Combination Treatment of Angiotensin-II Type I Receptor Blocker and New Oral Iron Chelator Attenuates Progression of Non-alcoholic Steatohepatitis in Rats

Kosuke Kaji, Hitoshi Yoshiji, Mitsuteru Kitade, Yasuhide Ikenaka, Ryuichi Noguchi, Yusaku Shirai, Yosuke Aihara, Tadashi Namisaki, Junichi Yoshii, Koji Yanase, Tatsuhiro Tsujimoto, Hideto Kawaratani and Hiroshi Fukui

Third Department of Internal Medicine, Nara Medical University, Kashihara, Nara, Japan

Running head: ARB and oral iron chelator suppress NASH progression

Correspondence should be addressed to:
Hitoshi Yoshiji, M.D., Ph.D.
Third Department of Internal Medicine, Nara Medical University,
Shijo-cho 840, Kashihara, Nara 634-8522, Japan.
Tel: 81-744-22-3051 (ext. 2314)
Fax: 81-744-24-7122
E-mail: yoshijih@naramed-u.ac.jp
**ABSTRACT**

Angiotensin-II type I receptor blocker (ARB) and iron chelator reportedly exert suppressive effects on non-alcoholic steatohepatitis (NASH) progression, including liver fibrosis and hepatocarcinogenesis. The aim of this study was to elucidate the combined effect of losartan (LOS), an ARB, and deferasirox (DSX), a newly developed oral iron chelator, on the progression of NASH in rats. To induce NASH, F344 rats were fed a choline-deficient L-amino acid-defined (CDAA) diet for 12 weeks, and the effects of LOS and DSX at clinically comparable low doses were elucidated in conjunction with oxidative stress, neovascularization, and hepatic stellate cells (HSC) activation, all known to play important roles in the progression of NASH. Treatment with both LOS and DSX suppressed CDAA-induced liver fibrosis development and hepatocarcinogenesis. This combination treatment exerted a stronger inhibitory effect in comparison with treatment with a single agent. These inhibitory effects occurred almost concurrently with the suppression of oxidative stress, neovascularization, and HSC activation. Our *in-vitro* study demonstrated that LOS and DSX inhibited angiotensin-II-induced proliferation, TGF-β1 expression of activated HSC, and *in-vitro* angiogenesis. These results indicated that dual inhibition by combined treatment of LOS and DSX...
attenuated the progression of NASH. Since both agents are widely
used in clinical practice, this combination therapy may represent a
potential new strategy against NASH in the near future.

Keywords: oxidative stress, angiogenesis, hepatic stellate cells,
VEGF.
INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is currently considered as one of the most common liver diseases in developed countries. It includes the spectrum of simple steatosis and non-alcoholic steatohepatitis (NASH) [2]. Whereas simple steatosis seems to be a benign and non-progressive condition, NASH is recognized as a potentially progressive disease that may cause cirrhosis, an end-stage liver disease, and hepatocellular carcinoma (HCC) [18, 27]. While a low-calorie diet has been recommended to treat obese NAFLD patients, it is sometimes difficult for many patients to change their lifestyle. Accordingly, although efforts are currently directed at overcome NASH, the optimal treatment for NASH has not been established in clinical practice.

The current concept in the pathogenesis of NASH is the so-called “two-hit” theory, in which an initial metabolic disturbance, such as insulin resistance (IR), causes steatosis, and then a second pathogenic stimulus causes oxidative stress and reactive oxygen species (ROS), leading to steatohepatitis [7, 30]. Several studies have shown that iron may be the substrate of oxidative stress and is probably responsible for the second hit in patients with NASH [4, 9, 14]. In steatotic livers, saturation of β-oxidation by excess free fatty acids ultimately leads to the production of hydrogen peroxide, which in turn can mutate into highly reactive hydroxyl radicals in the presence of free iron via the Fenton reaction [39].
Indeed, there is much evidence that hepatic iron overload may be a risk factor of HCC in patients with NASH [23, 24, 34]. Therefore, antioxidant therapy by iron reduction, such as dietary iron reduction, and phlebotomy for NASH have been reported [38, 44]. Moreover, injection therapy with deferoxamine (DFO), an iron chelator, may reportedly reduce pre-neoplastic lesions in the choline-deficient L-amino acid-defined (CDAA) rat model [31]. Although DFO is beneficial as an antioxidant therapy for NASH, it has limited efficacy due to its demanding therapeutic regimen requiring subcutaneous administration or intravenous infusions, leading to poor compliance by many patients. Recently, deferasirox (DSX), an oral iron-chelator taken once daily, has been developed as an effective alternative to DFO in the treatment of transfusional iron overload in patients with certain types of anemia, such as \( \beta \)-thalassemia, sickle cell disease and myelodysplastic syndrome in clinical practice [1]. However, the effect of DSX on NASH progression has yet to be clarified.

