Preclinical evaluation of an anti-interleukin 6 receptor antibody for Castleman’s disease and a serine palmitoyltransferase inhibitor as an antiviral agent for hepatitis C virus

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1. Background and Aims
2. Materials and Methods
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Preface

This thesis is based on the following publications.


Summary

Clarifying the preclinical profiles of drug candidates is essential for estimating their clinical performance. However, the applicability of preclinical research to clinical profiles varies a great deal depending on the availability of suitable experimental models. In this study, I attempt to assess two drug candidates for which the clinical potential was difficult to estimate from the preclinical research because of species specificity and the lack of appropriate models.

Firstly, the effects of interleukin 6 (IL-6) signal blocking agents are evaluated in genetically modified Castleman’s disease mice by administering anti-IL-6 receptor (IL-6R) monoclonal antibody (mAb). Castleman’s disease is a syndrome associated with lymph node hyperplasia, plasmacytosis, fever, anemia, and hypergammaglobulinemia with an abnormal augmentation of IL-6, and blocking the IL-6 function by using a mAb to human IL-6R results in beneficial effects in the patients. The H-2L\textsuperscript{d} human IL-6 transgenic mice (IL-6 tgm) develop various disorders associated with dysregulated IL-6 overproduction, and most of these disorders are identical with those in Castleman’s disease, so IL-6 tgm could be useful as a Castleman’s disease model. I prepared a rat mAb to mouse IL-6R that would block the IL-6 signal in IL-6 tgm, which, when administered, almost completely prevented these symptoms and prolonged the life of the mice without severe adverse events. Thus, the clinically proven efficacy of the IL-6R mAb against
Next, the therapeutic potential of a recently identified drug candidate to treat Hepatitis C virus (HCV) is assessed in preclinical examinations. HCV affects approximately 170 million people worldwide and can result in a persistent infection that leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Unfortunately, the interferon (IFN)-based current standard therapy has limited efficacy and significant side effects. Although combinations of direct-acting antiviral agents (DAAs) without IFN have been tested for clinical use as novel anti-HCV therapies, emerging data suggest that DAAs might select for resistance, which can become a primary cause of treatment failure in clinical studies. Additionally, differences in HCV genotypes can result in certain DAAs having reduced antiviral activity. Therefore, the development of additional antiviral agents with diverse resistance profiles and efficacy against a wide spectrum of HCV genotypes is necessary. To this end, I have identified a novel class of serine palmitoyltransferase (SPT) inhibitors that inhibited HCV replication by disrupting the host cell lipid rafts, which act as a scaffold for HCV replication. This unique mechanism of host enzyme-targeted viral inhibition is hypothesized to have potential for its high barrier to resistance and for its antiviral activity across different HCV genotypes.

Preclinical research of HCV replication has been limited by the lack of appropriate infection models. One model that has been reported as being possibly infected with HCV was produced by transplanting human primary hepatocytes into severe combined immunodeficient mice.
carrying the urokinase plasminogen activator transgene controlled by an albumin promoter. Here, I use these mice to assess the therapeutic potential of a SPT inhibitor, NA808, in clinical practice by investigating the efficacy across HCV genotypes, its synergistic effects with other antiviral agents, and the potential development of resistance.

These humanized liver chimeric mice infected with HCV genotypes 1a, 1b, 2a, 3a, and 4a could support long-term HCV infections at clinically relevant titers. Administration of NA808 results in approximately 1- to 3-log reductions in each genotype-infected group, which indicates that NA808 has a robust antiviral effect across HCV genotypes. In addition, NA808 has synergistic effects when combined with pegylated IFN (PEG-IFN), NS3/4A protease inhibitor, or NS5B polymerase inhibitors, regardless of the targeted enzyme. When a full-genome sequence analysis of HCV in NA808-treated chimeric mice was performed to evaluate the potential for resistance to develop, the viral sequences from NA808-treated mice were identical to those from untreated mice. This high barrier to resistance is also confirmed by an in vitro study using HCV sub-genomic replicon cells.

Thus, NA808 mediates potent anti-HCV activity in a variety of genotypes with an apparently high barrier to resistance and has synergistic effects with other anti-HCV agents. These findings suggest that NA808 has potential as a novel host-targeted drug in the treatment of HCV infection.
In conclusion, the results in the Castleman’s disease model treated with anti-IL-6R mAb suggest that preclinical assessment might be capable of predicting the clinical performance, when using appropriate models with clinical relevance. In HCV-infected humanized liver chimeric mice, viral replication occurred in human hepatocytes, and long-term infection could be supported at a clinically relevant titer; hence, these mice are considered a suitable model with clinical relevance for evaluating anti-HCV drug candidates. Therefore, beneficial profiles of NA808 as an anti-HCV drug in HCV-infected humanized liver chimeric mice were able to reflect the drug’s clinical potential and support the development of NA808 as a treatment for HCV infection or for use in combination with PEG-IFN alfa-2a or HCV polymerase or with protease inhibitors.
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Abbreviations

Alb : albumin
ALP : alkaline phosphatase
BUN : blood urea nitrogen
Ca : calcium
cDNA : complementary DNA
Cre : creatinine
DAA(s) : direct-acting antiviral agent(s)
DNP-KLH : dinitrophenol-conjugated keyhole limpet hemocyanin
Glu : glucose
GOT : glutamic-oxaloacetic transaminase
GPT : glutamic-pyruvic transaminase
HCV : hepatitis C virus
hIL-6 : human interleukin-6
IL-6 : interleukin-6
IL-6R : interleukin-6 receptor
IL-6 SCID : C.B-17^scid mice with human interleukin-6 transgene
<table>
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<th>Abbreviation</th>
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<tr>
<td>IL-6 tgm</td>
<td>H-2L&lt;sup&gt;d&lt;/sup&gt; human interleukin-6 transgenic mice</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<td>MH60</td>
<td>MH60.BSF2 cell line</td>
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<td>mIL-6R</td>
<td>mouse interleukin-6 receptor</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>ribavirin</td>
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<td>SCID</td>
<td>severe combined immunodeficient mice</td>
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<td>SPT</td>
<td>serine palmitoyltransferase</td>
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<td>TG</td>
<td>triglyceride</td>
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<td>TP</td>
<td>total protein</td>
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<tr>
<td>uPA/SCID</td>
<td>severe combined immunodeficient mice carrying the urokinase plasminogen activator transgene controlled by an albumin promoter</td>
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<tr>
<td>WBC</td>
<td>white blood cell</td>
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Introduction

Clarifying the preclinical profiles of drug candidates is essential for estimating their clinical performance. However, the applicability of preclinical research to clinical profiles varies a great deal depending on the availability of suitable experimental models. In this study, I attempt to assess two drug candidates whose clinical potential had been difficult to estimate from the preclinical research because of species specificity and the lack of appropriate models.

Firstly, to confirm the beneficial effects of IL-6 signal-blocking agents in patients with Castleman's disease, their effects are evaluated in genetically modified Castleman's disease mice by administering anti-IL-6 receptor (IL-6R) monoclonal antibody (mAb). IL-6 is a pleiotropic cytokine that acts on a wide variety of tissues and causes growth promotion, growth inhibition, differentiation, and induction of specific gene expression according to the type of target cells [2]. In addition to these multiple functions of IL-6, various types of biological activity could be significantly modulated by the existence of a soluble receptor that elicits agonistic signals through gp130 by forming a complex with IL-6. So, an abnormal augmentation of IL-6 has been suggested to be involved in the pathogenesis of certain disorders [6, 37]. Castleman's disease is a syndrome associated with lymph node hyperplasia, plasmacytosis, fever, anemia, and hypergammaglobulinemia that occur when IL-6 is abnormally augmented. Therefore, blocking the IL-6 signal transduction could provide a
therapeutic strategy for Castleman’s disease. Several researchers have attempted to block the IL-6 function using a mAb against IL-6 or IL-6R mAb to neutralize the IL-6 function and beneficial effects on patients with Castleman’s disease have been reported [5, 23].

