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Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model

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Abstract

Serine palmitoyltransferase (SPT) is a first-step enzyme in the sphingolipid biosynthetic pathway. Myriocin is an inhibitor of SPT and suppresses replication of the hepatitis C virus (HCV) replicon. However, it is still unknown whether this SPT inhibitor suppresses HCV replication in vivo. We investigated the anti-HCV effect of myriocin against intact HCV using chimeric mice with humanized liver infected with HCV genotype 1a or 1b. We administered myriocin into HCV infected chimeric mice and succeeded in reducing the HCV RNA levels in serum and liver to 1/10–1/100 of the levels prior to the 8 day treatment. Furthermore, combined treatment with pegylated interferon reduced the HCV RNA levels to less than 1/1000 of the control levels. We strongly suggest that suppression of SPT reduces HCV replication, and therefore that the SPT inhibitor is potentially a novel drug in the treatment of HCV infection.

Keywords: HCV; Myriocin; Serine palmitoyltransferase; Lipid raft; Chimeric mice with humanized liver

Hepatitis C virus (HCV) infection usually causes chronic hepatitis and often leads to cirrhosis of the liver or hepatocellular carcinoma [1,2]. The number of carriers now amounts to approximately 3% (~170 million) of the population worldwide. The most effective treatment against HCV infection is a combination of pegylated interferon (PEG-IFN) and ribavirin [3,4]. However, many people cannot tolerate the serious side effects and thus the number of patients able to receive the therapy is limited. The development of novel drugs to treat HCV with greater safety and better efficacy is therefore urgently required.

HCV is a single-stranded RNA virus that belongs to the Flaviviridae family [5]. The RNA genome produces at least 10 viral proteins, which include structural and non-structural (NS) proteins. The former are involved in the formation of the HCV particle. The latter play a key role in HCV genome replication [6]. It is generally accepted that a complex of NS proteins is associated with the lipid raft on the Golgi and endoplasmic reticulum membranes, where HCV replication occurs [7,8]. Thus, disruption of assembly of the lipid raft may lead to suppression of HCV replication.

Myriocin (ISP-1) is a specific inhibitor of serine palmitoyltransferase (SPT), a first-step enzyme in the sphingolipid biosynthetic pathway (Fig. 1A; [9,10]). Myriocin inhibits SPT activity due to its structural similarity to sphingosine (Fig. 1B), resulting in decreased intercellular sphingomyelin and its intermediates, dihydrosphingosine, sphingosine, ceramide, and sphingosine-1-phosphate (Fig. 1A). Inhibition of SPT by myriocin is thought to eventually lead to disruption of lipid raft assembly, as sphingomyelin is one of the major integral components of its assembly [11].

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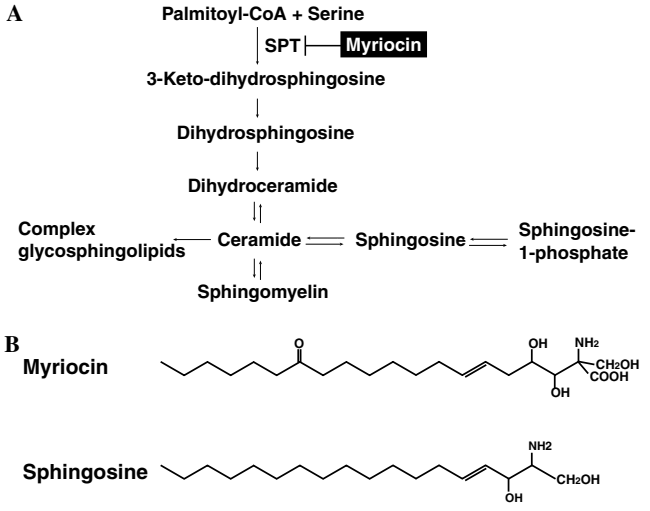

Fig. 1. Sphingolipid biosynthetic pathway. (A) Biosynthesis pathway of sphingolipid. (B) Structure of myriocin and sphingosine.

Previously, we isolated the compound NA255, which suppresses replication of the HCV subgenomic replicon [12]. NA255 is structurally similar to myriocin and inhibits the enzymatic activity of SPT, resulting in suppression of replication without affecting the enzyme activities of HCV NS3 (protease and helicase) or NS5B (RNA-dependent RNA polymerase). Thus, replication of the HCV subgenomic replicon was suppressed by NA255 in response to the decrease in amount of sphingolipid, ceramide, and sphingomyelin. These findings suggest that NA255 disrupts assembly of the lipid raft associated with HCV NS proteins. In the present study, we performed comparative and concomitant trials of one SPT inhibitor, myriocin, and PEG-IFN in chimeric mice with humanized liver (chimeric mice) infected with intact HCV. The results demonstrate for the first time that suppression of SPT inhibits replication of intact HCV in vivo.

Materials and methods

Inhibition assay of replication in HCV replicon cells by myriocin. Myriocin (Sigma, St. Louis, MO, USA) was added in the growth medium of HCV subgenomic replicon cells FLR3-1 (genotype 1b, Con-1; [12]) at a final concentration of 0.2, 1.0, 3.9, 15.6 or 62.5 nM. After 72 h incubation, we performed luciferase assays using the Bright-Glo luciferase assay kit (Promega, Madison, WI, USA).