The renin-angiotensin system (RAS) plays an important role in the regulation of local hemodynamics in several organs. It has been reported that angiotensin-II (AT-II), an octapeptide produced via enzymatic cleavage of angiotensin-I by an angiotensin-I converting enzyme (ACE), plays an important role in the progression of chronic liver diseases, including NASH [48]. We and another group have shown that suppression of AT-II by a
clinically used AT-II type-I (AT1) receptor blocker (ARB)
significantly attenuated liver fibrosis development in the rat
NASH model [15, 55]. ARB improved the fibrosis serum markers
of NASH not only in the animal model, but also in clinical
practice [46]. We previously reported that neovascularization
plays an important role in the progression of NASH both in
animal studies and clinical practice [20, 21]. In addition to its
effect against liver fibrosis, suppression of RAS alleviated
hepatocarcinogenesis in the CDAA rat model as well as inhibited
hepatic neovascularization and the vascular endothelial growth
factor (VEGF), a potent angiogenic factor [49]. Although ARB is
considered as an attractive therapeutic agent against NASH, we
previously reported that blockage of RAS by single treatment of
ACE-I did not exert sufficient effect on on-going fibrosis
development [53].

In this study, we examined the combined effect of ARB
and DSX on liver fibrosis and hepatocarcinogenesis in rats, which
was induced by feeding the rats on CDAA diet, in order to
evaluate the feasibility of future clinical applications. We also
attempted to investigate possibly related mechanisms.

MATERIALS AND METHODS

Animals and Reagents
A total of 50 male Fisher 344 rats, aged 6 weeks, were
purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). They were housed in stainless steel, mesh cages under controlled temperature (23 ± 3°C) and relative humidity (50 ± 20%), with 10-15 air changes per hour and illumination for 12 hours a day. The animals were allowed access to tap water *ad libitum* throughout the experimental periods. Losartan (LOS), an ARB, and DSX were provided by Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan) and Novartis Pharma K.K. (Basel Switzerland), respectively. CDAA and a choline-sufficient amino acid-defined (CSAA) diet were purchased from CLEA Japan Inc. (Tokyo, Japan). AT-II was purchased from WAKO Pure Chemical Industries (Tokyo, Japan).

**Animal Treatment**

We have previously shown that 30 mg/kg/day of LOS exerts an anti-fibrotic effect, and is comparable to the clinical practice dosage [55], and it has been reported that 100 mg/kg/day of DSX exerts an iron-chelating effect in rats [3]. Therefore, we used these respective dosages in this study. The experimental period of all experiments was 12 weeks. Rats in Groups 1-4 (G1-G4) were fed a CDAA diet. Rats in G1 received distilled water as a vehicle by gavage daily for 12 weeks (the control group). Rats in G2 and G3 received LOS and DSX, respectively, by gavage daily for 12 weeks. G4 was treated with a combination of LOS and DSX. Rats fed the CSAA diet were designated as the negative control group (G5). Each group consisted of 10 rats. At the end of the
experiments, all rats were anesthetized, and examined for the study items. Hemoglobin (Hb) content and several serum markers, such as alanine aminotransferase (ALT), albumin (Alb), total bilirubin (T-bil) and iron concentrations, were assessed by routine laboratory methods. Serum ferritin level was measured using enzyme-linked immunosorbent assay (ELISA) kit (Mitsubishi Chemical Safety Institute, Tokyo, Japan).

All animal procedures were performed according to standard protocols and in compliance with standard recommendations for the proper care and use of laboratory animals.