I have established H-2Ld human IL-6 transgenic mice (IL-6 tgm) which develop various disorders, including massive IgG1 plasmacytosis, mesangial proliferative glomerulonephritis, thrombocytosis, leukocytosis, anemia, and muscle atrophy, and which, upon aging, develop marked increases in serum IL-6 levels and die early from renal failure [15]. Most of these disorders are identical with those in Castleman’s disease, so IL-6 tgm could be useful as a Castleman’s disease model to examine its pathological features and evaluate the therapeutic efficacy of drug candidates. I also prepared a rat anti-mouse IL-6R mAb to block the IL-6 signal in these IL-6 tgm, since the anti-IL-6R mAb used in clinic is human specific. This rat anti-mouse IL-6R mAb was selected based on the in vivo antagonism it showed to IL-6’s biological activity in two experimental models.

Next, the therapeutic potential of a recently identified anti-Hepatitis C virus (HCV) drug candidate is assessed in preclinical examinations. HCV is a major cause of morbidity, affecting approximately 170 million people worldwide [35]. In many cases, HCV results in a persistent infection that evades the host immune response, leading to chronic liver disease, chronic hepatitis, cirrhosis, and hepatocellular carcinoma [3]. The current therapy for chronic hepatitis C is a combination of weekly injections of pegylated interferon alfa-2a (PEG-IFN) and twice-daily oral
doses of ribavirin (RBV). Unfortunately, this combination therapy has limited efficacy and
significant side effects [7, 20]. Although HCV NS3/4A protease inhibitors, telaprevir and boceprevir,
have been approved to treat chronic HCV infection, these compounds must be combined with the
current standard of care to be efficacious, and there are some infected individuals, including
IFN-intolerant patients, that they cannot cure [11, 17, 28]. Therefore, antiviral combinations that can
achieve a superior sustained virologic response without the use of IFN or RBV are needed. IFN-free
combinations of direct-acting antiviral agents (DAAs) have been tested for clinical use as novel
anti-HCV therapies [8, 26, 38]. Emerging data suggest that DAAs, including NS3/4 serine protease
inhibitors, NS5B RNA-dependent RNA polymerase inhibitors, and NS5A inhibitors, can achieve
significant antiviral activity when used in combination, but might select for resistance, which can
become a primary cause of treatment failure in clinical studies, especially in difficult-to-treat HCV
genotypes [26, 38]. Additionally, differences in HCV genotypes can result in reduced antiviral
activity by certain DAAs and DAA combinations [9]. Therefore, developing additional antiviral
agents with diverse resistance profiles and efficacy against a wide spectrum of HCV genotypes is
necessary. Major efforts are underway to identify novel inhibitors and DAA combinations with a
high barrier to resistance for the treatment of HCV infection.

I have identified a novel class of serine palmitoyltransferase (SPT) inhibitors derived from
fungal metabolites that exhibited HCV replication-inhibiting activity [27]. HCV replication occurs
on host cell lipid rafts that form a scaffold for the HCV replication complex. Sphingolipids, the
downstream products of SPT action, are essential components of lipid rafts associated with HCV
nonstructural proteins on this microdomain. By preventing the de novo synthesis of sphingolipids, an
SPT inhibitor disrupts the HCV replication complex and thereby inhibits HCV replication. This
unique mechanism of host enzyme-targeted viral inhibition was hypothesized as having a high
barrier to resistance and antiviral activity across different HCV genotypes. I identified a novel
compound, NA808, which is a derivative of the previously described compound NA255 with further
improved properties, including improved replicon potency from a 50% effective concentration of 2
nM for NA255 to a 50% effective concentration of 0.84 nM for NA808 [27].

Preclinical research of HCV replication has been limited by the lack of appropriate
infection models. The only animal species readily infected with HCV has been the chimpanzee,
which is difficult to use because of critical ethical problems, the necessity of labor-intensive work,
and high cost. One model that has been reported as being possibly infected with HCV was produced
by transplanting human primary hepatocytes into severe combined immunodeficient (SCID) mice
carrying the urokinase plasminogen activator transgene controlled by an albumin promoter [21]. In
these mice, HCV replication occurs in reconstituted human hepatocytes at a clinically relevant titer
[14]. Therefore, it is considered that this humanized liver chimeric mouse is suitable for evaluating
the anti-HCV activity of NA808. In this examination, I attempt to assess the effectiveness of NA808
alone and in combination with DAAs in humanized liver chimeric mice infected with HCV genotypes 1a, 1b, 2a, 3a, and 4a to evaluate the therapeutic potential of NA808 as a novel host-targeted HCV inhibitor, including efficacy across HCV genotypes, the potential development of resistance, and synergistic effects with other antiviral agents.
Chapter 1:

The effects of an anti-IL-6R antibody on human Castleman’s disease-like symptoms in IL-6 transgenic mice
1. **Background and Aims**

Castleman’s disease is a syndrome associated with lymph node hyperplasia, plasmacytosis, fever, anemia, and hypergammaglobulinemia occurring when IL-6 is abnormally augmented [37]. Administering a mAb against IL-6 or IL-6R to neutralize the IL-6 function results in beneficial effects in patients with Castleman’s disease [5, 23].

The IL-6 tgm that I established develop various disorders, including massive IgG1 plasmacytosis, mesangial proliferative glomerulonephritis, thrombocytosis, leukocytosis, anemia, and muscle atrophy with dysregulated IL-6 overproduction. Most of these disorders are identical with those in Castleman’s disease, so IL-6 tgm could be useful as a model for Castleman’s disease [15].

In Chapter 1, I describe the effects of IL-6 signal-blocking agents in IL-6 tgm to confirm the benefits in patients with Castleman’s disease, and I estimate whether preclinical evaluation using an animal model that has been artificially accommodated to human disease can predict the clinical performance of a drug. To block the IL-6 signal transduction in mice, I prepared a rat anti-mouse IL-6R (mIL-6R) mAb with potent neutralizing activity of the IL-6 signal, since the anti-IL-6R mAb used in clinic is specific to humans. The rat anti-mIL-6R mAb was selected because it showed *in vivo* antagonism to IL-6’s biological activity in two experimental models.
2. Materials and Methods

Preparation of rat anti-mIL-6R mAbs

Rat anti-mIL-6R mAbs (MR3-2, MR17-2, MR16-1, and MR44-10) were prepared as described previously [32], the isotypes of these antibodies were IgG2a (MR3-2 and MR17-1), IgG1 (MR16-1), and IgG2b (MR44-10). Hybridomas recognizing mIL-6R were injected intraperitoneally into pristan-pretreated BALB/c nu/nu mice (Nippon Clea, Tokyo, Japan) to produce ascites. Rat anti-mIL-6R mAbs were purified from the ascites fluids with a protein G column (Oncogene Science Inc., Uniondale, NY, USA).

Evaluate in vivo neutralizing activity of anti-mIL-6R mAbs

The in vivo neutralizing activity of anti-mIL-6R mAbs against the effects of IL-6 was confirmed as follows.