Measurement of cell viability using the Tetra Color One (WST-8) assay. Myriocin was added to FLR3-1 cells as described above. After 72 h incubation, cell viability was measured using the Tetra Color One kit (Seikagakukougyo, Tokyo, Japan) according to the manufacturer's instructions.

Immunoblotting analysis. Cells were harvested and lysed in lysis buffer (PBS containing 0.5% Triton X-100 and 0.5 mM PMSF), and then 5 μg of protein separated by 12% SDS–PAGE and electro-blotted onto a nitrocellulose membrane (Shleicher & Schuell, Dassel, Germany). A rabbit polyclonal anti-NS3 antibody [12] and anti-actin (20–33) antibody (Sigma, St. Louis, MO, USA) were used as the primary antibodies. The proteins were then detected by an anti-rabbit antibody HRP-linked IgG (Cell Signaling Technology, Beverly, MA, USA).

Immunofluorescent staining of HCV replicon cells. After treatment of 250 nM myriocin for 72 h, FLR3-1 cells were probed with a primary antibody, an anti-NS3 polyclonal antibody, after blocking with TNB blocking buffer (Perkin-Elmer, Wellesley, MA, USA). Next, an anti-rabbit IgG-Alexa-488 conjugate (Invitrogen, Carlsbad, CA, USA) was applied as the secondary antibody.

TLC analysis. Cells were incubated for 2 h with [^{14}C] serine (0.5 μCi/ml) in Opti-MEM (Invitrogen). After the cells were lysed with 0.1% SDS, and total lipids were extracted with chloroform/methanol (1:2 v/v). The extracts were spotted onto Silica Gel 60 thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) and chromatographed with methyl acetate/1-propanol/chloroform/methanol/0.25% KCl (25:25:25:10:9, v/v). Radioactive spots were detected by BAS 2000 (Fuji Film, Kanagawa, Japan).

Complementation of sphingolipid intermediates. FLR3-1 cells were incubated with 1 or 2.5 μM of sphingolipid intermediates (dihydrosphingosine, sphingosine, or sphingosine-1-phosphate) and sequentially diluted myriocin then added. After 72 h, the IC_{50} of each combination was measured by the luciferase assay.

Infection of HCV genotype 1a and 1b in chimeric mice. Chimeric mice were purchased from PhenixBio Co., Ltd. (Hiroshima, Japan). The mice were generated by transplanting human primary hepatocytes into SCID mice carrying the urokinase plasminogen activator transgene controlled by an albumin promoter (*Alb-uPA*) [13–15]. Overexpression of this transgene induces a profoundly hypofibrinogenemic state and accelerated hepatocyte death. HCG9 (genotype 1a) and HCR6 (genotype 1b, Accession No: AY045702), originally from patient serum, were intravenously injected at 10^6 copies/mouse at about 40 days after transplantation of human hepatocytes. After 4 weeks, the HCV 1a and 1b RNA levels had reached $\sim 10^8$ copies/ml and $\sim 10^7$ copies/ml, respectively, in the mice serum.

Administration of myriocin and/or PEG-IFN into chimeric mice infected with HCV 1b. Injections of myriocin or PEG-IFN (Chugai, Tokyo, Japan) or both were administered to HCV genotype 1b (HCR6) infected mice and blood then collected according to the protocol in Table 2.

Quantification of HCV RNA by real-time PCR. Total RNA was purified from 1 μl of serum or 50 μg of liver tissue from chimeric mice using the AGPC method. HCV RNA was quantified by real-time PCR as previously reported [16].

Measurement of human albumin in the serum. Human albumin concentration was measured in 2 μl of serum using the Alb-II kit (Eiken Chemical, Tokyo, Japan) according to the manufacturer's instructions.

Detection of core protein in live tissue. We used chimeric mice with a high RNA levels of HCV genotype 1a (HCG9) in the serum to easily detect HCV RNA and core protein in liver tissue. We administered 2 mg/kg myriocin daily for 6 days to a chimeric 1a-4 mouse and extirpated the liver. For comparison, the livers of non-treated (1a-1), non-infected (1a-2), and PEG-IFN treated (1a-3) mice were also extirpated. The liver tissues were homogenized in RIPA buffer and 100 μg of total protein was used for the detection of core protein using the Ortho HCV core protein ELISA kit (Eiken Chemical).

Immunofluorescent and histological staining of chimeric mouse liver tissue. Liver sections from 1a-1 and 1a-4 mice were probed by biotinylated anti-HCV core protein monoclonal antibody, and human hepatocyte monoclonal antibody (Dako, Glostrup, Denmark) as the primary antibodies, followed by streptavidin-Alexa-488 (Invitrogen) and anti-mouse-IgG-Alexa-546 (Invitrogen). The nuclei were stained using DAPI. Biotinylated normal mouse IgG (Ancell, Bayport, MN, USA) was used as the negative control. For histological analysis, liver sections from 1a-1 and 1a-4 mice were stained by hematoxylin-eosin (H&E staining).

Results

Anti-HCV effect of the SPT inhibitor, myriocin

We examined the anti-HCV effect and cell toxicity of myriocin in the HCV subgenomic replicon cells FLR3-1. Luciferase activity was greatly decreased by myriocin in a dose-dependent manner without affecting cell viability