAFLD is critical.

Hepatic stellate cells (HSCs), which reside in the liver sinusoids and contribute to the pathogenesis of chronic fibrotic liver disease [4, 5], are possible targets for novel NAFLD therapies [6]. HSCs are mesenchymal cells whose main function is to store 80–90% of the total body vitamin A (VA, retinol) pool as retinyl-esters (RE) [5, 7]. During hepatic injury HSCs lose their VA storage capacity, trans-differentiate into myofibroblasts, and orchestrate wound healing by secreting components of extra-cellular matrix (ECM), including type 1 collagen (coll1a1) and alpha-smooth muscle actin (α -SMA) [8]. The trans-differentiation of HSCs to fibrotic myofibroblasts is initiated by a number of paracrine stimulators, such as lipid peroxides, from injured hepatocytes, and inflammatory mediators, such as transforming growth factor beta-1 (TGF- β 1), secreted from Kupffer cells [8]. Sustained activation of HSCs in the progression of unchecked NAFLD leads to irreversible liver scarring and advanced fibrotic disease [8].

There is considerable evidence that in the early stages of NAFLD steatosis and its metabolic consequences, such as increased oxidative stress, lipid peroxidation, and inflammation, can initiate HSC activation in the absence of more advanced liver injury [9–13]. This suggests that early pharmacological interventions for attenuating the metabolic-inflammatory abnormalities that stimulate HSC activation represent a novel approach for preventing the progression of NAFLD to more advanced fibrotic liver disease. However, to date few researchers have examined the effects of pharmacological interventions on early HSC activation in dietary obesity models of NAFLD.

Retinoids are synthetic and natural analogues of VA, and there is interest in the potential of retinoids as anti-NAFLD agents because of their favorable effects on lipid metabolism [14, 15]. Our laboratory previously demonstrated an essential role for retinoids and for retinoic acid receptor- β (RAR β) in the regulation of pancreatic endocrine function and in the pathogenesis of obesity and NAFLD [16–18]. There are also data demonstrating that the VA metabolite, all-trans retinoic acid (RA), can mitigate HSC activation and fibrosis [19, 20], but the potential of synthetic agonists for specific retinoic acid receptors (RARs) for modulating HSC activation or altering the inflammatory and fibrogenic mediators involved in HSC activation is not known. Because of this lack of information, we tested the effects of synthetic agonists highly selective for RAR β 2 and another RAR gene, RAR γ , on mediators and markers of HSC activation using a high fat dietary model of NAFLD.

METHODS

Mice, diets, and drug treatments

Wild type (wt) male C57BL/6 mice were maintained on either a standard laboratory chow (Con) diet with 13 % kcal fat (diet# 5053, 15 IU/vitamin A-acetate/gram, Pico Diet, St. Louis, MO), or a high fat diet (HFD) with 45% kcal from fat (#58125, 4.7 IU vitamin A-acetate/gram, Test Diet, Inc., St. Louis, MO) for 4 months. We previously demonstrated that inducing obesity and NAFLD with chronic feeding of wt mice with HFD #58125 containing 4.7 IU vitamin A-acetate/gram resulted in significant reductions in tissue VA and VA signaling when compared to wt mice fed either the Weill Cornell Medical College (WCMC) vivarium standard chow diet (Pico Rodent Diet, #5053, with 15 IU/vitamin A-acetate) or a commercial control diet (Test-Diet, #58124, with 3.8 IU vitamin A-acetate/gram, Test Diet, Inc., St. Louis, MO)[17]. One month after the start of the HFD treatment, the Con chow and HFD groups were further split into 3 additional groups for 3 months to: i) remain on either Con chow (n=5) or HFD and drinking water containing 0.5% DMSO (n=5); ii) HFD and DMSO drinking water (0.5%) containing 1.5 mg/100 ml of AC261066 (n=5), a highly selective RAR β agonist [21], or iii) HFD and DMSO drinking water (0.5%) containing 2.5 mg/100 ml of CD1530 (n=4), a specific RAR γ agonist [22]. After 3 months of treatments, mice were sacrificed by cervical dislocation and liver samples were harvested for analysis.