IL-6-dependent cell growth in C.B-17<sup>scid</sup> mice with human IL-6 transgene (IL-6 SCID): A mouse-IL-6–dependent cell line, MH60.BSF2 (MH60), was inoculated subcutaneously (1 × 10<sup>6</sup> cells/mouse) into the left flank of IL-6 SCID mice [15]. One day after transplantation, the mice were injected subcutaneously with a single 0.5 mg/body dose of anti-mIL-6R mAb or control rat IgG (Cappel, Durham, NC, USA) followed by doses of 0.1 mg/body twice weekly from 4 to 29 days
after transplantation. Each group consisted of four mice. The tumor length and width were measured
to calculate tumor volume by the following formula:

\[
\text{tumor volume (mm}^3\text{)} = \text{length (mm)} \times \text{width (mm)}^2/2
\]

Production of antigen specific antibody: Male C3H/HeJ mice (Nippon Clea, Tokyo, Japan)
were injected with 0.1 mg of dinitrophenol-conjugated keyhole limpet hemocyanin (DNP-KLH)
intravenously, and implanted in the back with Alzet micro-osmotic pumps (model 1007D: Alzet
Corp., Palo Alto, CA, USA) loaded with recombinant human IL-6 (hIL-6) to deliver 6 \( \mu \)g of hIL-6
per day. These mice were injected with an anti-mIL-6R mAb or control rat IgG subcutaneously 1 day
after immunization, and serum anti-DNP specific antibody was detected by ELISA 7 days after
immunization using DNP-BSA and alkaline phosphatase conjugated goat anti-mouse IgG (ZYMED
Lab., South San Francisco, CA, USA). The serum from C3H/HeJ mice hyper-immunized with
DNP-KLH was used as a standard.

**Evaluating the effect of anti-IL-6R mAb in IL-6 tgm**

Male C57BL/6J hIL-6 tgm carrying hIL-6 cDNA fused with the H-2L\(^d\) promoter [15] were
used. These mice were maintained under specific-pathogen free conditions and fed standard
laboratory chow (CE-2, Nippon Clea, Tokyo, Japan) ad libitum. These mice were first injected with
a single dose of 2 mg/body of MR16-1 or an isotype-matched control rat IgG1 antibody (KH5)
intravenously at 4 weeks of age to induce tolerance to rat IgG in the mice, then subcutaneously with
0.1 mg/body twice weekly from 5 to 17 weeks of age. Antibodies against anti-rat IgG were not
detected in sera during the course of the study. From mice under anesthesia, peripheral blood
samples were collected from the retro-orbital sinus at 4, 8, 12, and 18 weeks of age; blood for serum
samples was collected from the inferior vena at 18 weeks of age. Left femur, kidney, spleen,
mesenteric lymph node, liver, lung, and heart were removed and fixed with 20% neutral buffered
formalin for histological examination. As control, normal littermate mice were used. Each group
consisted of 5 to 7 mice.

**hIL-6-specific ELISA**

The serum concentration of hIL-6 was determined by hIL-6-specific ELISA as described
in a previous report [21].

**Mouse IgG1-specific ELISA**

ELISA was performed utilizing affinity-purified goat anti-mouse IgG1 and alkaline
phosphatase conjugated affinity-purified rabbit anti-mouse IgG1 antibodies. Myeloma protein
(mouse monoclonal IgG1) was used as a standard. All reagents were purchased from ZYMED
Laboratories (South San Francisco, CA, USA).
Hematological evaluations of peripheral blood

Automated analysis was performed on a micro-cell counter (Sysmex® F-800, Toa Medical
Electronics, Kobe, Japan). The analysis included: white blood cell (WBC), red blood cell (RBC),
platelet counts, and hemoglobin levels.

Measurement of blood chemical parameters

Serum glucose (Glu), triglyceride (TG), total protein (TP), alkaline phosphatase (ALP),
albumin (Alb), blood urea nitrogen (BUN), calcium (Ca), creatinine (Cre), glutamic-oxaloacetic
transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were measured using a COBAS
FARA II automatic analyzer (Roche, Basel, Switzerland).

Statistical analysis

Statistical analysis of the data was performed by SPSS using the Student’s t-test.
3. Results

_In vivo_ neutralizing activity of anti-IL-6R mAbs

To validate the _in vivo_ inhibitory effect of anti-mIL-6R mAb upon IL-6 activity, one of four mAbs were each injected into MH60-bearing IL-6 SCID mice or C3H/HeJ mice immunized with DNP-KLH. The hIL-6 transgene accelerated the IL-6-dependent cell growth of MH60 (Fig. 1-1), but treatment with each of the four anti-mIL-6R mAbs inhibited it. After transplantation into SCID mice without the IL-6 transgene, the tumor nodules became palpable at 12.00 ± 3.6 (mean ± SD) days. After transplantation into IL-6 transgenic SCID mice, individually treated with either control rat IgG, MR3-2, MR17-2, MR16-1, and MR44-10, palpable tumor nodules were observed at 7.00 ± 1.6, 11.75 ± 2.1, 10.25 ± 2.5, 15.25 ± 2.5, and 13.75 ± 3.5 days, respectively. There were significant differences (P<0.01) between each anti-mIL-6R mAb-treated group and the control rat IgG-treated group. In C3H/HeJ mice immunized with DNP-KLH, the suppressive effect of each anti-mIL-6R mAb on exogenous IL-6-enhanced anti-DNP IgG production was observed (Fig. 1-2). In particular, MR16-1 blocked the effect of hIL-6 in a dose-dependent manner. Because MR16-1 antagonized the IL-6 activity most strongly in both _in vivo_ experimental models, it was selected for further examination.
Effect of MR16-1 on IgG1 plasmacytosis in IL-6 tgm

In KH5-treated IL-6 tgm, serum IgG1 levels increased markedly with aging, and the concentration amounted to over 100-fold more than that of normal littermates (Fig. 1-3a). Morphological examination revealed splenomegaly and an enlargement of the lymph nodes, with a large number of plasma cells (Fig. 1-4). Furthermore, plasma cell infiltration was abundant in liver and kidney. These mice also exhibited remarkable increases in serum hIL-6 concentration with aging (Fig. 1-3b). Treatment with MR16-1 completely prevented the increase in serum IgG1 levels and the development of splenomegaly; the average weight of spleens was $0.10 \pm 0.015 \text{ g}$ (mean $\pm$ SD) compared to those of the KH5-treated group ($0.93 \pm 0.28 \text{ g}$) and the appearance of the spleens was almost normal upon histological observation at the end of the experiment (Fig. 1-4b). The concentration of serum hIL-6 was as high as 200 pg/ml at the beginning of treatment, and the levels remained constant during the course of the study.

Suppression by MR16-1 of hematological abnormalities in IL-6 tgm

The KH5-treated IL-6 tgm had marked decreases in RBC counts and hemoglobin levels, and anemia became severe with aging (Fig. 1-5). The platelet count was markedly increased by 10 weeks of age compared with normal littermates, but subsequently underwent a dramatic decrease. In contrast, in IL-6 tgm treated with MR16-1, both anemia and thrombocytosis were mild and the RBC
count and hemoglobin levels remained almost constant during the course of the study.

**Suppression by MR16-1 of the abnormalities in blood chemical parameters in IL-6 tgm**

The serum levels of TG, Glu, ALP, and Alb significantly decreased, and the serum TP levels increased in KH5-treated IL-6 tgm (Table 1-1). These morbid phenomena were normalized by the treatment with MR16-1. There were no significant differences in GOT, GPT, BUN, Ca, or Cre between the MR16-1-treated and KH5-treated groups.
4. Discussion

In this examination, four anti-mouse IL-6R mAbs that neutralize the biological activity of IL-6 \textit{in vivo} were established. In particular, MR16-1 has the strongest activity and may effectively suppress endogenous IL-6 activity, because the levels of IL-6-dependent tumor growth or antigen-specific antibody production in mice treated with MR16-1 were lower than those in mice without the IL-6 transgene or an IL-6 infusion (Figs 1-1 and 1-2). When MR16-1 is administered to IL-6 tgm, various disorders associated with IL-6 overexpression are completely prevented. These observations demonstrate that MR16-1 is a potent inhibitor against the biological activity of IL-6 \textit{in vivo} and clearly confirm a significant role of IL-6 in the pathogenesis of a variety of disorders.