**Histological and Immunohistochemical Analyses**

In all experimental groups, one section of the liver was routinely stained with hematoxylin and eosin. Another section was stained with Sirius-red (S-R) and Perls’ Prussian blue to detect fibrosis and iron deposition, respectively. Immunohistochemical staining techniques of α-smooth muscle actin (α-SMA) (DAKO, Kyoto, Japan), enzyme-altered pre-neoplastic lesions; namely, the placental form of glutathione-S-transferase (GST-P) (MBL Co. Ltd., Nagoya, Japan), and 8-hydroxydeoxyguanosine (8-OHdG) (NIKKEN SEIL Co. Ltd., Tokyo, Japan), were performed as described previously [20, 50]. To determine neovascularization, we performed immunohistochemical detection of CD31 (BD Bioscience, CA, USA), which is widely used as a marker for
neovascularization, using frozen sections as previously described [52]. Semiquantitative analyses of fibrosis development and immunopositive cell area of α-SMA, GST-P, 8-OHdG and CD31 were carried out with Adobe Photoshop software and National Institutes of Health image software in 6 ocular fields (magnification ×40) per specimen of 10 rats as described elsewhere with minor modification [13, 48].

Hepatic Hydroxyproline, Transforming Growth Factor (TGF)-β1, Triglyceride (TG) and Lipid Peroxidation

Hepatic hydroxyproline content was determined as described previously with 200 mg of frozen liver samples [50]. After equalization of the protein contents, TGF-β1, TG and malondialdehyde (MDA) in the liver were determined by TGF-β1 ELISA kit (Bender MedSystems GmbH, Vienna, Austria), TG E-test (WAKO, Osaka, Japan) and MDA Assay kit (NWLSS, LLC, Vancouver, WA, USA), respectively, according to manufacturer instructions.

CD31, VEGF, TGF-β1 and α1(I)-procollagen mRNA Expressions

Total RNA was extracted by RNeasy mini kit (QIAGEN, Tokyo, Japan) from both liver tissue and isolated activated hepatic stellate cells (Ac-HSC) according to manufacturer instructions. Total RNA (2 μg) from each sample was reverse-transcribed to
complementary DNA (cDNA) by High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer instructions. The mRNA expressions of TGF-β1, α1(I)-procollagen, CD31 and VEGF from liver tissue, and TGF-β1 and α1(I)-procollagen from isolated Ac-HSC were estimated by quantitative real-time PCR using the StepOnePlus real-time PCR system (Applied Biosystems) with Fast SYBR Green master mix (Applied Biosystems). Relative gene expression was measured using glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as the internal control. The relative amount of target mRNA in each sample was determined by applying the threshold cycle to the standard curve. Primer sequences were as follows: CD31-forward, 5’-GGC GTC CTG TCC GGA ATC-3’ and reverse, 5’-AGA ACT CCT GCA CAG TGA CGT ATT-3’; VEGF-forward, 5’-GAG GAA AGG GAA AGG GTC AAA A-3’ and reverse, 5’-CAC AGT GAA CGC TCC AGG ATT-3’; TGF-β1-forward, 5’-CGG CAG CTG TAC ATT GAC TT-3’ and reverse, 5’-AGC GCA CGA TCA TGT TGG AC-3’; α1(I)-procollagen-forward, 5’-AAA GCA GAA ACA TCG GAT TTG G-3’ and reverse, 5’-CGT GTC ATC CCT TGT GCC GCA-3’; GAPDH-forward, 5’-GTA TGA CTC TAC CCA TAC GTA TGA TCA TCT-3’ and reverse, 5’-TTC CCG TTG ATG ACC AGC TT-3’.

In-vitro Assays

Primary HSC was isolated from the liver of F344 rats, as
described previously, with minor modification [42]. Cell viability
was over 95%, as determined by the Trypan blue exclusion test.
Freshly isolated HSC were plated at a density of $5 \times 10^5$ cells/ml on
uncoated plastic dishes. After 5-day culture, HSC became
myofibroblast-like with reduced lipid vesicles and increased
immunoreactive $\alpha$-SMA. After 7-day plating, all cells were well
spread and $\alpha$-SMA positive [33]. From day 10, culture media with
or without AT-II (1 $\mu$mol/L), LOS (10 $\mu$mol/L) and/or DSX (20
$\mu$mol/L) treatment were changed every 24 hours and cell culture
continued until day 12. The effects of LOS and DSX on the
proliferation of HSC were determined by tetrazolium, 3-(4, 5-
dimethylthiazol-2,5-yl)-2,5-diphenyltetrazolium bromide (MTT)
assay as described previously [53]. in-vitro angiogenesis was
assessed as the formation of capillary-like structures of human
umbilical vein endothelial cells co-cultured with human diploid
fibroblasts as described previously [20]. Experimental procedures
followed the instructions provided with the Angiogenesis Kit
(Kurabo, Tokyo, Japan). Computer-assisted quantitation of tubule
formation was performed as in the in-vivo assay.

