All trans-retinoic acid improves cholestasis in α-naphthylisothiocyanate treated rats and Mdr2−/− mice

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Running title: retinoic acid reduced liver injury in cholestatic rodents

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A list of nonstandard abbreviations: ANIT, α-naphthylisothiocyanate; atRA, all-trans retinoic acid; BA, bile acid; Bsep, bile salt export pump; Mdr2, multidrug resistance protein2; Mrp2, multidrug resistance-associate protein 2; NTCP/Ntcp, the Na+ taurocholate cotransporting polypeptide; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; PC, phosphatidylcholine; UDCA, ursodeoxycholic acid.

A recommended section assignment to guide the listing in the table of contents:
- Gastrointestinal, Hepatic, Pulmonary, and Renal
Abstract

Chronic cholestasis results in liver injury and eventually liver failure. Although ursodeoxycholic acid (UDCA) showed limited benefits in primary biliary cirrhosis, there is an urgent need to develop alternative therapy for chronic cholestatic disorders. Previous studies from our laboratory demonstrated that all-trans retinoic acid (atRA) is a potent suppressor of CYP7A1, the rate-limiting enzyme in bile acid synthesis. atRA also repressed the expression of TGFβ and collagen 1A1 in activated primary human stellate cells and LX2 cells. When administered together with UDCA to bile duct ligation rats, this combined therapy significantly reduced the bile acid pool size, and improved liver conditions. To further examine whether atRA alone or in combination with UDCA has greater beneficial effects than UDCA treatment alone, we assessed this treatment in two additional chronic cholestatic rodent models, α-naphthylisothiocyanate (ANIT) treated rats and the Mdr2−/− (Abcb4−/−) knockout mouse. atRA alone significantly reduced bile duct proliferation, inflammation and hydroxyproline levels in ANIT-rats, whereas the combination of atRA and UDCA significantly reduced plasma bile salt compared to UDCA treatment. atRA +/- UDCA significantly reduced plasma levels of ALP and bile salts in 12-week old Mdr2−/− mice. Reduced bile duct proliferation and inflammation were also observed in the livers of these mice. Together, atRA alone or in combination with UDCA significantly reduced the severity of liver injury in these two animal models, further supporting the combination treatment of atRA and UDCA as a potential new therapy for patients with chronic cholestatic liver disease who have not responded fully to UDCA.
Introduction

Chronic cholestasis results in bile duct proliferation, liver fibrosis, cirrhosis, and eventually liver failure. Currently, ursodeoxycholic acid (UDCA) is the only effective medical treatment for primary biliary cirrhosis (PBC), but is limited to early stages of the disease. In contrast there is no accepted medical treatment for primary sclerosing cholangitis (PSC) except liver transplantation. Therefore, there is an urgent need to develop alternative therapy for these chronic cholestatic disorders (Carey and Lindor, 2012).

Previous studies from our laboratory using primary human hepatocytes and HepG2 cells determined that all-trans retinoic acid (atRA) is a potent suppressor of CYP7A1, the rate-limiting enzyme in bile acid synthesis (Cai, et al., 2010). AtRA also repressed the expression of TGFβ and collagen 1A1 in activated primary human stellate cells and LX2 cells (He, et al., 2011). When administered together with UDCA to a cholestatic rat model induced by bile duct ligation for 14 days, this combined therapy significantly reduced the bile acid pool size, liver cell necrosis, inflammation, hepatic fibrosis and bile duct proliferation (He, et al., 2011). These dramatic findings suggest that atRA in combination with UDCA might be an effective alternative therapy for patients with chronic cholestasis who have not fully responded to UDCA. AtRA is already an FDA-proved medicine for treating patients with acne and acute promyelocytic leukemia (Baljevic, et al., 2011; Reichrath, et al., 2007).
To further examine whether atRA alone or in combination with UDCA has greater beneficial effects than UDCA treatment alone, we assessed this treatment in two additional chronic cholestatic rodent models, α-naphthylisothiocyanate (ANIT) treated rats (Ferreira, et al., 2003) and Mdr2−/− (Abcb4−/−) knockout mice (Fickert, et al., 2004; Fickert, et al., 2006; Mauad, et al., 1994; Smit, et al., 1993). The Mdr2−/− knockout mouse is a rodent model reminiscent of sclerosing cholangitis in humans. Our results demonstrate that atRA alone or in combination with UDCA also significantly reduced the severity of liver injury in these two animal models, further supporting the combination treatment of atRA and UDCA as a potential new therapy for patients with chronic cholestatic liver disease who have not responded fully to UDCA.