The critical role of IL-6 in Castleman’s disease has been reported [37]; that is, there is a strong correlation between serum IL-6 level and clinical abnormalities. In a patient with a solitary hyperplastic lymph node, a decrease in serum IL-6 level after surgical removal of the affected lymph node resulted in clinical improvement. In the present data, IL-6 tgm exhibit a variety of disorders, such as multiple plasmacytosis, hypergammaglobulinemia, anemia, lymphadenopathy, splenomegaly, and hypoalbuminemia. Furthermore, the expression of C-reactive protein and fibrinogen is reported as accelerated in IL-6 tgm [30]. These symptoms are quite similar to Castleman’s disease, and the severity is closely correlated with serum IL-6 level (Fig. 1-3b). Therefore, IL-6 tgm could be
regarded as a model of Castleman’s disease.

This examination indicated that treatment with anti-IL-6R mAb could block IL-6-mediated responses in vivo and completely eliminate various disorders in IL-6 tgm that are identical to those in Castleman’s disease, including inflammatory, immune, hematopoietic, and metabolic abnormalities. On the other hand, anti-IL-6R mAb showed no severe adverse effects, even when injected into non-transgenic littermate mice. These findings are consistent with the previous report by Nishimoto et al. indicating that administering anti-human IL-6R antibody improves various symptoms, such as anemia, hypoalbuminemia, hypergammaglobulinemia, and lymphadenopathy, and with the fact that treatment is well tolerated in Castleman’s disease patients [23]. Thus, the clinically proved efficacy of IL-6R mAb against Castleman’s disease could be reproduced in this assessment system. These results suggest that preclinical assessment using appropriate models that are clinically relevant might be capable of predicting clinical performance.
Figure 1-1. Tumor volume in MH60-bearing SCID IL-6 mice treated with anti-mIL-6R mAbs at 16 days after transplantation.

Cells ($1 \times 10^6$) were transplanted into the left flank of mice (n=4). Tumor volume was calculated as described in Materials and Methods. Data are presented as mean (±SD).
Figure 1-2. Anti-DNP antibodies in immunized C3H/HeJ mice.

Specific antibodies in sera were determined at 7 days after immunization by antigen-specific ELISA using hyper-immunized C3H/HeJ mice serum as a standard. C3H/HeJ mice were intravenously given 0.1 mg of DNP-KLH and implanted with hIL-6-loaded micro-osmotic pumps at 8 weeks of age, and anti-mIL6R mAbs were injected subcutaneously 1 day after immunization (n=5). Data are presented as the mean (±SD).
Figure 1-3. (a) Concentration of IgG1 in IL-6 tgm. Serum IgG1 levels were determined by mouse IgG1-specific ELISA using mouse myeloma protein as a standard. (b) Changes of serum hIL-6 levels in IL-6 tgm. Levels of hIL-6 were determined by hIL-6-specific ELISA. Data are presented as mean (± SE). n=7 for KH5-treated IL-6 tgm and n=6 for MR16-1-treated IL-6 tgm. (closed circle) KH5-treated IL-6 tgm: (open circle) MR16-1-treated IL-6 tgm.
Figure 1-4. Plasmacytosis in IL-6 tgm.
(a) Spleen weight of IL-6 tgm. Spleen weight was remarkably increased in IL-6 tgm treated with KH5. There were no significant differences in the MR16-1-treated IL-6 tgm or either littermate group. (b) Photomicrograph of spleen. Extensive proliferation of plasma cells in spleen was observed in IL-6 tgm treated with KH5, but not in IL-6 tgm treated with MR16-1. Original magnification $\times$200.
Figure 1-5. Changes of peripheral platelet counts (a), white blood cell counts (b), red blood cell counts (c), and hemoglobin levels (d) in IL-6 tgm. Hematological evaluations were performed on an automated micro-cell counter (Sysmex® F-800). Data are presented as the mean (±SE): (closed circle) KH5-treated IL-6 tgm: (open circle) MR16-1-treated IL-6 tgm: (closed square) KH5-treated littermates: (open square) MR16-1-treated littermates.
Table 1-1. Blood chemical parameters in MR16-1-treated IL-6 tgm at 18 weeks of age

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<th>TP (g/dl)</th>
<th>Alb (g/dl)</th>
<th>Glu (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>CRE (mg/dl)</th>
<th>BUN (mg/dl)</th>
<th>Ca (mg/dl)</th>
<th>ALP (IU/l)</th>
<th>GOT (IU/l)</th>
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<tr>
<td>KH5</td>
<td>mean</td>
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<td>77</td>
<td>20.7</td>
<td>0.40</td>
<td>24.8</td>
<td>8.60</td>
<td>27.7</td>
<td>33.3</td>
</tr>
<tr>
<td>(n=3)</td>
<td>S.D.</td>
<td>1.33</td>
<td>0.30</td>
<td>14.5</td>
<td>8.3</td>
<td>0.20</td>
<td>23.70</td>
<td>0.35</td>
<td>5.69</td>
<td>5.86</td>
</tr>
<tr>
<td>MR16-1</td>
<td>mean</td>
<td>5.7*</td>
<td>3.3*</td>
<td>199*</td>
<td>62.3*</td>
<td>0.51</td>
<td>28.3</td>
<td>8.67</td>
<td>156.5*</td>
<td>37.5</td>
</tr>
<tr>
<td>(n=6)</td>
<td>S.D.</td>
<td>0.30</td>
<td>0.20</td>
<td>29.5</td>
<td>10.9</td>
<td>0.22</td>
<td>4.08</td>
<td>0.58</td>
<td>14.31</td>
<td>8.22</td>
</tr>
<tr>
<td><strong>Littermates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KH5</td>
<td>mean</td>
<td>5.9</td>
<td>3.8</td>
<td>289</td>
<td>105.0</td>
<td>0.87</td>
<td>27.9</td>
<td>8.90</td>
<td>181.0</td>
<td>33.2</td>
</tr>
<tr>
<td>(n=5)</td>
<td>S.D.</td>
<td>0.65</td>
<td>0.46</td>
<td>98.9</td>
<td>28.8</td>
<td>0.22</td>
<td>6.32</td>
<td>0.74</td>
<td>21.24</td>
<td>8.90</td>
</tr>
<tr>
<td>MR16-1</td>
<td>mean</td>
<td>5.9</td>
<td>3.6</td>
<td>200</td>
<td>94.0</td>
<td>0.75</td>
<td>26.8</td>
<td>8.98</td>
<td>196.8</td>
<td>34.5</td>
</tr>
<tr>
<td>(n=6)</td>
<td>S.D.</td>
<td>0.53</td>
<td>0.34</td>
<td>25.5</td>
<td>20.0</td>
<td>0.20</td>
<td>5.26</td>
<td>0.82</td>
<td>21.68</td>
<td>4.89</td>
</tr>
</tbody>
</table>

Statistical significance of the difference was calculated by Student's t-test; *P<0.05 (KH5-treated IL-6 tgm vs MR16-1-treated IL-6 tgm).
Chapter 2:

Evaluation of the anti-HCV activity of a newly identified SPT inhibitor, NA808, \textit{in vitro}
1. Background and Aims

As described in the Introduction, the current treatment regimen for HCV infection is a combination therapy of PEG-IFN with RBV; however, this therapy has limited efficacy and is not well tolerated in many patients due to its systemic side-effect profile, even when combined with recently approved NS3/4 protease inhibitors [7, 20]. Therefore, the ultimate goal of developing a therapy for chronic hepatitis C is likely to combine HCV enzyme-targeting agents, without using IFN or RBV. Currently, combination therapies of DAAs, such as NS3/4 serine protease inhibitors, NS5B RNA-dependent RNA polymerase inhibitors, and NS5A inhibitors, are being tested in clinical trials; however, the emergence of resistance mutations limits the efficacy of these therapies [26, 38]. In addition, the antiviral activity of DAAs is reduced in certain HCV genotypes [9]. Additional antiviral agents with high barriers to resistance and potent antiviral activity against a wide variety of HCV genotypes are necessary to establish robust and effective antiviral combination therapy without the use of IFN or RBV.

