SUPPLEMENT MATERIAL

**KRP-203 induces persistent lymphopenia in LDL-R⁻/⁻ mice** – KRP-203 is a 480 Da synthetic compound with a molecular structure similar to FTY720. KRP-203 displays functional selectivity for S1PR1 (EC₅₀ of 0.84 nmol/L) with weak agonist activity towards S1PR4 and no agonist towards S1PR2 and S1PR3, and can be chronically administered to mice without systemic toxicity [1-3]. Here, KRP-203 was administered 3-weekly via intraperitoneal injections to 8-week-old LDL-R⁻/⁻ mice at a dose of 3.0 mg/kg/day, which in previous studies significantly improved heart allograft survival in rats and is close to what was used to ameliorate autoimmune myocarditis in rat (1.0 mg/kg/day), concanavalin A-induced hepatic injury in mice (1.0 mg/kg/day), or renal injury in MRL/lpr mice (6.0 mg/kg/day) [4-7]. To verify the dosage scheme in LDL-R⁻/⁻ mice, the lymphocyte count in peripheral blood was determined at different time points after administration of KRP-203 and compared to FTY720. As shown in Fig. I, both KRP-203 and FTY720 reduced peripheral lymphocytes by 63.1% and 58.2%, respectively, 6 h after a single dosage and in case of KRP-203 this effect was persistent even after 48 hours.

**KRP-203 fails to affect smooth muscle cell content and distribution in the lesion** – To assess the smooth muscle cell content in the lesion, immunohistochemical analysis using anti-smooth muscle α-actin antibody was performed. As shown in Fig. IIA, atherosclerotic lesions from control mice and mice treated with KRP-203 for 6 or 16 weeks were characterized by a comparable content of smooth muscle cells. In addition, lesions from control and treated animals displayed similar distribution of smooth muscle cell relative to lesional macrophages as visualized using immunofluorescent double-staining with anti-MoMa and anti-α-actin antibodies (Fig. IIB). Because the lesional macrophage content in mice treated with KRP-203 for 6 weeks was considerably lower than in control mice, these animals were characterized by on average higher smooth muscle cell/macrophage ratio (1.13 vs. 0.71). However, this difference was almost equalized in animals treated with KRP-203 for 16 weeks (0.25 vs. 0.22).

**KRP-203 fails to affect plasma lipid profile, body weight, and liver and renal function parameters** – To assess potentially anti-atherogenic effects of KRP-203 on lipid metabolism, we determined plasma lipid profiles in LDL-R⁻/⁻ mice at sacrifice. As shown in Fig. III, treatment with KRP-203 failed to alter plasma concentrations of total and HDL-cholesterol and triglycerides. In addition, both groups showed similar body weight gains, when fed a cholesterol-enriched Western type diet (Fig. III). Parameters reflecting potential adverse effects of KRP-203 on liver and kidney functions (ALT, creatinine, blood urea nitrogen (BUN)) did not significantly differed between KRP203-treated and control animals after 6 weeks of treatment (Table I).
**Table I.** Liver and renal function parameters in KRP-203-administered and control LDL-R⁻/⁻ mice after 6 weeks of treatment.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>KRP-203 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALT (U/L)</strong></td>
<td>164 ± 38</td>
<td>134 ± 44</td>
</tr>
<tr>
<td><strong>BUN (mg/dL)</strong></td>
<td>19.5 ± 1.8</td>
<td>18.7 ± 2.1</td>
</tr>
<tr>
<td><strong>Creatinin (mg/dL)</strong></td>
<td>0.26 ± 0.03</td>
<td>0.31 ± 0.04</td>
</tr>
</tbody>
</table>

Data represent mean ± SD.