Materials and Methods

Animal experiments. All animal experimental protocols were approved by the local Animal Care and Use Committee, according to criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences, as published by the National Institutes of Health (NIH publication 86-23, revised 1985). Male Sprague-Dawley rats (200-230 g) were obtained from Charles River (Wilmington, MA). ANIT (dissolved in corn oil) was administered to 24 male rats (80mg/kg, s.c. weekly) for 8 weeks. After 4 weeks, when pilot experiments indicated that ANIT had produced chronic liver injury, the animals were divided into 4 groups. Group A received PBS (the treatment control and solvent); Group B, 15mg/kg UDCA (suspended in PBS);
Group C, 5mg/kg atRA; and Group D, combinations of UDCA (15mg/kg) and atRA (5mg/kg) daily by gavage for the additional 4 weeks. For experiments with \textit{Mdr2}^{+/-} mice, two month old male mice were randomly divided into four groups (n=6-8) and fed diets containing 100 mg/kg UDCA and/or 33 mg/kg atRA or a control diet for one month. In addition, age matched \textit{Mdr2}^{+/-} and \textit{Mdr2}^{+/-} male mice were also fed the control diet as additional healthy control groups (n=6 each). Body weight was measured weekly. The animals were sacrificed in random order between 9:00 am – 11:00 am after an overnight fast. Samples of plasma, liver, kidney, urine (from bladder) and feces were collected for further analyses.

**Plasma biochemistry and liver histology.** Plasma liver alanine aminotransferase (ALT), and alkaline phosphatase (ALP) enzymes were analyzed by the Analytical Core in the Mouse Metabolic Phenotyping Center at Yale University. 3α-hydroxy bile salt concentrations were determined in plasma, urine, feces and liver tissue using a kit from Trinity BioTech (Newark, NJ) or Diazyme Laboratories (Poway, CA). Formalin fixed tissue was embedded in paraffin and sections were stained with hematoxylin and eosin and Sirius Red. Liver histology was blindly assessed for inflammation, necrosis, bile duct proliferation and fibrosis on a 1-4+ scale, as previously described (He, et al., 2011). Liver hydroxyproline content was measured as described (Soroka, et al., 2010).

**Immunohistochemistry.** Immunohistochemistry was performed using an antibody against cytokeratin 19 (CK19) (Troma-III) developed by R. Kemler and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA using a DAB peroxidase kit (Vector Laboratory, Burlingame, CA). Images
were acquired using an Olympus BX2 microscope. Quantitation of CK19 labeling was performed using ImageJ software (NIH open source) with thresholding. Data are presented as a percentage of the total area that is positive for CK19.

**Quantitative real-time PCR and Western blot analysis.** As described previously (Soroka, et al., 2010), gene mRNA expression was detected using TaqMan real-time PCR in an ABI7500 system and protein was analyzed by Western blotting. GAPDH gene was used as reference to normalize data. The TaqMan primer/probes and antibodies are as described in our previous publications (Soroka, et al., 2010; He, et al., 2011).

**Statistical Analysis.** Data are presented as means ± standard deviation (SD). Differences between experimental groups were assessed for significance using one-way ANOVA. Two-tailed Student t-test was used to calculate the p value. A p value of < 0.05 was considered to be statistically significant.

**Results**

There was no significant difference in body weight and liver/body ratio among groups after 4-week treatment with or without atRA and/or UDCA in ANIT treated rats. All four groups had normal levels of plasma ALT and GGT.

**UDCA and atRA in combination significantly reduced plasma bile salt concentrations in ANIT-rats:** Significantly lower levels of plasma bile acid were detected in rats treated with both atRA and UDCA when compared to UDCA treated group, whereas the liver bile acid levels were not significantly different (Figure 1). In
addition, urine bile acid levels were undetectable in all four groups and fecal bile acids did not differ among these groups.