The infection and replication process of HCV uses not only HCV proteins, but also several known and unknown host protein factors. Drugs that target host protein factors could provide therapies against HCV with a high barrier to resistance and with efficacy against a wide spectrum of HCV genotypes. I have identified a novel class of SPT inhibitors that inhibit HCV replication by
disrupting host cell lipid rafts, which act as a scaffold for HCV replication [27]. Based on the
mechanism of host enzyme-targeted viral inhibition, the newly identified SPT inhibitor, NA808, is
hypothesized to have potential for its high barrier to resistance and for antiviral activity across
different HCV genotypes. In this chapter, I describe the *in vitro* characteristics of NA808 to estimate
its potential for further investigation as an anti-HCV drug candidate.
2. Materials and Methods

Compounds

NA808 and telaprevir were synthesized by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan).

Inhibition assay of replication in HuH7 cells harboring HCV subgenomic replicon cells

The HCV subgenomic replicon cell FLR3-1 [27] (genotype 1b, Con-1) or R6 FLR-N [36] (genotype 1b, HCV-N) was seeded in 96-well tissue culture plates containing GlutaMax-I (DMEM-GlutaMax-I; Invitrogen, Carlsbad, CA, USA) with 5% FBS. After overnight incubation at 37°C (5% CO2), serial dilutions of the reagents were added to the growth medium. Luciferase activity was determined after 72 h with a Bright-Glo luciferase assay kit (Promega, Madison, WI, USA). The viability of the replicon cells was determined using a cell counting kit (Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions.

Anti-HCV assay of NA808 in HCVcc (JFH-1/K4 cells)

Cured K4 (HuH7-K4) cells that continuously produce HCV viral particles (JFH-1/K4) were maintained in DMEM containing 10% fetal calf serum. JFH-1/K4 cells were seeded onto
24-well tissue culture plates in DMEM supplemented with 10% fetal calf serum, and cultivated overnight at 37°C (5% CO2). Several concentrations of NA808 were added to the growth medium. After 72 hrs incubation, total RNA was extracted from the cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method. HCV RNA was quantified by real-time PCR, as described previously [31].

**Development of drug-resistant mutations in HCV subgenomic replicon cells**

The HCV subgenomic replicon cell line R6 FLR-N [36] (genotype 1b, HCV-N) was cultured with GlutaMax-I (DMEM GlutaMax-I; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum in the presence of 0.5 mg/mL G418 and 48 to 72 nM NA808 or 1.8 to 2.7 μM telaprevir at a concentration of 4 to 6 times the 50% inhibitory concentration (IC₅₀) value for 14 passages. For the replicon assay, cells were seeded in 96-well tissue culture plates, and a 3-fold gradual dilution of NA808 or telaprevir in GlutaMax-I supplemented with 5% fetal bovine serum was added. Serial dilutions of both compounds were prepared from the stock solutions dissolved in dimethyl sulfoxide at a concentration of 1 mM for NA808 and 50 mM for telaprevir. Luciferase activity was determined with a Steady-Glo luciferase assay kit (Promega, Madison, WI).

**Deep sequencing of HCV genomes from genotype 1b replicon cells**
Deep sequencing of the HCV coding sequences was performed using the GS Junior System (Roche Diagnostics, Mannheim, Germany), according to the manufacturer’s instructions. First, the acid guanidinium thiocyanate-phenol-chloroform extraction method was used to extract total RNA from the R6 FLR-N replicon cells after 14 passages with telaprevir or NA808 at a concentration of 6 times the IC_{50} value. Complementary DNA (cDNA) was then synthesized from the total RNA with random primers using Superscript III Reverse Transcriptase (Invitrogen, Life Technologies, Carlsbad, CA). The sequence of nucleotides 3429 to 9727 of the HCV genotype 1b replicon (R6NRz) genome, which includes all of the HCV protein coding sequence, was divided into several segments of 1.5 to 3 kb with overlapping regions. Four segments of the genotype 1b replicon genome were amplified from the cDNA by polymerase chain reaction (PCR) with specific primers (Table 2-1) using PrimeSTAR GXL DNA Polymerase (TaKaRa Bio, Shiga, Japan). The amplified segments of HCV cDNA were purified from 1% agarose gels using a MinElute Gel Extraction Kit (Qiagen, Valencia, CA) and quantified by measuring absorbance at 260 nm with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). The cDNA segments covering the coding sequence of HCV were then pooled together at approximately equimolar ratios. The Covaris S220 system (Covaris, Woburn, MA) was used to shear 500 ng of the pooled cDNA into 700- to 800-bp fragments. The sheared cDNA fragments were purified with the MinElute PCR Purification Kit (Qiagen), ligated with RL MID adaptors (Roche Diagnostics) to prepare the multiple cDNA libraries,
and further purified with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). The quality and quantity of the libraries were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the KAPA Library Quantification Kit (Nippon Genetics, Tokyo, Japan), respectively. The libraries were then subjected to emulsion PCR, and enriched DNA beads (approximately 10% recovery) were loaded onto a picotiter plate and pyrosequenced with a GS Junior sequencer using titanium chemistry (Roche Diagnostics). Several libraries derived from the HCV genomes generated by different treatments were sequenced in a single GS Junior run. The data obtained were analyzed by the GS Reference Mapper software (Roche Diagnostics) to identify resistant mutations.

**SPT assay**

Crude extracts of the HCV subgenomic replicon cell line FLR3-1 [27] (genotype 1b, Con-1) were used as a source of SPT in this assay. Briefly, FLR3-1 cells were suspended in HSS buffer (10 mM HEPES-KOH, 25 mM sucrose, and 0.1% sucrose monolaurate) containing 1/100 volume of protease inhibitor cocktail (Sigma, St Louis, MO) and sonicated 10 times with short pulses. After centrifugation at 10,000 rpm for 10 minutes, the supernatant was stored at -80°C until use. Crude extract of FLR3-1 cells was added to 0.015 mL of a reaction mixture containing 200 mM HEPES buffer (pH 8.0), 5 mM EDTA, 10 mM dithiothreitol, 0.05 mM pyridoxal 5-phosphate, 0.05
mM palmitoyl-CoA, and 0.06 mM L-[\(^{14}\)C]serine in the presence of NA808. After a 15-minute incubation at 37°C, 0.3 mL chloroform/methanol (1:2, v/v), 0.1 mL phosphate-buffered saline, and 0.1 mL chloroform were added and mixed well. The extracts were spotted on TLC plates and chromatographed. Radioactive spots were evaluated using a Bio-imager.

**Statistical analysis**

Statistical analysis was performed using the Student t test. A P value <.05 was considered statistically significant.
3. Results

**In vitro characteristics of NA808**

NA808 (Figure 2-1A), a derivative of NA255 isolated from fungal metabolites of Fusarium incarnatum F1476, demonstrated potent antiviral activity in HCV genotype 1b replicon cells with no apparent cellular toxicity under the assay conditions (Figure 2-2A) and decreased HCV propagation in genotype 2a HCVcc-producing cells (Figure 2-2C). NA255 is a selective inhibitor of SPT that inhibits HCV replication by suppressing the biosynthesis of sphingolipids that are required for HCV replication in replicon cells [27]. NA808 also inhibited the de novo synthesis of sphingolipids (Figure 2-2B). According to the Lineweaver-Burk plot of SPT inhibition in a replicon cell lysate, NA808 exhibited a noncompetitive inhibition pattern (Figure 2-1B). These findings suggest that NA808 inhibits HCV replication activity by preventing sphingolipid biosynthesis by a mechanism that inhibits SPT noncompetitively.