Immunohistochemistry

Paraffin-embedded liver sections were deparaffinized and rehydrated, and antigen retrieval was performed using an antigen unmasking solution (Vector Laboratories, H-3300). The tissue sections were then incubated with the following antibodies overnight at 4 °C, 4-HNE (1:1000; Abcam, ab48506), or TGF- β 1 (1:100; Santa Cruz, sc-146). After incubation with secondary antibodies (SuperPicture™ Polymer. Detection Kit, Invitrogen), signals were visualized based on a peroxidase detection mechanism with 3,3-diaminobenzidine (DAB) used as the substrate.

Oil Red O Staining

Frozen liver tissue sections were fixed in 4% paraformaldehyde (PFA) for 15 minutes, followed by staining with Oil Red O reagent (Rowley Biochemical, H-503-1B) according to the manufacturers' protocol.

Liver Histopathology Evaluation

Paraffin-embedded liver samples were stained with hematoxylin and eosin (H&E) using standard protocols and Mason's trichrome kit for collagen detection (Poly Scientific, Bayshore, NY), according to the manufacturer's protocol. H&E and trichrome stained liver samples then underwent a complete histopathology evaluation for evidence of steatohepatitis, steatosis, and fibrosis according to the Brunt criteria [23].

Immunofluorescence Microscopy

Frozen mouse liver tissue sections were fixed in 4% PFA for 1 hour, followed by blocking with 2% bovine serum albumin (BSA) for 30 min, and then incubation overnight at 4 °C

with the following antibodies against: LRAT (1:300, Santa Cruz, sc-99015), α -SMA (1:500, Dako, clone 1A4), F4/80 (1:500, Santa Cruz Inc sc-52664), and TGF- β 1 (1:500; Santa Cruz, sc-146). Liver sections were then incubated with alexa-fluor 594 and 488 conjugated anti-rabbit (1:500), anti-mouse (1:500), anti-goat (1:500), and anti-rat (1:500) secondary antibodies (Invitrogen, Carlsbad, CA) for immunofluorescence labeling of targets, followed by visualization using a Nikon TE2000 inverted fluorescence microscope (Nikon, Inc). Oil Red O/ α -SMA double immunofluorescence staining was performed as described [24].

RNA Isolation and Quantitative RT-PCR (Q-RT-PCR)

Total RNA was isolated from mouse livers using TRIzol reagent (Life technologies) and 1 μ g was used to synthesize cDNA. Q-RT-PCR was performed as previously described [25], using gene specific primers (Table S1) to amplify mRNA target genes. cDNAs from 3–4 mouse samples per experimental group were analyzed for relative mRNA fold changes, calculated using the delta CT method, and these changes were normalized to hypoxanthine guanine phosphoribosyl transferase (Hprt), an internal control gene.

Statistics

All histograms are reported as the mean $\pm$ standard error of the mean (S.E.M) with 3–5 mice per experimental group. Significant differences are defined as p-values less than an alpha of 0.05 and were calculated using one-way and two-way analysis of variance, followed by Bonferroni multiple comparison post-hoc analysis with * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. All statistical analyses were performed using GraphPad Prism 6.0 statistical software (GraphPad Software, Inc).

RESULTS

RAR β 2 Agonist Reduces Hepatic Steatosis and Oxidative Stress

Consistent with reported HFD models of NAFLD in rodents [26, 27], we found that when compared to chow-fed mice, four months of HFD-feeding increased body weights (BW) (Fig. 1A) and caused hepatic steatosis. The livers were marked by macrovesicular lipid droplets (Fig. 1D [a,b]) and increased hepatic lipid content (Fig 1B, E [a,b]), a clinical hallmark of NAFLD steatosis [28]. AC261066, an RAR β 2 agonist, and CD1530, an RAR γ agonist, had no effect on BW in HFD-fed mice compared to HFD alone controls (Fig. 1A), but treatment with AC261066 resulted in a ~48% reduction in hepatic steatosis (Fig 1B, D[b,c], E[b,c]). In contrast, CD1530 treated, HFD-fed mice showed no decreases in hepatic lipid accumulation compared to HFD-controls (Fig 1B, D[b,d], E[b,d]).

