Prevention of Hepatic Fibrosis in a Murine Model of Metabolic Syndrome with Nonalcoholic Steatohepatitis

The endocannabinoid pathway plays an important role in the regulation of appetite and body weight, hepatic lipid metabolism, and fibrosis. Blockade of the endocannabinoid receptor CB1 with SR141716 promotes weight loss, reduces hepatocyte fatty acid synthesis, and is antifibrotic. D-4F, an apolipoprotein A-1 mimetic with antioxidant properties, is currently in clinical trials for the treatment of atherosclerosis. C57BL/6J mice were fed a high-fat diet for 7 months, followed by a 2.5-month treatment with either SR141716 or D-4F. SR141716 markedly improved body weight, liver weight, serum transaminases, insulin resistance, hyperglycemia, hypercholesterolemia, hyperleptinemia, and oxidative stress, accompanied by the significant prevention of fibrosis progression. D-4F improved hypercholesterolemia and hyperleptinemia without improvement in body weight, steatohepatitits, insulin resistance, or oxidative stress, and yet, there was significant prevention of fibrosis. D-4F prevented culture-induced activation of stellate cells in vitro. In summary, C57BL/6J mice given a high-fat diet developed features of metabolic syndrome with nonalcoholic steatohepatitis and fibrosis. Both SR141716 and D-4F prevented progression of fibrosis after onset of steatohepatitits, i.e., a situation comparable to a common clinical scenario, with D-4F seeming to have a more general antifibrotic effect. Either compound therefore has the potential to be of clinical benefit.

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idemia when fed a Western diet, a high-fat diet formulated to approximate the typical human diet in North America and Europe. We demonstrate that C57BL/6J mice given 9.5 months of a Western diet develop both steatohepatitis and significant fibrosis. Thus the high saturated fat diet model presented here represents a model of metabolic syndrome with advanced fatty liver disease. We have examined the effect of 2.5 months of treatment with either SR141716 or D-4F in this model.

**Materials and Methods**

**Reagents**

SR141716 was a kind gift from Sanofi-Aventis, Paris, France. SR141716 was dissolved in water with 0.1% Tween and given daily by gavage for 10 weeks. D-4F is an 18-mer of D-amino acids (Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH2) that was commercially synthesized (A & A Laboratories, San Diego, CA) with 80% purity. D-4F was dissolved in the drinking water at a concentration of 125 µg/ml of water for a calculated dose corrected for drug purity of 125 µg of D-4F/mouse/day. The high-fat diet used was the RD Western diet (D12079B; Research Diets, New Brunswick, NJ), which contains 41% of calories from fat, 43% calories from carbohydrate, and 17% of calories from protein; this diet was designed to approximate the typical human diet of North America and Europe.

**Animal Model**

Studies were performed in male C57BL/6J mice (The Jackson Laboratory, Bar Harbor ME). Six groups were studied (n = 3 to 5; Figure 1) with four mice per group at the outset, except for group 2, which was started with n = 5; one mouse died in group 1. Groups 1, 2, and 3 received the Western diet for 9.5 months; during the last 2.5 months of the 9.5 months, group 2 received SR141716 10 mg/kg/day i.g., and group 3 received D-4F, 125 µg/mouse/day, in the drinking water. Groups 4, 5, and 6 received the Western diet for 7 months followed by standard chow for 2.5 months; during the 2.5 months on standard chow, group 5 received SR141716 and group 6 received D-4F as described for groups 2 and 3, respectively. Additional control groups were studied: 6-month-old C57BL/6J mice as age-matched controls (n = 5) and C57BL/6J mice that received the Western diet for 6 months (n = 6). All protocols dealing with animals were reviewed and approved by the Animal Care and Use Committees at the University of Southern California and University of Arizona to ensure ethical and humane treatment of the animals. This study followed the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Revised 1985) prepared by the National Academy of Sciences.

**Sirius Red Staining**

Sirius red staining was performed by the Morphology Core of the USC-UCLA Research Center for Alcoholic Liver and Pancreatic Diseases.