**NA808 shows a high barrier to resistance in vitro**

To evaluate the potential development of resistance to NA808, replicon cells (R6 FLR-N) were cultured in the presence of both G418 and NA808 at a concentration of 4 to 6 times the IC₅₀ for 14 passages. Obvious changes in sensitivity to NA808 were not observed in these continuously
treated replicon cells (Figure 2-2D), and the IC₅₀ values were 18.9 nM (no treatment), 14.3 nM (treatment with 4 times the IC₅₀), and 19.8 nM (treatment with 6 times the IC₅₀). In contrast, there was a 5- to 17-fold increase of the IC₅₀ values for telaprevir, an NS3/4 serine protease inhibitor, in replicon cells treated with 4 to 6 times the IC₅₀ of telaprevir for the same duration (Table 2-2). The coding sequences of NS3 to NS5B from the replicon system after 14 passages with telaprevir or NA808 were determined by using deep sequencing. The sequences obtained at the 14th passage with telaprevir contained 3 known protease inhibitor resistance mutations (V36A, T54V, and A156T) [10] and NS5 region (Q181H, P223S, and S417P) (Table 2-3), suggesting that the increase in IC₅₀ with telaprevir was accompanied by a shift in viral sequence. In contrast, no significant mutations were found in the 14th passage with NA808. Continuously treated replicon cells developed resistance to telaprevir, but not to NA808.
4. Discussion

In these experiments, the anti-HCV activity of NA808, a novel host SPT inhibitor, was evaluated \textit{in vitro}. The HCV replication in HCV genotype 1b subgenomic replicon cell lines, FLR3-1 and R6 FLR-N, were suppressed by NA808 in a dose-dependent manner with no cell toxicity (Fig. 2-2A). In addition, NA808 decreased HCV RNA in genotype 2a HCVcc producing cells in a dose-dependent manner (Fig. 2-2C). These findings indicate that NA808 suppresses both the genotype 1b HCV-RNA replication and genotype 2a HCV propagation \textit{in vitro}.

The inhibitory activity of NA808 is attributed to its inhibition of the cellular enzyme SPT, which is necessary for viral replication. The mode of action of NA255, a lead compound of NA808, is to disrupt the scaffold where the HCV replication complex forms on host cellular lipid rafts [27]. NA808 potently inhibits the de novo biosynthesis of cellular sphingolipids, such as ceramide and sphingomyelin, in a dose-dependent manner (Figure 2-2B). NA808 also influences sphingolipid metabolism in host cells, and the metabolism of sphingomyelin, a type of sphingolipid, plays a multifaceted role in the HCV life cycle [12]. Additionally, altering the sphingolipid metabolism contributes to virion maturation and infectivity in the JFH-1 culture system [1]. These results suggest that NA808 affects many phases of the life cycle, including entry, replication, and maturation.

The expected higher barrier of NA808 to developing resistant clones on the basis of its
host-targeted unique mode of action is well confirmed. Obvious changes in drug sensitivity to
NA808 are not observed in replicon cells treated continuously with NA808 (Table 2-2).

Deep-sequencing analysis also showed no evidence for the development of NA808 resistance after
14 passages in HCV replicon cells, while telaprevir treatment resulted in the selection of known
protease resistance mutations (V36A, T54V, and A156T) (Table 2-3).

These in vitro findings suggest that NA808 has potent anti-HCV activity across the HCV
genotypes with a high barrier to developing resistance.
Figure 2-1. Characteristics of NA808.

(A) Chemical structures of the compounds used in this study. (B) Scheme of de novo sphingolipid biosynthesis and Lineweaver-Burk plot of SPT assay results. Crude extract of FLR3-1 cells was incubated with L-[14C]serine in the presence of NA808. After incubation at 37°C, the extracts were spotted on TLC plates and chromatographed. 3-Ketodihydrosphingosine (3KDS) generated from a bacterial SPT reaction is shown as a positive control marker.
Figure 2-2. In vitro activities of NA808

(A) Luciferase activity (circles) and cell viability (triangles) of FLR3-1 or R6 FLR-N subgenomic replicon cell lines in the presence of NA808. The data represent the mean values and the bars indicate the standard deviation (S.D.) of triplicate determinations. (B) Inhibition of de novo sphingolipid synthesis by NA808. The replicon cells were cultured with NA808 for 48 h and then labeled with $[^{14}C]$serine for 18 h, and the newly synthesized lipids were analyzed by thin-layer chromatography (TLC). Cer, ceramide; SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine. (C) Cellular HCV-RNA levels (closed circles) and cell viability (open circles) of HCVcc-producing cells, named JFH-1/K4 cells, in the presence of NA808. (D) Activity on replicon cells cultured with NA808. Drug-resistant HCV replicons were selected in the presence of G418 and NA808 at a concentration of 4 to 6 times the IC$_{50}$. Changes in drug sensitivity were examined after the 14$^{th}$ passage. The data represent the mean values and the bars indicate the standard deviation (S.D.) of triplicate determinations.
Table 2-1. PCR primers used for deep sequencing of HCV genotype 1b from replicon cells

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6 FLR-N replicon</td>
<td></td>
</tr>
<tr>
<td>Segment 1</td>
<td>R6NRz-3429-S19 5’-AACAAGGGGCTGAAGGATG-3’</td>
</tr>
<tr>
<td></td>
<td>R6NRz-5782-R19 5’-AAAGCTCGCCACTTGACT-3’</td>
</tr>
<tr>
<td>Segment 2</td>
<td>R6NRz-4707-S19 5’-AAGTGCAGAGCTTGCCG-3’</td>
</tr>
<tr>
<td></td>
<td>R6NRz-7401-R19 5’TGTAATCCGGCCGTGCCCA-3’</td>
</tr>
<tr>
<td>Segment 3</td>
<td>R6NRz-6043-S19 5’TGGGAAGGTGCTATGGA-3’</td>
</tr>
<tr>
<td></td>
<td>R6NRz-9063-R21 5’-ATGGAATCATCAATCGCC-3’</td>
</tr>
<tr>
<td>Segment 4</td>
<td>R6NRz-7627-S20 5’TGGACGACCTCGGACAAC-3’</td>
</tr>
<tr>
<td></td>
<td>R6NRz-9727-R20 5’-TCGACTAGCGTGGACTAGG-3’</td>
</tr>
</tbody>
</table>

Data are indicated as the mean ± S.D.