Hepatic steatosis is associated with increased oxidative stress and the accumulation of reactive oxygen species (ROS), which can stimulate HSC activation and contribute to the pathogenesis of NAFLD [29–31]. Therefore, we next determined if livers from HFD-fed showed evidence of increased hepatic oxidative stress by measuring the levels of 4-hydroxynonenal (4-HNE), a hydroxyalkenal produced by ROS driven lipid peroxidation and a marker of oxidative stress [32]. Compared to chow-fed mice, livers from HFD-fed mice exhibited a higher percentage of 4-HNE positive cells (Fig. 1C, F[a,b], ~11% vs 32%, $p < 0.05$). We detected 4-HNE positive areas around or near lipid-laden hepatocytes (Fig. 1C, F,

[b, inset, yellow arrow]). HFD-fed mice treated with AC261066 compared to HFD-fed controls (Fig. 1C, F,[b,c]) showed reductions in the mean hepatic 4-HNE positive regions, with notable reductions in 4-HNE around lipid-filled hepatocytes (Fig. 1C, F[c, inset, yellow arrow]). We did not detect reductions in hepatic oxidative stress in the livers of HFD-fed mice treated with CD1530 (Fig. 1C, F,[b,d]).

The RAR β 2 Agonist Reduces Expression of Pro-inflammatory Mediators

Steatosis and excessive oxidative stress are associated with increased inflammatory responses by resident liver immune cells, which can further promote the expression of pro-inflammatory mediators, such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), the activation of HSCs and, if unchecked, the progression of NAFLD [31]. We examined hepatic mRNA levels of a panel of inflammatory mediators relevant to the pathogenesis of NAFLD and HSC activation, including monocyte chemoattractant protein-1 (MCP-1/CCL2, gene ID: 20296); its cognate receptor, chemokine (C-C) receptor 2 (CCR2, gene ID:12772); tumor necrosis factor (TNF- α gene ID:21926), interleukin-1 β (IL-1 β , gene ID:16176); and IL-6 (gene ID:16193) by Q-RT-PCR. Transcripts of the inflammatory mediators MCP-1, CCR2, TNF- α , IL-1 β and IL-6 were markedly elevated in livers of HFD-fed compared to chow-fed mice (Fig. 2A–E). We detected 30–40% reductions in MCP-1, CCR2, TNF- α , and IL-1 β transcripts (Fig. 2A, B, C, D) and a 73% reduction in IL-6 transcripts in livers of AC261066 treated HFD-fed mice (Fig. 2E). In contrast, MCP-1, CCR2, TNF- α , IL-1 β , and IL-6 transcripts were not reduced in livers of CD1530 treated, HFD-fed mice (Fig. 2A–E).

RAR β 2 Agonist Reduces Hepatic Stellate Cell (HSC) Activation

Hepatic steatosis and inflammation can increase the risk for progression of NAFLD and promote early phases of HSC activation, marked by increased HSC expression of α -SMA [8, 12]. To examine the effects of AC261066 on steatosis-associated HSC activation in HFD-fed mice, we performed double immunofluorescence staining of HSCs using antibodies against LRAT, a marker of HSCs [4], and the HSC activation marker α -SMA. The livers of HFD-fed mice showed increases in LRAT (green)/ α -SMA (red) double positive HSCs (yellow/orange) (Fig. 3A white arrows [a,b]; B) compared to chow-fed mice; however, the percentages of total LRAT positive HSCs (Fig. 3C) and LRAT transcripts (Fig. 3D) were similar across all experimental groups. In contrast to the HFD-fed mice, the livers of AC261066 treated, HFD-fed mice showed reductions in the percentages of LRAT/ α -SMA double positive HSCs (Fig. 3A white arrows [b,c]; B). The percentages of activated HSCs in the livers of CD1530 treated, HFD-fed mice were the same as those in HFD-fed mice (Fig. 3A [b,d]; B). Liver sections co-stained with the neutral lipid stain Oil Red O (red) and α -SMA (green) showed staining patterns similar to those seen with 4-HNE staining in HFD-fed mice (Fig. 1F [a,b]), demonstrating that activated HSCs in HFD-fed mice clustered in areas with hepatocyte lipid accumulation (Fig. 3A [e, f]). We did not detect this clustering pattern of α -SMA positive HSCs in lipid positive (red) regions in livers of AC261066 treated HFD-fed mice (Fig. 3A [g]) but we did observe this pattern in CD1530 treated, HFD-fed mice (Fig. 3A [h]). Coll1a1 α is a major contributor to the ECM matrix during wound healing [8]. However, as others have reported in similar HFD-rodent models of early stages of NAFLD [33, 34] and in human NAFLD [35], we did not detect increased type 1 collagen (coll1a1, gene ID:12842) mRNA (Fig. 3E) or collagen deposition and fibrosis in the livers of