**α-Smooth Muscle Actin (α-SMA)**

Seven-µm-thick sections of formalin-fixed, paraffin-embedded liver were used for α-SMA immunofluorescence staining. Slides were deparaffinized in xylene and serially rehydrated in graded ethanol (100 to 70%). Slides were treated with 0.1 mol/L Tris-HCl (pH 7.6)/0.05 Tween-20 for 5 minutes, incubated with 1 µg/ml of proteinase K (Sigma, St. Louis, MO) for 15 minutes at room temperature, and then rinsed in water for 10 minutes. Slides were rinsed in 0.1 mol/L Tris-HCl with 2% goat serum for 5 minutes, incubated with monoclonal anti-α-SMA (1:5000, Sigma) for 30 minutes at room temperature, followed by a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:75, Sigma) secondary antibody for 45 minutes at room temperature. After rinsing in 0.1 mol/L Tris-HCl/0.05% Tween-20 for 5 minutes followed by 0.1 mol/L Tris-HCl for 5 minutes, nuclear staining was performed with 4,6-diamidino-2-phenylindole diluted in methanol (1:1000, Sigma) for 5 minutes. Negative controls were performed by omitting the primary antibody.

**Evaluation of Steatosis and/or Fibrosis**

The slides were coded, without the pathologist knowing the specific treatment group that the slides represented. The
histology was graded according to a number of histological features. The degree of fat was graded as to the approximate percentage of hepatocytes containing fat: 0, absent; +/-, less than 5%; 1+, 5 to 25%; 2+, 25 to 50%; 3+, 51 to 75%; 4+, >75%. The fat was also assessed as to whether it was totally macrovesicular, predominantly macrovesicular, totally microvesicular, predominantly microvesicular, or mixed macro- and microvesicular. In addition, the zone was assessed as to whether the fat was perivenular, perportal, diffuse, or focal. Inflammation was graded as absent, scanty (1 to 3 foci per slide), present (scattered, with 4 to 10 foci per slide), present + (scattered, >10 foci per slide but not numerous), and present ++ (numerous). The type of inflammation was graded as totally mononuclear, predominantly mononuclear, totally neutrophilic, predominantly neutrophilic, and mixed mononuclear-neutrophilic. The hydropic change of hepatocytes was graded as absent, mild, and moderate/severe, with the location noted as perivenular, focal, or diffuse.

Whole Section-Scanning Morphometric Image Analysis

Tissue sections stained with Sirius red were scanned by a third-generation automated cellular imaging system (ACIS III), a highly automated microscope-based digital imaging system (Carl Zeiss MicroImaging AIS Inc., Aliso Viejo, CA). The color of the Sirius red staining in the digitized tissue sections was determined and applied to a pre-existing algorithm that provides the area of a target color in μm². The outline of each digitized tissue section was manually traced using the computer’s mouse. Areas of large portal tracts and central veins were also outlined manually. The total area (μm²) of each tissue section and of the total area of large portal tracts and central veins were determined by the image analysis system. The area of fibrosis was calculated as the total area stained by Sirius red minus perivascular staining as a percentage of the size of the tissue section. This method has the advantage of scanning the entire section, rather than measuring fibrosis in randomly chosen fields.

Electron Microscopy

In a separate set of experimental animals (n = 3), routine methods were used to prepare liver specimens for transmission and scanning electron microscopy. Briefly, the livers were perfused through the portal vein with 0.1 mol/L Na-cacodylate buffer to aid in washing out blood. This was immediately followed with a fixative containing 1.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4. For transmission electron microscopy, minced pieces of liver were further fixed for 2 hours, then washed in buffer, postfixed with 1% osmium tetroxide in 0.1 mol/L cacodylate buffer at 4°C, dehydrated through graded alcohols, and embedded in Spurr. Thin sections were cut on a Reichert Ultracut microtome. (Reichert-Jung Ultracut microtome, Vienna, Austria) examined and photographed using a Philips CM-12S electron microscope (Philips Electronic Instruments, Eindhoven, The Netherlands). For scanning electron microscopy, pieces of perfused-fixed livers were dehydrated in a graded ethanol series, fractured, dried with hexamethyldisilozane, sputter-coated with 10-nm gold, and examined using a Philips XL30 scanning electron microscope.