**AtRA treatment alone reduced bile duct proliferation, inflammation and liver hydroxyproline in ANIT-rat livers:** Blinded examination of liver histology revealed that treatment with atRA alone significantly reduced bile duct proliferation and inflammation. No histological differences in fibrosis were noted given that the liver injury was relatively mild in these 8-week ANIT treated rats (Figure 2). However hepatic levels of hydroxyproline were significantly less after atRA compared to the control group. Liver histology also showed less inflammation in the UDCA treated group, although this difference was not maintained in the atRA + UDCA group. Histologic evidence of necrosis was absent in all 4 groups.

**Expression of bile acid transporter genes in the liver and kidney of ANIT-rats:** Analyses of mRNA and/or protein expression of liver genes involved in bile acid synthesis and transport revealed no significant differences among the 4 groups, including expression levels of Bsep (Abcb11), Mrp2 (Abcc2), Mrp3 (Abcc3), Mrp4 (Abcc4), Ntcp (Slc10a1), Oatp2 (Slco1a2), Ostα, and Cyp7a1 (data not shown). There were also no differences in mRNA expression of liver TGFβ, Tnfa, and IL-1β (data not shown). In contrast, both atRA and UDCA single treatments significantly increased the expression of Mrp4 mRNA and protein in the kidney while only atRA increased the expression of Mrp3 mRNA in the kidney (Figure 3).

**atRA +/- UDCA significantly reduced plasma levels of ALP and bile salts in 12-week old Mdr2^-/- mice.** Previous studies have established the Mdr2^-/- mouse as an animal model for PSC because the typical histological features of onion skin fibrosis
develops around the bile ducts in these mice at approximately two months of age (Fickert, et al., 2004). To test whether atRA +/- UDCA improved liver injury in these mice, we treated two month old $Mdr2^{-/-}$ male mice for 4 weeks. Analysis of plasma biochemistry confirmed the homozygous knockout ($Mdr2^{/-}$) mice developed cholestatic liver injury as levels of ALT, ALP and bile acids (including liver levels) were elevated compared to age matched wild-type ($Mdr2^{+/+}$) and $Mdr2^{+/-}$ heterozygotes (Table1).

Interestingly, atRA treatment alone or in combination with UDCA significantly lowered the plasma levels of ALP but not ALT (Table 1), suggesting less injury to the bile duct epithelium in the groups treated with atRA alone or in combination with UDCA. Lower plasma bile acid levels were also detected in animals treated with UDCA and atRA alone and in combination, suggesting that these treated mice were less cholestatic than the control groups.

**AtRA +/- UDCA reduced bile duct proliferation and inflammation in $Mdr2^{-/-}$ livers:**

Blinded examination of liver histology detected less bile duct proliferation in the liver of $Mdr2^{-/-}$ groups treated with atRA alone or in combination with UDCA (Figure 4A). Less inflammation was also observed in the atRA treated group. No significant difference was detected in liver fibrosis among $Mdr2^{-/-}$ groups. This was also confirmed by measuring liver hydroxyproline content. There was also no histologic evidence of necrosis in the livers of these mice. To further confirm the H&E histological assessment on bile duct proliferation, we performed immunohistochemical labeling of CK19, a cytokeratin specifically expressed in bile duct cells in liver. As shown in Figure 4B, CK19 positive staining was significantly reduced in the $Mdr2^{-/-}$ mice treated with atRA alone or in combination with UDCA, in agreement with our histological assessment. Lower liver bile
acid levels were also detected in these animals treated with the combination of atRA and UDCA, as well as UDCA alone but not atRA alone due to greater variation in this data.

**Hepatic expression of genes involved in inflammation, bile acid synthesis and transport in Mdr2⁻/− mice:** No significant changes in mRNA expression of Bsep, Mrp2, Mrp3, Mrp4, Ntcp and Cyp7a1 were noted in any of the groups. There was also no difference in expression levels of hepatic Tnfα and IL-1β mRNA. Liver CK19 mRNA levels in the mice treated with atRA alone or the combination groups tended to be lower, but did not reach statistical significance due to great variation in the data among animals in each treatment group (data not shown). Western blot analysis also did not detect differences in liver protein expression of Bsep, Mrp2, and Oatp2 (data not shown).