4-month HFD-fed mice (Fig. 3F) despite the increased percentages of activated HSCs (Fig. 3A).

RAR β 2 Agonist Reduces Kupffer TGF- β 1 Expression

TGF- β 1 is a multifunctional cytokine involved in HSC activation and a potential therapeutic target in NAFLD and other fibrotic liver diseases [36, 37]. We measured hepatic transcripts of TGF- β 1 and α -SMA, a direct transcriptional target of TGF- β 1 and a marker of TGF- β 1 signaling [38]. We detected approximately four-fold and three-fold increases in TGF- β 1 (gene ID: 21803) and α -SMA (gene ID: 11475) mRNA levels, respectively, in HFD-fed compared to chow-fed mice (Fig 4A, B). In contrast, we found that hepatic TGF- β 1 and α -SMA transcripts were ~38–40% lower in AC261066 treated, HFD-fed mice than in HFD-fed and CD1530 treated, HFD-fed mice (Fig 4A, B). By immunohistochemistry (IHC) we found that TGF- β 1 protein immunopositive areas were increased in livers of HFD-fed relative to chow-fed mice (Fig 4C, [a,b]) and that TGF- β 1 positive regions were localized near lipid enriched hepatocytes (Fig 4C, [b, inset, yellow arrow]). We observed reductions in hepatic TGF- β 1 protein levels in AC261066-treated, HFD-fed, but not in CD1530 treated, HFD-fed mice relative to HFD-fed mice (Fig 4C [b,c,d]), consistent with our Q-RT-PCR data (Fig. 4A).

TGF- β 1 is secreted by liver macrophages (Kupffer cells) in response to liver injury and is a key signaling protein in initiating the transdifferentiation of quiescent HSCs to activated myofibroblasts [8]. Therefore, we performed double immunofluorescence staining with antibodies against F4/80, a Kupffer cell specific marker [39], and TGF- β 1 to determine if AC261066 treatment resulted in reductions in Kupffer cell TGF- β 1 expression. The livers of HFD-fed mice displayed increased percentages of F4/80 positive Kupffer cells (Fig. 4E blue bars [+ = $p < 0.05$ vs. con]), and F4/80/TGF- β 1 double positive cells (Fig. 4D [a,b], [yellow/orange, white arrows], E grey bars [* = $p < 0.05$ vs. con]) compared to chow-fed mice. Livers of AC261066-treated, HFD-fed mice exhibited an ~38% reduction in F4/80 positive Kupffer cells (Fig. 4E, blue bars [# = $p < 0.05$ vs. HFD]), and a ~55% reduction in F4/80/TGF- β 1 double positive Kupffer cells (Fig. 4D [b,c], [yellow/orange, white arrows], E) compared to HFD-fed mice. CD1530-treated, HFD-fed mice showed no significant reductions in the percentages of F4/80 positive cells (Fig. 4E) and, consistent with our IHC staining, no reductions in the percentages of F4/80/TGF- β 1 double positive Kupffer cells (Fig. 4D [b,d], [yellow/orange, white arrows], E), compared to HFD-fed mice.

DISCUSSION

Activated HSCs have a central role in hepatic fibrogenic response to injury [5]; thus, development of anti-NAFLD therapies has focused on identifying novel molecules capable of modulating HSC activation and the pathways that drive HSC fibrogenic responses [40]. Here we examined the effects of highly selective agonists for RAR β 2 and RAR γ on HSC activation *in vivo*.