In Vitro Studies

Murine HSCs were isolated by collagenase/pronase digestion and Stratcan density gradient centrifugation. For α-SMA staining the HSCs were cultured on glass coverslips and fixed with 10% formaldehyde (EM Science, Gibbstown, NJ) for 20 minutes at room temperature. Cells were incubated with a mouse monoclonal anti-α-SMA (1:250 or 1:50, as indicated), followed by a rabbit anti-mouse IgG tetramethyl-rhodamine isothiocyanate conjugate (1:50; Santa Cruz Scientific, Santa Cruz, CA). Slides were examined using a Nikon (Melville, NY) PCM-2000 confocal microscope with a Nikon Eclipse TE300 microscope with a plan Apo 60×/1.4 aperture oil immersion objective, 543-nm laser, and Simple PCI software from the C-Imaging series from Nikon/Compix Inc. (Cranberry Township, PA). Values were obtained by counting the number of α-SMA-positive cells in 15 randomly selected fields in triplicate. Oil red O staining was performed by the Histology Core of the USC Research Center for Liver Disease and slides were stained with oil red O for 10 minutes, rinsed briefly with 70% ethanol, counterstained with Harris’ hematoxylin, and then treated with saturated sodium bicarbonate solution.

Biochemical Assays

Serum aspartate amino transferase (AST) and alanine aminotransferase (ALT) were measured with the Infinity AST and ALT liquid assay reagent (catalog number TR70121 and TR71121; Thermo Electron Corporation, Louisville, CO). Serum triglycerides and cholesterol were measured with the Infinity cholesterol assay reagent (catalog number TR22321 and TR71121; Thermo Electron Corporation, Gibbstown, NJ). Serum insulin was measured with the Infinity cholesterol assay reagent (catalog number TR71121; Thermo Electron Corporation). Serum glucose was measured with a glucose (HK) assay kit (catalog number GAHK-20, Sigma). Serum insulin was measured with the Mercodia UltraSensitiv mouse insulin enzyme-linked immunosorbent assay kit (catalog number RD 293023100; Biovendor LLC, Candler, NC). Serum leptin was measured with the mouse adiponectin kit (catalog number RD 293023100; Biovendor LLC, Candler, NC). Thio-barbituric acid reactive substance (TBARS) was measured as previously described, in brief, liver was homogenized and diluted 1:1 in 10% ice cold trichloroacetic acid. Supernatant or a standard solution of 1,3,3,3-tetraethoxypropane (0.5 ml) was mixed with 0.5 ml of...
0.6% (w/v) 4,6-dihydroxy-2-thiopyrimidine. The solution was boiled for 30 minutes, cooled, and centrifuged. Absorbance was measured at 532 nm with a Shimadzu (Kyoto, Japan) UV-2101 PC spectrophotometer. To measure hepatic glutathione (GSH), liver was homogenized and diluted 1:1 in 10% ice-cold trichloroacetic acid. GSH in the supernatant was measured according to the recycling assay of Tietze.21

### Statistical Analysis

Groups were compared by analysis of variance with post hoc analysis by least significant difference. P < 0.05 was considered statistically significant.

### Results

#### Western Diet Model

Group 1 (Western diet for 9.5 months) had a 43% increase in body weight, hypercholesterolemia, hyperglycemia, hyperinsulinemia, marked insulin resistance (elevated HOMA-IR), and hyperleptinemia (Table 1). Liver weight was twice that of controls, AST and ALT were elevated 5- to 12-fold, and there was evidence of oxidative stress in the liver with increased tissue TBARS and decreased GSH. Liver histology showed diffusely distributed marked steatosis, foci of mononuclear inflammatory cells, and increased fibrosis (Figure 2). There was marked fibrosis with 4.6% of the surface area positive for Sirius red staining by -SMA staining (Figure 4).

There were substantial differences in the model after 9.5 months on the Western diet versus 6 months: more prominent hyperinsulinemia and insulin resistance (HOMA-IR), more evidence of oxidative stress in the liver with higher tissue TBARS and lower hepatic GSH, and higher leptin levels at 9.5 months (Table 1). The major histological difference (Table 2 and Figure 2) was that there was significantly less fibrosis after 6 than after 9.5 months of the Western diet. The surface area of fibrosis by whole section-scanning microscopy was 0.76 ± 0.03% after 6 months on the Western diet; P < 0.01 for 6-month control versus 6 months of the Western diet followed by 2.5 months of D-4F; P = 0.01 for 6-month control versus 6 months of the Western diet followed by 2.5 months of SR141716.