**Discussion**

The pathogenesis of cholestasis varies greatly among patients with different cholestatic disorders, but there are some common features, including elevated levels of plasma bile acids and alkaline phosphatase, and histologic evidence of bile duct proliferation and liver fibrosis. Unfortunately, there is no single animal model that fully resembles all of the clinical and pathological features seen in cholestatic patients. We have previously demonstrated that the combination of atRA and UDCA significantly improved plasma biochemistry and histologic features of cholestasis compared to UDCA or atRA alone in an obstructive model of 14 day bile duct ligation in rats, in which reduced bile acid pool
size, duct proliferation, liver necrosis and fibrosis were observed (He, et al., 2011). In
the present report, we tested these treatments in two additional chronic cholestatic
rodent models. We find that in ANIT-rats, a chemical induced liver injury model, the
combination treatment of atRA and UDCA significantly reduced plasma bile acid levels
(Figure 1), whereas plasma ALT and GGT levels remained in normal range in all
groups, (indicating that the liver injury was relatively mild in these animals). Gene
expression analyses also did not detect changes in liver bile salt transporters. However,
an increase in kidney Mrp4 expression was observed in atRA treated rats (Figure 3).
This adaptation might be beneficial as it could result in enhanced renal excretion of toxic
compounds, including conjugated bile salts.

In the Mdr2−/− knockout mice, both atRA alone and in combination with UDCA
significantly reduced plasma levels of bile acid and ALP. Reduced plasma bile acid
levels were also seen in UDCA treated animals (Table 1). Liver histology revealed that
atRA alone and in combination with UDCA reduced bile duct proliferation. This
reduction was further confirmed by CK19 immunohistochemical labeling, also indicating
that the liver injury was improved in groups treated with atRA +/- UDCA in the Mdr2−/−
mice.

The reductions in plasma bile acids and bile duct proliferation seen in these two
experimental animal models are consistent with our earlier findings that atRA has a
profound inhibitory effect on bile acid synthesis both in human and rodent hepatocytes
(He, et al., 2011). While atRA might be expected to have anti-inflammatory effects as
well (as seen in the Mdr2\(^{-/-}\) mice, Figure 4), the primary beneficial effects in these animal models appears related to the reduction in the bile acid pool size as reflected in the reduced plasma and liver bile acid levels seen with atRA and UDCA.

When we compare the findings in the present study with the beneficial effects of atRA +/- UDCA treatment seen in the liver of bile duct ligated rats (He, et al., 2011), there was a less dramatic effect on features of cholestasis seen in these ANIT-rats and Mdr2\(^{-/-}\) knockout mice. Biochemical and histological features of cholestasis in these two models were also much less severe than seen after 14 days of bile duct ligation in the rat, so less overall benefit might be expected. However, to our surprise, there were no additive beneficial effects in the combination therapy in both ANIT-rats and Mdr2\(^{-/-}\) knockout mice, as was seen in bile duct ligated rats. It is possible that the stimulation of bile flow that is associated with UDCA resulted in increased clearance of atRA since vitamin A and its metabolites are cleared from the blood into bile (Boyer, 2013). This would reduce the systemic effects of atRA’s when in combination with UDCA in these two cholestatic rodent models where bile flow is not obstructed, in contrast to the situation after bile duct ligation. This also may explain why lower plasma bile acid levels were detected with combination therapy in the current two rodent models, even though there were no differences in Cyp7A1 expression among the different treatment groups.

In summary, atRA +/- UDCA treatment reduced plasma levels of ALP and bile acids, and histologic features of bile duct proliferation in chronic cholestatic rodent models due to ANIT treatment in rats and the Mdr2\(^{-/-}\) mouse. These findings support our prior findings in bile duct ligated rats and provide further suggestion that atRA +/- UDCA
may be an effective alternative therapy for patients with chronic cholestatic liver disease.

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Authorship Contributions:

Participated in research design: Cai, and Boyer.

Conducted experiments: Cai, Mennone, and Soroka and Boyer.

Performed data analysis: Cai, Mennone, Soroka and Boyer.