The early phase of HSC activation is initiated by a number of paracrine stimulators, such as lipid peroxides, from injured hepatocytes, and by inflammatory mediators from immune cells [8]. Histological evaluation and gene expression profiling of livers from AC261066 or

CD1530 treated, HFD-fed mice showed that administration of AC261066, but not CD1530, markedly reduced hepatic steatosis (Fig. 1B, E [b,c,d]) and oxidative stress (Fig. 1C, F[b,c,d]). In the current study we administered AC261066 at a dose of 1.5 mg/100 ml. However, unlike previous studies from our lab in which AC261066 at a dose of 3.0 mg/100 ml corrected hyperglycemia in genetic and high fat diet mouse models of obesity [41], we did not observe a glucose lowering effect in HFD-fed mice treated with AC261066 at 1.5 mg/100 ml, or in mice treated with CD1530 (Fig. S1 A–D). We also have shown that CD1530, provided in the drinking water, is active in reducing oral cavity carcinogenesis [42]. Taken together, these data demonstrate that the anti-NAFLD properties of AC261066 are independent of its glucose lowering properties. These data also indicate that different RAR selective agonists have effects on different organs and cell types.

Consistent with our previous studies demonstrating that obesity and NAFLD are associated with marked reductions in VA and VA signaling in multiple organs, including liver [17], we detected a 25-fold and 12-fold reduction in hepatic VA (retinyl plamitate and retinol, respectively) (Fig S2 A,B), and ~1.5 to 5.8 fold reductions in transcripts of the VA signaling [43] and metabolism [44] relevant genes, including retinoic acid receptors RAR α 2, RAR β 2, RAR γ 2 and cytochrome P450, 26A1 (CYP26A1) in HFD-fed mice compared to chow-fed mice (Fig S2 C–F). Hepatic VA levels were not markedly different in HFD-fed mice treated with AC261066 or CD1530 compared to untreated HFD-fed mice (Fig S2, A,B). However, among the three RAR mRNAs measured by Q-RT-PCR, hepatic RAR β 2 transcripts were not reduced in HFD-fed mice treated with AC261066 compared to chow-fed mice (Fig S2 D), and were ~6–10 fold higher than those detected in HFD-untreated and HFD-CD1530 treated mice respectively (Fig S2 C–E). These data demonstrate that AC261066 does not restore hepatic VA levels in NAFLD, but can maintain hepatic RAR β signaling, as RAR β 2 is a VA-responsive gene [45] and an indicator of endogenous VA signaling [46].

Livers from AC261066-treated, HFD-fed mice also exhibited reduced hepatic expression of a panel of genes that mediate both Kupffer cell chemotaxis, including CCL2/MCP-1 and CCR2, and inflammatory responses relevant to NAFLD, such as increases in IL-1 β , TNF- α , and IL-6 (Fig. 2A–E). We did not detect these changes in CD1530 treated, HFD-fed mice (Fig 2A–E). We also detected reduced percentages of hepatic F4/80 positive Kupffer cells (Fig. 4E [blue bars]), and reductions in F4/80/TGF- β 1 double positive Kupffer cells in mice treated with AC261066 (Fig. 4E, D [b,c]). TGF- β 1 is a central mediator of the multi-step signaling cascade that primes HSCs for further stimulation by injury-specific paracrine signaling [8] and is an important therapeutic target for NAFLD [37, 40]. Our data demonstrate that compared to CD1530, AC261066 uniquely suppressed the inflammatory TGF- β 1 axis and reduced the percentages of HSCs expressing the activation and myofibroblast marker α -SMA (Fig. 3A [b, c,], B), but the RAR γ agonist, CD1530, did not (Fig. 3A [b,d], B). Despite the increase in percentages of activated HSCs (co-expressing LRAT and α -SMA) (Fig 3A [a,b], B), livers of HFD-fed mice showed no evidence of increased colla1 mRNA levels, collagen deposition, or fibrosis (Fig. 3E, F), suggesting that HFD-driven NAFLD can promote "semi-activated" HSC populations that are not fully fibrogenic. This interpretation is consistent with findings from similar HFD-models of early stages of NAFLD [33], and is in line with evidence that HSC activation can occur during the

early stages of human NAFLD [47] and that HSC activation is not binary (quiescent or fibrogenic), but results in a range of pre- and poised states of activation [4, 12, 48].