**Statistical Analyses**

To assess statistical significance of differences in the
quantitative data between groups, Bonferroni’s multiple
comparison test was used after one-way ANOVA. This was
followed by Barlett’s test to determine homology of variance.
RESULTS

General Findings

Data from all experimental groups are shown in Table 1. Final body weight of CDAA-treated rats (G1) were less than CSAA-treated rats (G5), while relative liver weights of G1 were greater than those of G5. Serum ALT was significantly higher in G1-G4 than in G5, whereas there was no significant difference between in Alb and T-bil. No marked differences were observed in Hb between experimental groups, although serum concentrations of iron and ferritin were significantly higher in rats given the CDAA diet. Among the CDAA-treated groups, the levels of ferritin in G2 and G3 were less than in G1, and the level in G4 was less than in G2 and G3. Hepatic iron deposition almost matched the serum levels of iron and ferritin. Moreover, LOS and/or DSX did not affect liver function and several indices of serum biochemistry in CSAA-fed normal rats. In addition, neither histological inflammation nor hepatic TG content was significantly altered by treatment with LOS and DSX (data not shown).

Effects of LOS and DSX on the Liver Fibrosis Development

We first examined the effects of clinically comparable doses of LOS and DSX on liver fibrosis induced by a CDAA diet. As shown in Fig. 1, liver fibrosis development was significantly
suppressed by treatment with LOS (G2) and DSX (G3) as compared to the control group (G1). The combination treatment of both agents (G4) exerted a more potent inhibitory effect than that of either single treatment. No fibrosis development could be observed in the CSAA-treated control rats (G5). We next carried out an immunohistochemical analysis of α-SMA to examine the effects of LOS and DSX on HSC activation during liver fibrosis development. α-SMA immunopositive Ac-HSC cells drastically decreased by treatment with LOS and DSX (Fig. 2A). Computer-assisted semi-quantitative analysis showed that α-SMA-positive cells in the LOS- and DSX-treated groups significantly decreased in comparison with the control group (Fig. 2B). We also found that LOS and DSX markedly suppressed both hepatic TGF-β1 and hydroxyproline content in comparison with the control group (Fig. 3A and B, respectively). These inhibitory effects closely agreed with the changes in TGF-β1 and α1(I)-procollagen mRNA (Fig. 3C and D, respectively) expressions in the liver. Similar to the effect on liver fibrosis, the combination treatment with both agents exerted a more potent inhibitory effect on the α-SMA, TGF-β1, hydroxyproline and collagen expressions than that of either single treatment, and these inhibitory effects were almost parallel to the fibrosis area reduction.

To elucidate whether LOS and DSX directly affected Ac-HSC, we next examined the effects of LOS and DSX on AT-II-
induced proliferation of Ac-HSC *in vitro*. As shown in Fig. 4A, proliferation of Ac-HSC increased by treatment with AT-II. Both LOS and DSX significantly suppressed this stimulating effect, and combination treatment with both compounds exerted a stronger inhibitory effect on AT-II-induced Ac-HSC proliferation. We also examined TGF-β1 and α1(I)-procollagen mRNA expression in Ac-HSC. Similar to the relation of α-SMA-positive cells in the liver, the suppressive effect of treatment with LOS and DSX on Ac-HSC proliferation closely agreed with the reduction of TGF-β and α1(I)-procollagen mRNA expression *in vitro* (Fig. 4B and 4C).

**Effects of LOS and DSX on the Oxidative Stress in the Liver**

Since ROS plays an important role in the progression of NASH, we examined markers of oxidative DNA-damage and lipid peroxidation; namely, 8-OHdG and MDA, respectively. As shown in Fig. 5A, the number of 8-OHdG-immunopositive cells markedly decreased in the LOS-(G2) and DSX-(G3) treated groups than in G1. MDA content in the liver was also lower in G2 and G3 than in G1 (Fig. 5B). The inhibitory effects of LOS and DSX on 8-OHdG and MDA were of similar magnitude, and combination treatment of LOS and DSX (G4) exerted a much stronger inhibitory effect than either single agent.