### Table 1. Biochemical Findings and Liver and Body Weights

<table>
<thead>
<tr>
<th></th>
<th>6-Month control</th>
<th>6-Month Western diet</th>
<th>Western diet</th>
<th>Western diet + D-4F</th>
<th>Western diet + SR141716</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>28.1 ± 1.1</td>
<td>45.9 ± 1.2</td>
<td>40.2 ± 5.2</td>
<td>39.5 ± 0.8</td>
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<td>Liver weight (g)</td>
<td>1.34 ± 0.05</td>
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<td>2.42 ± 0.04</td>
<td>1.54 ± 0.1</td>
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<td>AST (UL)</td>
<td>54.7 ± 10.0</td>
<td>250.7 ± 27.1</td>
<td>268.2 ± 29.6</td>
<td>211.9 ± 19.1</td>
<td>109.8 ± 10.4</td>
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<tr>
<td>ALT (UL)</td>
<td>18.8 ± 4.0</td>
<td>300.0 ± 37.1</td>
<td>235.2 ± 23.5</td>
<td>277.3 ± 18.2</td>
<td>54.8 ± 12.4</td>
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<tr>
<td>Cholesterol (mg/dl)</td>
<td>90.0 ± 5.1</td>
<td>274.1 ± 22.1</td>
<td>288.0 ± 5.4</td>
<td>146.8 ± 2.1</td>
<td>112.2 ± 13.7</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>50.7 ± 7.7</td>
<td>32.4 ± 5.6</td>
<td>38.0 ± 4.4</td>
<td>40.0 ± 6.7</td>
<td>33.1 ± 2.2</td>
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<td>Glucose (mg/dl)</td>
<td>65.0 ± 1.2</td>
<td>261.0 ± 24.2</td>
<td>270.2 ± 10.0</td>
<td>231.2 ± 18.0</td>
<td>136.3 ± 29.2</td>
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<td>Insulin (ng/ml)</td>
<td>0.47 ± 0.06</td>
<td>2.21 ± 0.09</td>
<td>3.27 ± 3.0</td>
<td>3.7 ± 1.1</td>
<td>0.81 ± 0.15</td>
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<td>HOMA-IR</td>
<td>2.2 ± 0.3</td>
<td>40.9 ± 4.2</td>
<td>62.8 ± 5.1</td>
<td>63.3 ± 23.5</td>
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<td>TBARS (nmol/g tissue)</td>
<td>18.7 ± 1.2</td>
<td>16.4 ± 0.4</td>
<td>27.1 ± 4.0</td>
<td>29.4 ± 2.4</td>
<td>17.1 ± 0.7</td>
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<td>GSH (nmol/mg protein)</td>
<td>91.8 ± 3.8</td>
<td>71.2 ± 3.9</td>
<td>39.6 ± 4.8</td>
<td>50.1 ± 5.8</td>
<td>68.7 ± 2.0</td>
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<tr>
<td>Leptin (ng/ml)</td>
<td>1.89 ± 0.29</td>
<td>24.0 ± 2.2</td>
<td>32.62 ± 1.86</td>
<td>17.49 ± 1.41</td>
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<td>Adiponectin (µg/ml)</td>
<td>22.9 ± 0.3</td>
<td>31.1 ± 1.9</td>
<td>17.3 ± 0.8</td>
<td>15.3 ± 1.0</td>
<td>18.4 ± 1.8</td>
</tr>
</tbody>
</table>

- 7-Month Western diet followed by 2.5 months of
- 7-Months Western diet followed by 2.5 months of