Wrote or contributed to the writing of the manuscript: Cai, Soroka and Boyer.

Financial Disclosure: All authors have nothing to disclose.


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Figure legends

Figure 1. UDCA and atRA in combination significantly reduced plasma bile salt concentrations in ANIT-rats. (Mean ± SD; * \( p < 0.05 \) to Ctrl; \( n=6 \)).

Figure 2. atRA treatment alone but not in combination with UDCA reduced bile duct proliferation, inflammation and liver hydroxyproline in ANIT-rat livers. (Mean ± SD; * \( p < 0.05 \) to Ctrl; \( n=6 \)). Of note: paired t-test indicate hydroxyproline values correlate to histological scores of fibrosis, \( p = 0.034 \).

Figure 3. atRA altered the expression of bile salt transporters in kidney. A, Mrp3 and 4 mRNA expression were significantly increased in atRA treated rat kidneys compared to untreated controls (\( * p < 0.05, n=6 \)). B, Western blots demonstrate that Mrp4 protein expression was significantly increased in atRA and UDCA treated rat kidneys. \( * p < 0.05, n=6 \), to no treatment controls.

Figure 4. atRA treatment alone or with UDCA reduced bile duct proliferation and inflammation in \( Mdr2^{-/-} \) mouse livers. (Mean ± SD; * \( p < 0.05, \) \# \( p < 0.01 \) to Ctrl; \( n=11-13 \)).

Figure 5. Reduced bile duct proliferation in \( Mdr2^{-/-} \) mice by atRA and combination treatment was confirmed using CK19 labeling. (Mean ± SD; * \( p < 0.05 \) to Ctrl; \( n=6-8 \)).
Table 1: Plasma biochemistry in wild-type and Mdr2 knockout mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mdr2&lt;sup&gt;++&lt;/sup&gt;</th>
<th>Mdr2&lt;sup&gt;+-&lt;/sup&gt;</th>
<th>Mdr2&lt;sup&gt;--&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Ctrl diet n=7</td>
<td>Ctrl diet n=7</td>
<td>Ctrl diet n=12</td>
</tr>
<tr>
<td></td>
<td>UDCA n=11</td>
<td>atRA n=12</td>
<td>UDCA &amp; atRA n=12</td>
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<tr>
<td>ALT (U/L)</td>
<td>42±7</td>
<td>52±17</td>
<td>294±86&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>72±6</td>
<td>78±8</td>
<td>204±29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma BA (µM)</td>
<td>17±15</td>
<td>12±9</td>
<td>46±16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver BA (µM)</td>
<td>118±94</td>
<td>102±72</td>
<td>329±47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: mean±SD, <sup>a</sup>p<0.05 to Mdr2<sup>++</sup> (wild-type), <sup>b</sup>p<0.05 to Mdr2<sup>--</sup> Ctrl diet.
Figure 1

Plasma

Liver

BA conc. (µM)

Ctrl  UDCA  atRA  UDCA&atRA

Ctrl  UDCA  atRA  UDCA&atRA
Figure 2

Duct proliferation

Fibrosis

Inflammation

Hydroxyproline

Score

Score

Score

μg/g liver tissue

Ctrl  UDCA  atRA  UDCA&atRA

Ctrl  UDCA  atRA  UDCA&atRA

Ctrl  UDCA  atRA  UDCA&atRA
Figure 3A

A graph showing the relative mRNA amounts of Mrp2, Mrp3, Mrp4, Osta, and Asbt under different conditions: Ctrl, UDCA, AtRA, and UDCA+AtRA. The graph includes error bars indicating variability.
Figure 3B

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>UDCA</th>
<th>atRA</th>
<th>UDCA+atRA</th>
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<td>Mrp2</td>
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<tr>
<td>Mrp3</td>
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<td>0.8±0.4</td>
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<td>Mrp4</td>
<td>1±0.2</td>
<td>1.3±0.2*</td>
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<tr>
<td>SH-PTP</td>
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</tbody>
</table>
Figure 4

Duct proliferation

Inflammation

Score

Ctrl  UDCA  atRA  UDCA&atRA

Ctrl  UDCA  atRA  UDCA&atRA

* indicates statistical significance.