**Effects of LOS and DSX on the Development of Pre-neoplastic Lesions**

We next examined the effects of LOS and DSX on GST-P-
positive pre-neoplastic lesions in conjunction with neovascularization. As shown in Fig. 6, GST-P-positive pre-neoplastic lesions were significantly suppressed by treatment with LOS (G2) and DSX (G3). Combination treatment of LOS and DSX (G4) exerted a stronger inhibitory effect than either single agent. On the other hand, neither LOS nor DSX affected the number of GST-P-positive lesions, and no GST-P-positive lesions developed in CSAA-treated rats (G5). To determine whether the inhibitory effects of LOS and DSX on pre-neoplastic lesions development were accompanied by suppression of neovascularization, we next examined the expression level of CD31 in the liver. Treatment with LOS and DSX significantly attenuated CD31 mRNA expression in the liver along with the inhibition of GST-P-induced pre-neoplastic lesions (Fig. 7A). To elucidate whether the suppressive effects on neovascularization were accompanied by the inhibition of VEGF or not, we also examined VEGF mRNA expression in the liver. As shown in Fig. 7B, VEGF expression in the liver was suppressed by treatment with LOS and DSX. Both CD31 and VEGF expressions were more potently suppressed by the combination treatment with both agents than with either single agent. We also performed immunohistochemical analysis of CD31. CD31-positive neovessels significantly increased in areas of GST-P-positive lesions of the CDAA-treated liver. Furthermore, the inhibitory
effects of LOS and DSX closely agreed with the changes in CD31 and VEGF mRNA expressions (data not shown). We also investigated *in-vitro* endothelial cells (EC) tubule formation to elucidate the direct effects of LOS and DSX on angiogenesis. AT-II significantly stimulated EC tubule formation, and LOS and DSX individually suppressed AT-II-induced EC tubular formation. Combination treatment with LOS and DSX exerted a stronger suppression than either single treatment (Fig. 8 A to E, left panel). Semi-quantitative analysis confirmed that the total length of tubules formed in the combination-treated culture was almost similar to the untreated control culture (Fig. 8, right panel).

**DISCUSSION**

Several investigators have revealed that the pathogenesis of NASH most likely involves multiple steps. The “two-hit theory” is widely recognized to explain the pathogenesis of NASH [7]. The second hit, which sequentially occurs after the first hit, is based on several metabolic disorders such as IR, and leads to the progression of NASH [2, 30]. Several studies suggested that ROS is the leading culprit of the second hit [32, 35, 45]. ROS has been focused on as a common pathogenic mechanism in various liver diseases, including NAFLD. It has been reported that increased production of ROS occurs very early in the histological spectrum
of NAFLD [26]. It is well known that iron plays a pivotal role in
the development of ROS [12]. As a catalyst of the Fenton reaction,
excessive free iron in the liver contributes to redox activation and
ROS formation, resulting in lipid peroxidation [39]. So far,
several lines of evidences have emerged, and one convincing
candidate for the source of ROS in NASH is excessive
accumulation of iron in the liver [19, 36]. Iron overload is
associated with hepatic ROS damage to DNA in NASH [11].
Accordingly, phlebotomy or DFO have been applied as therapies
for NASH [31, 38]. However, these therapeutic modalities are not
always favorable for the patient due to accompanying pain or
severe anemia in the clinical practice in some cases. In this study,
we used DSX, a newly developed oral iron chelator, which
improves compliance in clinical practice compared to either
phlebotomy or DFO. We observed that DSX reduced hepatic ROS,
as represented by MDA and 8-OHdG, as well as decreased the
serum ferritin level and hepatic iron deposition without causing
severe anemia. In addition, previous experiments showed that AT-
II induced hepatic iron deposition and accumulation of ROS in the
liver [16, 41]. Similar to DSX treatment, hepatic iron deposition
and ROS were suppressed in the LOS-treated group. A previous
report showed that continuous AT-II infusion induced iron
deposition in the liver possibly due to the activation of the
monocytes and macrophages [16]. Moreover, another study
demonstrated that AT-II induced ROS production via NADPH oxidase-2 (NOX2) [10]. We - as well as another group - previously reported that either DFO or iron deficiency could suppress pre-neoplastic lesions in CDAA-treated rats along with the inhibition of ROS [31, 47]. CDAA diet and LOS treatment may have induced alteration of metabolism or absorbance of iron via genetic modulation of hepcidin and ferroportin, although the exact mechanism are still not clear at this time. We hope to elucidate this issue in another study in the near future.

In this study, we observed that combination treatment with LOS and DSX exerted a stronger inhibitory effect than either single agent. Since several researchers have suggested that treatment with a single agent may not be sufficient to completely suppress fibrosis and carcinogenesis, it may be difficult to really suppress the cumulative development of NASH with a single agent in clinical practice. For future clinical applications, a combination treatment with LOS and DSX would likely be required to exert a clinically beneficial effect against NASH progression.