What remains unclear is the question of whether AC261066 specifically reduces hepatic inflammatory responses through modulation of Kupffer and immune cell activities or if the reductions in steatosis by AC261066 indirectly mitigate inflammation. Chronic accumulation of hepatic triglyceride (TG) can stimulate oxidative stress and inflammatory responses in hepatocytes, Kupffer cells and HSCs [11, 12, 29, 31]. However, the convergence between metabolic and inflammatory pathways in NAFLD is not unidirectional [49], as acute phase cytokines, such as TNF- α , can also promote steatosis in NAFLD [50, 51], raising the possibility that the reduction in inflammation by AC261066 inhibits hepatocyte TG accumulation.

Discerning the molecular pathways that are drivers versus bystanders in the propagation and attenuation of steatosis and inflammation in the complex response of liver injury remains a challenge. Nevertheless, it is noteworthy that 4-HNE (Fig. 1F [b], α -SMA (activated HSCs) (Fig. 3A[e,f]), and TGF- β immunostaining (Fig. 4C [b, yellow arrows]) all showed a similar pattern of clustering around areas rich with lipid filled hepatocytes. These data suggest that ectopic lipids are a primary source of the immunogenic stimuli for HSC activation [12, 52], which is consistent with data showing that the metabolic consequences of simple steatosis can promote inflammation and HSC activation [9–11, 13].

Our double immunofluorescence data demonstrate that AC261066 treatments lead to a reduction in Kupffer cell TGF- β 1 inflammatory responses (Fig. 4D,E). However, as RARs can exert pleiotropic effects on metabolic and anti-inflammatory pathways in humans and rodents [14, 53] and all of the liver parenchymal (hepatocytes) and non-parenchymal cells (HSCs, Kupffer cells, endothelial cells, and immune cells) involved in the pathogenesis of NAFLD [54] express RAR β [55, 56], it is likely that the anti-NAFLD effects of AC261066 occur through modulation of multiple cell types involved in the metabolic and inflammatory axes. Taken together, our experiments demonstrate for the first time that a highly specific agonist for RAR β 2 [21] can improve HFD-driven NAFLD and possibly an early fibrogenic response of HSCs, strongly indicating a potential therapeutic role for AC261066 in preventing the progression of early stages of NAFLD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Messages

- Hepatic stellate cells (HSCs) are an important pharmacological target for the prevention of non-alcoholic fatty liver diseases (NAFLD).
- Retinoids and retinoic acid receptors (RARs) possess favorable metabolic modulating properties.
- We show that an agonist for retinoic acid receptor- β 2 (RAR β 2), but not RAR γ , mitigates HSC activation and NAFLD.

Figure 1. Retinoic Acid Receptor β (RAR β 2) Agonist Reduces Hepatic Steatosis and Oxidative Stress in NAFLD

A) Body weights (BW) of wt C57BL/6 male mice after 16 weeks of being fed: a standard chow (13% Kcal/fat) diet (Con, n=4); a high fat (45% Kcal/fat) diet (HFD, n=5); a HFD with the RAR β 2 agonist, AC261066 (1.5mg/100 ml) (HFD + AC261066, n=4) or an RAR γ agonist, CD1530 (2.5 mg/100 ml) (HFD + CD1530, n=4), in their drinking water. **B)** Quantitation of steatosis (% Oil Red O lipid positive fields), and **C)** 4-hydroxynonenal (4-HNE) stained livers from mice described in A. Errors bars represent $\pm$ SEM of n=3–4 animals per experimental group *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$. **D–F) [a–d]**, Representative images of: D) Hematoxylin and Eosin (H&E), E) Oil Red O and, F) 4-HNE stained liver sections from mice described in A. Scale bar = 50 μ m.