#### 6-Month control

<table>
<thead>
<tr>
<th></th>
<th>6-Month Western diet</th>
<th>Chow</th>
<th>Chow + D-4F</th>
<th>Chow + SR141716</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>28.1 ± 1.1</td>
<td>45.9 ± 1.2</td>
<td>34.6 ± 1.2</td>
<td>35.9 ± 3.2</td>
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<tr>
<td>Liver weight (g)</td>
<td>1.34 ± 0.05</td>
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<td>1.67 ± 0.19</td>
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<tr>
<td>AST (UL)</td>
<td>54.7 ± 10.0</td>
<td>250.7 ± 27.1</td>
<td>107.2 ± 20.5</td>
<td>104.2 ± 22.4</td>
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<tr>
<td>ALT (UL)</td>
<td>18.8 ± 4.0</td>
<td>300.0 ± 37.1</td>
<td>44.0 ± 4.0</td>
<td>69.1 ± 21.5</td>
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<tr>
<td>Cholesterol (mg/dl)</td>
<td>90.0 ± 5.1</td>
<td>274.1 ± 22.1</td>
<td>108.6 ± 4.7</td>
<td>120.8 ± 2.9</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>50.7 ± 7.7</td>
<td>32.4 ± 5.6</td>
<td>36.2 ± 6.6</td>
<td>49.1 ± 1.4</td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>65.0 ± 1.2</td>
<td>261.0 ± 24.2</td>
<td>137.2 ± 18.6</td>
<td>159.2 ± 2.4</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.47 ± 0.06</td>
<td>2.21 ± 0.09</td>
<td>1.78 ± 0.39</td>
<td>1.46 ± 0.2</td>
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<tr>
<td>HOMA-IR</td>
<td>2.2 ± 0.3</td>
<td>40.9 ± 4.2</td>
<td>18.1 ± 5.1</td>
<td>16.5 ± 2.2</td>
</tr>
<tr>
<td>TBARS (nmol/g tissue)</td>
<td>18.7 ± 1.2</td>
<td>16.4 ± 0.4</td>
<td>20.4 ± 0.3</td>
<td>20.0 ± 0.6</td>
</tr>
<tr>
<td>GSH (nmol/mg protein)</td>
<td>91.8 ± 3.8</td>
<td>71.2 ± 3.9</td>
<td>85.1 ± 3.8</td>
<td>82.8 ± 5.2</td>
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<td>Leptin (ng/ml)</td>
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<td>9.36 ± 0.76</td>
<td>8.70 ± 1.43</td>
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<td>Adiponectin (µg/ml)</td>
<td>22.9 ± 0.3</td>
<td>31.1 ± 1.9</td>
<td>19.3 ± 1.1</td>
<td>18.52 ± 1.5</td>
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</table>

- 7-Month Western diet followed by 2.5 months of D-4F
Western diet). Electron microscopy of mice after 6 months on the Western diet demonstrated early capillarization with defenestration of sinusoidal endothelial cells (Figures 5 and 6) and formation of a basal lamina (Figure 6). Scanning electron microscopy demonstrated marked distortion of the sinusoids by fat-laden hepatocytes (Figure 6).

SR141716

In group 2 (Western diet with addition of SR141716 for the last 2.5 months), body weight decreased back to the normal range within 1 week (data not shown) and remained stable until mice were sacrificed. Compared to group 1 (9.5 months Western diet), group 2 demonstrated improvement in hyperglycemia, hyperinsulinemia and insulin resistance (HOMA-IR), and hypercholesterolemia (Table 1). Liver weight returned to near normal, AST and ALT improved, leptin levels dropped markedly, and evidence of oxidative stress (hepatic TBARS and GSH) improved. Histologically, inflammation was reduced. Hepatic steatosis was decreased in three of five mice and was more commonly predominantly microvesicular in pattern, but the marked decrease in liver weight in all mice treated with SR141716 suggests that histological assessment underestimates the decline in steatosis (Table 2). The area of fibrosis was reduced from 4.6 ± 1.9% in group 1 to 1.2 ± 0.3% in group 2 (P < 0.005; Figures 2 and 3) and α-SMA staining was decreased (Figure 4).

When compared to group 4 (switch to chow), group 5 (switch to chow plus SR141716) demonstrated greater decreases in body weight and liver weight, lower AST levels, greater improvement in hyperglycemia, hyperinsulinemia and insulin resistance (HOMA-IR), and in hyperleptinemia. Steatohepatitis was comparable, but the histological assessment underestimates the improvement in steatosis because the chow plus SR141716 group had normalization of liver weight and the group on chow alone did not. The switch to chow alone (group 4) did not significantly reduce progression of fibrosis unless SR141716 was added (group 5) (Figures 2 and 3).