In addition to ROS reduction, we focused on the role of neovascularization in the progression of NASH. Angiogenesis is now recognized to play an important role in many physiological and pathological events [5]. We previously reported that angiogenesis significantly increased during liver fibrosis...
development and hepatocarcinogenesis, including the CDAA-fed rat NASH model [20]. Among the identified angiogenic factors, VEGF is the most potent factor in angiogenesis. Furthermore, VEGF expression stepwise increased during liver fibrosis development and hepatocarcinogenesis, and suppression of the VEGF signaling cascade attenuated these pathological sequences [50, 51]. We previously reported that VEGF-mediated neovascularization played a pivotal role in the progression of NASH in the animal model as well as in clinical practice [20, 21].

It has been reported that both AT-II and ROS induced neovascularization [37, 52], and we observed that LOS and DSX suppressed VEGF-mediated neovascularization in this study. The anti-angiogenic effects of LOS and DSX should also contribute to their suppressive effects on the progression of NASH.

However, several reports have indicated a contradictory effect of iron on angiogenesis. It has been reported that DFO enhanced the hypoxia inducible factor (HIF)-1α, which is known to be one of the strong promoters of VEGF transcription [43]. Moreover, iron deficiency may increase the risk of breast cancer via stabilization of the HIF-1α-mediated VEGF expression [6]. The reason for the contradiction in the role of iron in neovascularization is not clear at this time. It has been reported that there is an alternative pathway to induce VEGF in the tumor apart from HIF-1α dependent cascade, such as mammalian target
of rapamycin (mTOR) [22]. A recent study on HCC has demonstrated that mTOR, especially TORC2, is involved in hepatocarcinogenesis [40]. It may be possible that HCC-specific pathways between iron metabolic routes and VEGF expression through mTOR pathway exist, but are different from those involved in breast cancer. Also, several investigators have shown the adverse effects of neovascularization on fibrosis development. [17, 28] However, in the CDAA model, we previously noticed that angiogenesis plays a positive important role in liver fibrogenesis [54]. In the CDAA model, \textit{in-vivo} effects closely agreed with the \textit{in-vitro} results, including the tube formation assay. Further studies are needed to elucidate the exact mechanism of this phenomenon.

Nevertheless, several other pathways are also likely to be involved in the inhibitory effects of LOS and DSX. It is well known that the pathological sequences of NASH progression are based on IR [2, 30]. Iron depletion reportedly up-regulates glucose uptake, and increases insulin receptor activity in the liver, with consequent improvement in IR status [8]. ARB also has been shown to improve the AT-II-mediated IR [29]. These results indicate that the effects of LOS and DSX on IR play an important role in suppression of NASH progression; i.e., liver fibrogenesis and hepatocarcinogenesis. In addition, we observed that neither LOR nor DSX affected the inflammation process or TG
accumulation in the liver, indicating that the inhibitory effects of these agents were not predominantly due to suppression of liver inflammation, lipid accumulation or hepatic steatosis (data not shown).

In this study, we used the CDAA model to elucidate the effects of LOS and DSX on the progression of NASH. This CDAA model has several critical disadvantages as a NASH model, although the histological progression features are very similar to those in human NASH. This CDAA diet model does not induce typical features of human NASH such as obesity, glucose intolerance, and IR. We originally developed this CDAA model in 1992, and reported that ROS was significantly increased in conjunction with the histological progression [25]. Since ROS plays a pivotal role in human NASH, this CDAA model would be suitable to elucidate how anti-ROS agents inhibit the progression of similar histological features of human NASH. We previously reported that LOS exerted a marked inhibitory effect on liver fibrosis development under conditions of IR in Otsuka Long-Evans Tokushima Fatty (OLETF) rats [55]. We will perform further studies to elucidate the effects of this regimen in OLETF rats in the near future.