Figure 2. Retinoic Acid Receptor β 2 (RAR β 2) Agonist Reduces Hepatic Gene Expression of Pro-inflammatory Mediators

A) Semi-quantitative PCR of hepatic transcripts of *MCP-1* and *HPRT* (internal control) from wt C57BL/6 male mice after 16 weeks of being fed: a standard chow (13% Kcal/fat) diet (Con, n=4); a high fat (45% Kcal/fat) diet (HFD, n=5); a HFD with the RAR β 2 agonist, AC261066 (1.5mg/100 ml) (HFD + AC261066, n=4) or an RAR γ agonist CD1530 (2.5 mg/100 ml) (HFD + CD1530, n=4), in their drinking water. **B)** Quantitative Real-time PCR (Q-PCR) measurements of relative hepatic transcript levels of genes for pro-inflammatory mediators from mice described in A. Errors bars represent $\pm$ SEM of (n=3–4) animals per experimental group $\pm$ p<0.05 vs. con mice, $\ast$ =p < 0.05, $\ast\ast$ =p < 0.01.

Figure 3. Retinoic Acid Receptor $\beta 2$ (RAR $\beta 2$) Agonist Reduces Hepatic Stellate Cell (HSC) Activation in NAFLD

A) [a–d] Representative images of HSCs, double immunofluorescence stained with antibodies against the HSC marker LRAT (green) and the activation marker, α -SMA (red) [LRAT/ α -SMA double labeled HSCs yellow/orange positive fields, white arrows], and **[e–h]** HSCs double stained with an antibody against α -SMA (green) and the neutral lipid stain Oil Red O (red) from wt C57BL/6 male mice after 16 weeks of being fed: a standard chow (13% Kcal/fat) diet (Con, n=4); a high fat (45% Kcal/fat) diet (HFD, n=5); a HFD with the RAR $\beta 2$ agonist, AC261066 (1.5mg/100 ml) (HFD + AC261066, n=4) or a HFD with an RAR γ agonist CD1530 (2.5 mg/100 ml) (HFD + CD1530, n=4), in their drinking water. Scale bar = 50 μ m. **B)** Quantitation of HSC activation (% of LRAT/ α -SMA double positive HSCs) from mice described in A. **C)** Quantitation of % of LRAT positive HSCs from mice described in A. Quantitative Real-time PCR (Q-PCR) measurements of relative hepatic transcript levels of **D)** LRAT and **E)** Col1a1 from mice described in A. Errors bars represent $\pm$ SEM of (n=3–4) animals per experimental group $\pm$ p<0.05 vs. con mice, *p < 0.05. **F)** Representative images of trichrome stained liver sections from mice described in A. Scale bar = 50 μ m.

Figure 4. Retinoic Acid Receptor $\beta 2$ (RAR $\beta 2$) Agonist Reduces Kupffer Cell Expression of TGF- $\beta 1$ in NAFLD

Quantitative Real-time PCR (Q-PCR) measurements of relative hepatic transcript levels of **A)** TGF- $\beta 1$ and **B)** α -SMA from wt C57BL/6 male mice 16 weeks after being fed: a standard chow (13% Kcal/fat) diet (Con, n=4); a high fat (45% Kcal/fat) diet (HFD, n=5); a HFD with the RAR $\beta 2$ agonist, AC261066 (1.5mg/100 ml) (HFD + AC261066, n=4) or an RAR γ agonist CD1530 (2.5 mg/100 ml) (HFD + CD1530, n=4), in their drinking water. **C)** Representative images of TGF- $\beta 1$ stained livers from mice described in A. Scale bar = 50 μ m. **D)** Representative images of Kupffer cells double immunofluorescence stained with antibodies against the macrophage marker F4/80 (green) and TGF- $\beta 1$ (red) (F4/80/TGF- $\beta 1$ double labeled Kupffer cells yellow/orange positive fields, white arrows). Scale bar = 50 μ m. **E)** Quantitation of percent F4/80 and F4/80/TGF- $\beta 1$ double positive Kupffer cells in livers from mice described in A. Errors bars represent $\pm$ SEM of (n=3–4) animals per experimental group + = p<0.05 vs. con mice, * = p<0.05 vs. con mice, and # = p<0.05 vs. HFD-fed mice.