**KRP-203 inhibits endothelial activation in vitro** – As reduction of the lesion macrophage content in KRP203-treated animals might be a consequence of reduced monocyte transendothelial migration, in the last step we assessed the effect of KRP-203 on *in vitro* monocyte adhesion and endothelial barrier functions. To this aim, bEnd.5 murine endothelial cells were pre-incubated for 24 h with 5.0 µmol/L KRP-203 and afterwards exposed for 4h to TNFα at a concentration of 20 ng/mL, which has previously been shown to effectively stimulate expression of adhesive molecules in this cell line [8]. As shown in Fig. IVA, TNFα potently induced expression of the adhesion molecule VCAM-1 in the absence but not in the presence of KRP-203. The extent of monocyte adhesion to endothelium was examined next using an *in vitro* cytoadherence assay. Fig. IVB demonstrates the increased adhesion of U937 monocytes to bEnd.5 endothelial cells exposed for 4h to 50 ng/mL of TNFα and its almost complete reversal by pre-treatment of cells with KRP-203 (5.0 µmol/L). To investigate the influence of KRP-203 on endothelial permeability we quantified the flux of FITC-dextran (20 kDa) and the migration of U937 cells across bEnd.5 endothelial cells cultured in a Boyden chamber on 0.4 µm and 8.0 µm pore-size inserts, respectively. As shown in Fig. IVC, pretreatment of endothelial cells for 24 h with 5.0 µmol/L KRP-203 significantly reduced the permeability to FITC-dextran and the migration of U937 cells, when compared to untreated cells.

**REFERENCES TO SUPPLEMENT MATERIAL**


FIGURES TO SUPPLEMENT MATERIAL

**Figure I.** Effect of single dose KRP-203 or FTY720 administration on peripheral lymphocyte counts in LDL-R<sup>−/−</sup> mice - LDL-R<sup>−/−</sup> mice were intraperitoneally administered with KRP-203 (3.0 mg/kg; n=3; ○) or FTY720 (2.0 mg/kg; n=3; △) as a single dose. Blood was collected by retroorbital puncture at four different time points (0h - 6h - 24h - 48h) and relative lymphocyte counts were determined. Values are means ± SD.
Figure II. Effect of KRP-203 on lesional content and distribution of smooth muscle cells in LDL-R⁻/⁻ mice – KRP-203 (3.0 mg/kg) or saline were administered to Western-type diet-fed LDL-R⁻/⁻ mice for 6 or 16 weeks. Animals were euthanized, bled and aortic roots were fixed, stained and used for morphometric analysis or stained for smooth muscle cells (α-actin) or macrophages (MOMA-2). A. Left and middle panels – representative anti-α-actin staining from control and KRP-203-treated mice. Right panel – bar graph showing the content of smooth muscle cells in the atherosclerotic plaque expressed as stained area (mm²). Images taken at 10 X magnification. Values represent mean ± SD. B. Representative immunofluorescence double-stainings visualizing smooth muscle cells (green fluorescence) and macrophages (red fluorescence) distribution within atherosclerotic lesions of animals treated with KRP-203 for 16 weeks. Images taken at 40 X magnification. Sections were counterstained with DAPI for nuclear morphology (blue fluorescence).
**Figure III. Effect of KRP-203 on body weight and plasma lipid levels in LDL-R−/− mice** – Body weight was measured and plasma was obtained from LDLR−/− mice after 0, 6 or 16 weeks of control (○) or KRP-203 treatment (■). Total cholesterol, triglyceride and HDL-cholesterol levels were determined using routine laboratory procedures. Right lower panel: Demonstration of chromatographic lipoprotein profile in plasma obtained from animals treated for 6 weeks with KRP-203 or in untreated animals.

**Figure IV. KRP-203 inhibits murine endothelial cell activation in vitro** – **A** and **B.** bEnd.5 murine endothelial cells were stimulated with TNFα (20.0 ng/mL) for 4 h. VCAM-1 expression was determined by Western blot (A) and the adherence of calcein-loaded U937 monocytes was observed under fluorescence microscope (B). Data are representative for three independent experiments. Numbers above blots show increases in band intensities relative to controls as determined by densitometry. **C.** FITC-dextran (20 kDa) or calcein-loaded U937 cells were placed in the apical compartment of the Boyden chamber and the influx or migration across the endothelial layer was quantified by determining fluorescence in the basolateral compartment. Results are expressed as the percentage of influx/migration across untreated monolayers. Data are representative for 3 independent experiments. ∗ - p<0.05, ∗∗ - p<0.01 (–KRP-203 vs. +KRP-203).