In conclusion, we could herein show that treatment with clinically used LOS and DSX markedly inhibited liver fibrosis development and hepatocarcinogenesis in the CDAA-fed rat
NASH model, and that these effects, at least partly, were mediated via suppression of ROS and neovascularization. The combined treatment with both agents markedly attenuated these processes than treatment with either single agent. It is noteworthy that the inhibitory effects of LOS and DSX on the progression of NASH could be achieved at clinically comparable low doses. Since these agents are already widely used in clinical practice with great safety, they may represent a potential new therapeutic strategy against the progression of NASH in the near future.
### Table 1

Characteristics of the experimental groups in the rat NASH model.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>G1 (n=10)</th>
<th>G2 (n=10)</th>
<th>G3 (n=10)</th>
<th>G4 (n=10)</th>
<th>G5 (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>259±14</td>
<td>281±17</td>
<td>266±18</td>
<td>282±20</td>
<td>291±18</td>
</tr>
<tr>
<td>Liver weight (% body)</td>
<td>4.10±0.41</td>
<td>3.19±0.21</td>
<td>3.51±0.19</td>
<td>3.02±0.11</td>
<td>2.98±0.09</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>12.9±1.2</td>
<td>13.1±2.1</td>
<td>12.1±2.7</td>
<td>11.9±3.2</td>
<td>13.5±3.2</td>
</tr>
<tr>
<td>Serum Fe (μg/dl)</td>
<td>329±19</td>
<td>319±33</td>
<td>271±21</td>
<td>269±28</td>
<td>154±8</td>
</tr>
<tr>
<td>Serum ferritin (μg/ml)</td>
<td>3.26±1.10</td>
<td>1.95±0.51</td>
<td>1.46±0.39</td>
<td>1.13±0.24</td>
<td>0.55±0.18</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>266±46</td>
<td>251±31</td>
<td>249±51</td>
<td>253±29</td>
<td>53±7</td>
</tr>
<tr>
<td>Alb (g/dl)</td>
<td>3.5±0.4</td>
<td>3.7±0.2</td>
<td>3.8±0.1</td>
<td>3.5±0.2</td>
<td>3.6±0.4</td>
</tr>
<tr>
<td>T-Bil (mg/dl)</td>
<td>0.16±0.03</td>
<td>0.19±0.05</td>
<td>0.13±0.02</td>
<td>0.09±0.02</td>
<td>0.11±0.03</td>
</tr>
</tbody>
</table>
a: Data represents the mean ± SD.

b, c: Statistically significant as compared to G1 and G5, respectively (p< 0.01)
FIGURE LEGENDS

Figure 1.
A: Characteristics of microphotographs of liver sections with Sirius-red staining. CDAA-treatment (G1) showed an extensive fibrosis development accompanied with fatty accumulation, and individual treatment with either ARB (G2) or DSX (G3) showed significant inhibitory effect. Combination treatment with both agents (G4) exerted a stronger inhibitory effect than in G2 and G3. No fibrosis development was observed in CSAA-fed control rats (G5) (Original magnification ×40). B: Semi-quantitative analysis confirmed histological findings. Data represents the mean ± SD (bars; n =10). * indicates statistically significant difference between indicated experimental groups ($p < 0.01$). G1: CDAA with vehicle-treated group. G2: CDAA with ARB-treated group. G3: CDAA with DSX-treated group. G4: CDAA with ARB and DSX-treated group. G5: CSAA-treated group.

Figure 2.
A: Immunohistochemical analysis of α-SMA expression in the liver. B: α-SMA positive cells were measured by an image-analyzing system. Treatment with either ARB (G2) or DSX (G3) showed a marked inhibitory effect on α-SMA expression in the liver in comparison with the CDAA-diet group (G1). Combination
treatment with both agents (G4) exerted a stronger inhibitory effect. No α-SMA positive cells were observed in the CSAA-fed control rats (G5). Data represents the mean ± SD (bars; n =10). * indicates statistically significant difference between the indicated experimental groups (p < 0.01). G1: CDAA with vehicle-treated group. G2: CDAA with ARB-treated group. G3: CDAA with DSX-treated group. G4: CDAA with ARB and DSX-treated group. G5: CSAA-treated group.

Figure 3.

TGF-β1 (A) and hydroxyproline contents (B) in the liver. TGF-β1 and hydroxyproline contents were significantly higher in the CDAA-fed rats (G1) than in the CSAA-fed control rats (G5). Treatment with either ARB (G2) or DSX (G3) showed significant inhibitory effect on both contents in the liver in comparison with G1. Combination treatment with both agents (G4) exerted a stronger inhibitory effect. These inhibitory effects closely agreed with the changes in TGF-β1 (C) and α1(I)-procollagen mRNA (D) expressions in the liver. Data represents the mean ± SD (bars; n =10). * indicates statistically significant difference between the indicated experimental groups (p < 0.01). G1: CDAA with vehicle-treated group. G2: CDAA with ARB-treated group. G3: CDAA with DSX-treated group. G4: CDAA with ARB and DSX-treated group. G5: CSAA-treated group.
Figure 4.