Table 2-2. Changes in sensitivity to drug of HCV replicon cells after the 14th passage in the presence of NA808 or telaprevir

<table>
<thead>
<tr>
<th>Drug</th>
<th>No treatment</th>
<th>IC_{50}×4</th>
<th>IC_{50}×6</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA808 (nM)</td>
<td>18.9 ± 2.82</td>
<td>14.3 ± 5.52</td>
<td>19.8 ± 7.86</td>
</tr>
<tr>
<td>Telaprevir (μM)</td>
<td>0.39 ± 0.022</td>
<td>2.14 ± 0.019</td>
<td>6.48 ± 1.30</td>
</tr>
</tbody>
</table>

Data are indicated as the mean ± S.D.
Table 2-3. Summary of mutation frequency detected using deep sequencing for NA808- or telaprevir-treated replicon cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Treatment</th>
<th>Mutation</th>
<th>Region</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telaprevir</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;×6, 14 passages</td>
<td>V36A</td>
<td>NS3</td>
<td>18.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T54V</td>
<td>NS3</td>
<td>26.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A156T</td>
<td>NS3</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q181H</td>
<td>NS5A</td>
<td>25.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P223S</td>
<td>NS5A</td>
<td>23.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S417P</td>
<td>NS5A</td>
<td>15.8</td>
</tr>
<tr>
<td>NA808</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;×6, 14 passages</td>
<td>not detected</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nucleotide sequences based on deep sequencing of the NS3 to NS5B region from NA808- or telaprevir-treated replicon cells are compared to untreated controls, and amino acid mutations are shown.
Chapter 3:

Evaluation of anti-HCV activity of NA808 in humanized liver chimeric mice

infected with HCV
1. Background and Aims

In Chapter 2, I describe *in vitro* anti-HCV activity of NA808, which has potent anti-HCV activity on HCV-RNA replication in genotype 1b HCV subgenomic replicon cells and on HCV propagation in genotype 2a HCVcc with no evidence of developing resistance clones. These findings suggested that NA808 has potent anti-HCV activity across the HCV genotypes with a high barrier to developing resistance. Next, these beneficial profiles of NA808 as a novel drug candidate for anti-HCV treatment are confirmed *in vivo* to estimate its therapeutic potential in clinical practice.

Preclinical research on HCV replication has been limited by a lack of appropriate infection models. The chimpanzee is known as the only animal species to permit HCV infection; however, the experimental use of chimpanzees is difficult because of ethical problems. To overcome this problem, Mercer et al. developed a chimeric SCID mouse carrying the albumin promoter controlled transgene that activates urokinase plasminogen and containing primary human hepatocytes in which infection and replication of intact HCV occurs [22]. Mercer’s humanized liver chimeric mouse model was improved by Tateno et al. to have a higher substitution rate of human hepatocytes [33]. The use of this humanized liver chimeric mouse model is considered to bridge the gap between the replicon system and human liver and is suitable for evaluating the anti-HCV activity of NA808.
Here, the therapeutic potential of NA808 as an anti-HCV agent is assessed using HCV-infected humanized liver chimeric mice, in terms of efficacy across HCV genotypes, synergistic effects with other antiviral agents, and the potential development of resistance.
2. Materials and Methods

Compounds

NA808 and telaprevir were the same as in Chapter 2. PEG-IFN was purchased from Chugai Pharmaceutical Co., Ltd. Non-nucleoside polymerase inhibitor, HCV-796, and nucleoside polymerase inhibitor, RO-9187 [16], were synthesized by F. Hoffmann-La Roche Ltd. (Basel, Switzerland).

Infection of HCV genotypes 1a, 1b, 2a, 3a, and 4a in humanized liver chimeric mice

Chimeric mice were purchased from PhoenixBio 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 (uPA/SCID). HCG9 (genotype 1a, GenBank accession number AB520610), HCR6 (genotype 1b, AY045702), HCR24 (genotype 2a, AY746460), HCV-TYMM (genotype 3a, AB792683), and HCV genotype 4a/KM (genotype 4a, AB795432) were intravenously injected into the chimeric mice with humanized liver at $10^4$ (for HCR6, HCR24, HCV-TYMM, and HCV genotype 4a/KM) or $10^6$ (for HCR6 and HCG9) copies/mouse. After 4 weeks, the HCV RNA levels in the mice sera had reached approximately $10^8$ copies/mL for HCG9 and HCV-TYMM and approximately $10^7$ copies/mL for HCR6, HCR24, and
HCV genotype4a/KM. The protocols for animal experiments were approved by ethics committee at my institution. The animals received humane care according to National Institutes of Health guidelines. Patients gave written informed consent before blood or tissue samples were collected.

**Administration of NA808 and/or PEG-IFN, telaprevir, HCV-796, RO-9187 into HCV-infected humanized liver chimeric mice**

Treatment was started 12 weeks after HCV inoculation and continued for 14 days. Each treatment group contained at least 3 animals. NA808, PEG-IFN, RO-9187, HCV-796, and telaprevir were administered alone or in combination to chimeric mice infected with HCV genotype 1a (HCG9), genotype 1b (HCR6), genotype 2a (HCR24), genotype 3a (HCV-TYMM), or genotype 4a (HCV genotype4a/KM). Blood samples and liver samples were collected according to the protocols shown in Table 3-1. All DAAs were used at suboptimal doses to demonstrate the synergy when administered in combination therapy.

**Quantification of HCV RNA by real-time reverse transcription PCR**

Total RNA was purified from 1 μL chimeric mouse serum using SepaGene RV-R (Sanko Junyaku Co., Ltd., Tokyo, Japan) and total RNA was prepared from liver tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction method. HCV RNA was quantified by
quantitative real-time PCR using techniques reported previously [31]. This technique has a lower limit of detection of approximately 4000 copies/mL for serum. Therefore, all samples in which HCV RNA was undetectable were assigned this minimum value.

Deep sequencing of HCV genomes from genotype 1a-infected chimeric mice

The acid guanidinium thiocyanate-phenol-chloroform extraction method was used to extract total RNA from the liver tissue of HCV-infected chimeric mice that were treated with or without NA808 for 14 days. The sequence of nucleotides 325 to 9381 of the HCV genotype 1a (HCG9) genome, including all of the HCV protein coding sequence, was divided into several segments of 1.5 to 3 kb with overlapping regions. Seven segments of the genotype 1a (HCG9) genome were amplified from the cDNA by nested PCR with the indicated primers (Table 3-2). Other procedures were the same as Chapter 2.

Statistical Analysis

Statistical analysis was performed using the Student $t$ test. A $P$ value $<.05$ was considered statistically significant.
3. Results

Anti-HCV activity of NA808 in humanized liver chimeric mice infected with HCV

To evaluate the anti-HCV effect of NA808 in vivo, humanized liver chimeric mice infected with HCV genotype 1a (HCG9) or 1b (HCR6) were used. The mice were transgenic uPA/SCID mice with reconstituted human livers; this mouse model supports long-term HCV infections at clinically relevant titers [14]. NA808 was administered via intravenous injection according to the schedule shown in Table 3-1. In mice infected with HCV genotype 1a, NA808 (5 mg/kg/d) led to a rapid decrease in serum HCV-RNA (approximately a 2-log decrease within 14 days) (Figure 3-1A). A similar decrease in serum HCV-RNA occurred in mice infected with HCV genotype 1b that were treated with NA808 (5 mg/kg/d) (Figure 3-1C). NA808 also reduced hepatic HCV-RNA at the end of the treatment period in a dose-dependent manner (Figure 3-1B and D). These results indicate that NA808 has a robust antiviral effect in humanized liver chimeric mice infected with HCV genotype 1a or 1b. The most effective dose was 5 mg/kg/d in both genotype 1a- and genotype 1b-infected mice; therefore, this dose was used for further experiments.

To address whether NA808 had antiviral activity across HCV genotypes, chimeric mice infected with various strains of HCV were treated with 5 mg/kg of NA808 for 14 days, and then the HCV-RNA levels in sera were evaluated. Inoculation with several HCV strains, HCG9 (genotype 1a),
HCR6 (1b), HCR24 (2a), HCV-TYMM (3a), and HCVgenotype4a/KM(4a), resulted in HCV titers in the sera of mice of approximately $10^8$ (HCG9 and HCVTYMM) and $10^7$ (HCR6, HCR24, and HCVgenotype4a/KM) copies/mL, respectively (Figure 3-2). At 14 days after administration, NA808 treatment resulted in approximately 1- to 3-log reductions of serum HCV-RNA in each genotype-infected group (Figure 3-1E). Human serum albumin levels were not changed during the administration period, suggesting that the anti-HCV activity of NA808 against several genotypes occurred without any overt toxicity. NA808 was effective and well tolerated in chimeric mice with humanized liver infected with several genotypes of HCV.