Comparison of group 2 (Western diet, SR141716) versus group 4 (switch to chow) demonstrated that SR141716 more markedly reduced evidence of hyperinsulinemia and insulin resistance than the change in diet did (Table 1). Liver weight, AST and ALT, and histological evidence of inflammation were comparable in both groups. Even though group 2 had slightly

<table>
<thead>
<tr>
<th>7 Months of Western diet followed by 2.5 months of</th>
<th>Fatty change</th>
<th>Inflammatory clusters†</th>
</tr>
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<tbody>
<tr>
<td>Western diet</td>
<td>4 Macrovesicular</td>
<td>4</td>
</tr>
<tr>
<td>Western diet</td>
<td>4 Mixed</td>
<td>3</td>
</tr>
<tr>
<td>Western diet + D-4F</td>
<td>4 Macrovesicular</td>
<td>3</td>
</tr>
<tr>
<td>Western diet + SR141716</td>
<td>3 Mixed</td>
<td>2</td>
</tr>
<tr>
<td>Chow</td>
<td>2 Mixed</td>
<td>2</td>
</tr>
<tr>
<td>Chow + D-4F</td>
<td>2 Mixed</td>
<td>2</td>
</tr>
<tr>
<td>Chow + SR141716</td>
<td>3 Mixed</td>
<td>2</td>
</tr>
</tbody>
</table>

*The type of fatty change listed indicates the predominant type present. Steatosis was diffusely distributed in mice that remained on the Western diet with or without D-4F, and perivenular in mice that switched to chow with or without D-4F; steatosis was diffuse or perivenular in mice on SR141716.

†Inflammatory clusters were composed of mononuclear cells.
higher leptin levels, somewhat greater evidence of oxidative stress, and somewhat more steatosis, there was less progression of fibrosis in group 2: when contrasted with the Western diet (group 1), group 4 did not have a significant decrease in fibrosis and group 2 did (Figures 2 and 3).

D-4F

When mice received the Western diet for 9.5 months with addition of D-4F for the last 2.5 months (group 3), total cholesterol and leptin levels were decreased by half compared to mice on Western diet alone (group 1). There was no improvement in body weight, liver weight, serum transaminases, hyperglycemia, hyperinsulinemia, insulin resistance, or overall hepatic oxidative stress with D-4F (Table 1). Histologically, there was no effect on steatosis and some evidence of a reduction in inflammation (Table 2). Nevertheless, D-4F reduced fibrosis from 4.6 ± 1.9% in group 1 to 1.25% in group 3 ($P < 0.005$; Figures 2 and 3).

Comparison of group 4 (switch to chow for the last 2.5 months) with group 6 (switch to chow plus D-4F) demonstrated less fibrosis in the group receiving D-4F: 0.9 ± 0.2% fibrosis in group 6 versus 2.8 ± 0.7% fibrosis in group 4 ($P = 0.05$) and perhaps a small decrease in inflammation (Table 2). No other effect was observed with chow plus D-4F compared to chow alone (Table 1).

Comparison of group 3 (Western diet, D-4F) with group 4 (switch to chow) demonstrated that group 4 had greater improvement in body and liver weight, steatohepatitis (AST, ALT, histological steatosis), hypercholesterolemia, hyperglycemia, insulin resistance, hyperleptinemia, and overall hepatic oxidative stress (TBARS, GSH). However, group 3 had a significant reduction in progression of fibrosis and group 4 did not (Figures 2 and 3).

To examine the mechanism of action of D-4F, murine HSCs were cultured on plastic for 7 days in the absence or presence of 10 μg/ml of D-4F. Without D-4F, 87 ± 2% of cells expressed α-SMA after 7 days as assessed by

![Figure 4](image-url)  
**Figure 4.** α-SMA staining. **A:** Control; **B:** 9.5 months of Western diet; **C:** 7 months of Western diet followed by 2.5 months of chow; **D:** 9.5 months of Western diet with addition of D-4F during the last 2.5 months; **E:** 9.5 months of Western diet with addition of D-4F during the last 2.5 months. α-SMA (green) stains activated HSCs. Erythrocytes (red) are visible in venules and sinusoids.

![Figure 5](image-url)  
**Figure 5.** Transmission electron micrograph of the liver of a mouse that received 6 months of the Western diet, illustrating thickened sinusoidal endothelium (E), and a subendothelial basal lamina (arrow) and collagen deposition (CF) in the space of Disse. S, sinusoid lumen. Original magnification, ×6000.
confocal microscopy. In contrast, in the presence of D-4F 0% of HSCs expressed α-SMA after 7 days (see Figure 7). HSCs cultured in the presence of D-4F also maintained cytoplasmic fat droplets, in contrast to HSCs cultured without D-4F (Figure 7).