Effects of ARB and DSX on AT-II-induced proliferation, TGF-β1 and α1(I)-procollagen mRNA expressions of Ac-HSC. Cells were incubated in the presence of AT-II (1 μmol/L), ARB (10 μmol/L), and/or DSX (20 μmol/L) for 24 hr, and proliferation, TGF-β1 and α1(I)-procollagen mRNA expressions of Ac-HSC were measured by MTT assay and real-time RT-PCR, respectively. AT-II-induced Ac-HSC proliferation (A) was suppressed by treatment with either ARB or DSX. Combination treatment with both agents exerted a stronger suppressive effect in comparison with treatment with either single agent. Similar to the changes in Ac-HSC proliferation, TGF-β1 (B) and α1(I)-procollagen mRNA (C) expressions in Ac-HSC were suppressed by treatment with ARB and DSX. Each bar represents the mean ± SD (n=12). * and ** indicate statistically significant differences between the indicated experimental groups (p < 0.01 and < 0.05, respectively). Control: untreated control group. Both: ARB and DSX-treated group.

Figure 5.

Effects of ARB and DSX on ROS production in the liver. A: The number of 8-OHdG immunopositive cells significantly increased in the liver of the CDAA-diet group (G1) as compared with the CSAA-fed control group (G5), and decreased in both the ARB-
(G2) or DSX- (G3) treated group in comparison with G1. Combination treatment with both agents (G4) exerted a stronger inhibitory effect as compared with G2 and G3. B: Similar to the changes in 8-OHdG, hepatic lipid peroxidation as measured by MDA was markedly suppressed in G2 and G3, and a stronger suppressive effect was observed in G4. Data represents the mean ± SD (bars; n =10). * indicates statistically significant difference between the indicated experimental groups (p < 0.01). G1: CDAA with vehicle-treated group. G2: CDAA with ARB-treated group. G3: CDAA with DSX-treated group. G4: CDAA with ARB and DSX-treated group. G5: CSAA-treated group.

Figure 6.

A: Representative photomicrographs of GST-P positive pre-neoplastic lesions in the liver. B: Semi-quantitative analysis revealed that treatment with either ARB (G2) or DSX (G3) exerted significant inhibitory effects on the size of the pre-neoplastic foci in comparison with the CDAA-treated group (G1). Combination treatment with both agents (G4) exerted a stronger inhibitory effect. No pre-neoplastic lesions were observed in the CSAA-fed control rats (G5). Data represents the mean ± SD (bars; n =10). * indicates statistically significant difference between the indicated experimental groups (p < 0.01). G1: CDAA with vehicle-treated group. G2: CDAA with ARB-treated group.

Figure 7.
CD31 (A) and VEGF (B) mRNA expressions in the liver were measured by real-time PCR. Expressions of both CD31 and VEGF significantly increased in the CDAA-diet group (G1) as compared with the CSAA-fed control group (G5). Treatment with either ARB (G2) or DSX (G3) markedly suppressed the expressions of both CD31 and VEGF in comparison with G1. Combination treatment with both agents (G4) exerted a stronger inhibitory effect in comparison with either single agent. Data represents the mean ± SD (bars; n =10). * indicates statistically significant difference between the indicated experimental groups (p < 0.01).


Figure 8.
Characteristics and index of in-vitro EC tubular formation (Left and right panels, respectively). Treatment with AT-II (1 μmol/L) (B) increased EC tubule formation in comparison with the phosphate-buffered saline-treated control group (A). Treatment with either ARB (10 μmol/L) (C) or DSX (D) (20 μmol/L)
suppressed AT-II-induced EC tubular formation, and combination treatment with ARB and DSX (E) exerted a stronger suppression in comparison with treatment with a single agent. Semi-quantitative analysis by image-analyzing system confirmed the above-mentioned findings (Right panel). Data represents the mean ± SD (bars; n = 6). * indicates statistically significant difference between the indicated experimental groups (p < 0.01).
References


7. **Day CP and James OF.** Steatohepatitis: a tale of two "hits"?


35. **Sumida Y, Nakashima T, Yoh T, Furutani M, Hirohama A,


A

G1  G2  G3  G4  G5

B

Firosis area (%)

G1  G2  G3  G4  G5
A: Control
B: AT-II
C: AT-II + ARB
D: AT-II + DSX
E: AT-II + ARB + DSX