Discussion

C57BL/6J mice given 9.5 months of a high saturated fat diet formulated to approximate the typical human diet in North America and Europe developed nonalcoholic steatohepatitis with significant fibrosis and signs of the metabolic syndrome (obesity, insulin resistance, hyperglycemia, and hypercholesterolemia). This provides a model to examine interventions that prevent progression of fibrosis and to correlate the degree of fibrosis with features of steatohepatitis and the metabolic syndrome.

In mice remaining on the Western diet, SR141716 markedly improved body weight, liver weight, serum transaminases, insulin resistance, hyperglycemia, hypercholesterolemia, and oxidative stress. This was accompanied with significant prevention of progression of fibrosis. In mice remaining on the Western diet and treated with D-4F there was improvement in hypercholesterolemia and hyperleptinemia, no improvement in body weight, steatohepatitis, insulin resistance, or oxidative stress, and yet there was significant prevention of fibrosis.

From a clinical perspective, the most important comparison may be the effect of switching to chow versus the use of either SR141716 or D-4F with continuation of the Western diet, ie, comparing the effect of switching to a healthier diet with the effect of medication and a continued high-fat diet. Treatment with SR141716 with continuation of the Western diet significantly prevented progression of fibrosis, whereas the switch to chow somewhat decreased fibrosis but not to a statistically significant degree. SR141716 is known to prevent fibrosis by blocking CB1 in disparate models of experimental fibrosis. The reduction in steatohepatitis and markers of oxidative stress was similar in mice receiving treatment with SR141716 with continuation of the Western diet and mice that were switched to chow. SR141716 more markedly reduced hyperinsulinemia and insulin resistance and nearly normalized leptin levels. The fall in leptin may have contributed to the antifibrotic effect. Adiponectin levels did not increase in mice treated with SR141716, in contrast to the marked increase in adiponectin seen in obese fa/fa rats treated with the drug; the difference in adiponectin response to SR141716 may reflect differences between the models used.

D-4F prevented progression of fibrosis to a comparable degree to SR141716 in mice continued on the Western diet. D-4F had no effect on steatohepatitis, and no effect on overall oxidative stress in liver tissue. The only extrahepatic effects of D-4F that were observed were reduction in leptin and cholesterol in the mice continued on the Western diet. However the combination of D-4F and a switch to chow reduced fibrosis more than chow alone and in this group D-4F had no additive effect on cholesterol and leptin compared to the switch to chow alone. The in vitro studies demonstrated that D-4F prevented HSC activation in vitro, which suggests that D-4F prevented fibrosis in the nonalcoholic fatty liver disease model through a more general antifibrotic effect.

Fat-laden hepatocytes are swollen, and further swelling occurs in nonalcoholic steatohepatitis because of hydropic change (ballooning) of hepatocytes. As can be appreciated in the scanning electron microscopy (Figure 6), such swelling causes sinusoidal distortion. In vivo microscopy of fatty liver demonstrates sinusoidal distortion with reduced intrasinusoidal volume and microvascular blood flow. Involvement of other cell types (sinusoi-
concentrations of \( /H9251 \) cells cultured in the presence of D-4F express steatohepatitis.\(^{27,28}\) Liver injury and disease progression in nonalcoholic such microvascular damage could accentuate further normally perfused sinusoids. It has been proposed that changes is a marked reduction of sinusoidal space (fibrosis). In animal models, the net effect of such inflammatory cells and perisinusoidal matrix deposition dysregulation of microvascular blood flow by adherent recruitment of inflammatory cells and platelets lead to dal endothelial cells, Kupffer cells, stellate cells) and recruitment of inflammatory cells and platelets lead to dysregulation of microvascular blood flow by adherent inflammatory cells and perisinusoidal matrix deposition (fibrosis). In animal models, the net effect of such changes is a marked reduction of sinusoidal space (~50% of control), and a decrease in the number of normally perfused sinusoids. It has been proposed that such microvascular damage could accentuate further liver injury and disease progression in nonalcoholic steatohepatitis.\(^{27,28}\)

In summary, both SR141716 and D-4F prevented progression of fibrosis after onset of steatohepatitis, ie, a situation comparable to a common clinical scenario, and either compound therefore has the potential to be of clinical benefit. SR141716 promoted weight loss, improved steatohepatitis, decreased insulin resistance, decreased leptin levels, and prevented fibrosis through antagonism of CB1. D-4F did not alter steatohepatitis, but prevented stellate cell activation \textit{in vitro} and may therefore be a more general antifibrotic agent.

**References**


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