**Deep sequencing of HCV genotype 1a from humanized liver chimeric mice**

Full-genome sequence analysis of HCV in the humanized liver chimeric mouse model after 14 days of NA808 administration was performed. The viral RNA was extracted from liver tissues of humanized liver mice, amplified using the primer sets shown in Table 3-2, and sequenced with the Roche/454 GS Junior sequencer using titanium chemistry. A total of 43,911 and 68,272 sequence reads for HCV genomes were obtained from untreated mice and from NA808-treated mice, respectively. The sequences were determined by comparing them with the HCG9 reference sequence (GenBank accession number AB520610), and, as a result, the viral sequences from NA808-treated mice were identical to those from untreated mice.
Synergistic effects of NA808 with PEG-IFN or DAAs in chimeric mice infected with HCV

The *in vivo* synergistic effects of NA808 combined with PEG-IFN on HCV replication were investigated using humanized liver chimeric mice infected with HCV genotypes 1a, 2a, and 4a. For 14 days, NA808 was administered intravenously with or without a subcutaneous injection of PEG-IFN. In mice infected with HCV genotype 1a, the combination therapy of NA808 with PEG-IFN led to a rapid decrease in serum HCV-RNA of about 4 log within 10 days (Figure 3-3A), and monotherapy with NA808 or PEG-IFN achieved about a 2-log or 1-log decrease, respectively (Figure 3-4A). The levels of serum HCV-RNA were also significantly reduced in genotype 2a- and 4a-infected chimeric mice that received the combination treatment (Figure 3-3A). Although sensitivity to the combination treatment varied according to HCV genotype (1a, 2a, or 4a), the serum HCV-RNA level eventually fell below the level of detection in all mice treated with the combination of NA808 and PEG-IFN (Figure 3-3A); this result was consistent with the significant reductions in hepatic HCV-RNA levels at day 14 (Figure 3-3B). To determine if NA808 has a synergistic effect with DAAs, combination treatment with NS5B nucleoside inhibitor, RO-9187 [16], NS5B polymerase non-nucleoside inhibitor, HCV-796, or NS3/4A protease inhibitor, telaprevir, in HCV genotype 1a- or 1b-infected chimeric mice was examined. Oral administration of once-daily 1000 mg/kg RO-9187, 100 mg/kg HCV-796, or 400 mg/kg telaprevir had only very limited or no apparent
effect on serum HCV-RNA levels during the 14 days of treatment (Figure 3-4B, C, and D). However, the combination therapy of NA808 with RO-9187, HCV-796, or telaprevir led to a decrease in serum HCV-RNA levels of about 2.6 log, 3.5 log, and 2.5 log, respectively, within 14 days (Figure 3-4B, C, and D); these reductions were all in excess of the viral load reduction achieved by treatment with NA808 (5 mg/kg) alone. These data suggest that NA808 has synergistic antiviral effects with HCV enzyme-targeted drugs in vivo, regardless of the targeted enzyme. The combination therapy of NA808 with telaprevir and HCV-796 resulted in up to 4.7-log reduction of serum HCV-RNA within 14 days (Figure 3-4D). At the end of the treatment, hepatic HCV-RNA levels were also reduced, correlating with the reduction of serum HCV-RNA (Figure 3-4E).
4. Discussion

In Chapter 2, the potent *in vitro* anti-HCV activity of NA808 across several HCV genotypes with no evidence of developing resistance clones is described. Following these findings, *in vivo* anti-HCV activity of NA808 is evaluated in human primary hepatocytes transplanted into *uPA/SCID* mice to assess the therapeutic potential of NA808 in clinical practice as an anti-HCV agent. These humanized liver chimeric mice infected with HCV genotypes 1a, 1b, 2a, 3a, and 4a could support long-term HCV infections at clinically relevant titers, hence these mice are considered a suitable model in which to evaluate the anti-HCV activity of NA808.

Based on the mechanism of action, NA808 could be anticipated to have antiviral activity against a wide variety of HCV genotypes and have a high barrier to developing resistance. These hypotheses are also confirmed *in vivo*; the humanized liver chimeric mice infected with HCV genotypes 1a, 1b, 2a, 3a, and 4a and treated with NA808 show significant reductions of serum HCV-RNA in each genotype-infected group (Figure 3-1E), and the full-genome sequences of HCV obtained from HCV-infected humanized liver chimeric mice treated with NA808 for 14 days show no evidence of selection of resistance mutations, which is consistent with the viral load kinetics (Figure 3-1A).
To estimate the effects of NA808 in DAA combination therapy without the use of IFN or RBV, a combination treatment of NA808 with NS5B polymerase inhibitors and/or NS3/4A protease inhibitors was evaluated in chimeric mice with humanized liver infected with HCV. Both non-nucleoside inhibitors and nucleoside inhibitors of NS5B are currently being tested in clinical trials, and the most advanced protease inhibitors, such as telaprevir, are linear compounds that are currently on the market, while several macrocyclic compounds are being tested in clinical trials [9, 18, 19, 29, 34]. Monotherapy with the non-nucleoside polymerase inhibitor, HCV-796, showed a reduction of less than 0.5 log in serum HCV RNA levels, while a combined treatment with NA808 reduced the HCV titer by 1000-fold from the initial serum levels. This effect was higher than the effect of NA808 alone and higher than the totaled effects of NA808 and HCV-796 monotherapies, suggesting a synergistic antiviral efficacy. A similar effect was observed by combining NA808 with nucleoside polymerase inhibitor, RO-9187, as shown in Figure 3-4B. The maximum reductions in HCV RNA are mediated by triple combination treatment and the significant in vivo anti-HCV activity with combination treatment is also observed when NA808 is combined with PEG-IFN. These observations suggest that NA808 may have synergistic antiviral activity with various classes of anti-HCV agents, regardless of their inhibition mechanism, because of its unique mechanism of action that targets the host enzyme. NA808 influences sphingolipid metabolism in host cells, which contributes to many phases of the HCV life cycle [1, 12]. It seems that these diverse working points
in the HCV life cycle along with the differentials of the target site could be a reason for the enhanced anti-HCV activity of combination treatment with NA808 and other anti-HCV agents.

Host enzyme inhibition is sometimes associated with mechanism-related toxicity or side effects. Although more thorough analyses of toxicity with NA808 are warranted, it was well-tolerated in vivo at the efficacious dose used. Besides, NA808 did not affect host cell viability in vitro under the assay conditions described in Chapter 2 (Figure 2-2A). Homozygous knockout mice for sptlc1 and sptlc2, subunits of SPT, were embryonic lethal; however, heterozygous mice showed no phenotype [13], and the short term deficiency of sptlc2 in conditional knockout mice induced no severe phenotypes except necrotic lesions in gastrointestinal cells [24]. Thus, inhibiting SPT would not result in severe adverse events except during fetal life and in the case of complete systemic elimination. In addition, the potential systemic toxicity associated with SPT inhibition by NA808 may be limited by the highly selective distribution of NA808 to the liver [25].

Thus, NA808 mediates potent anti-HCV activity in a variety of genotypes with an apparently high barrier to resistance. Synergistic effects with PEG-IFN, HCV protease, and/or polymerase inhibitors are observed in humanized liver chimeric mice infected with HCV. These findings suggest that NA808 has potential as a novel host-targeted drug in the treatment of HCV infection. NA808 is considered a promising candidate for DAA combination treatment that does not
use IFN or RBV and that prevents the development of drug resistance while effectively inhibiting a wide spectrum of HCV genotypes.
Figure 3-1. Antiviral effects of NA808 in various HCV genotypes.

(A, C) Time course of serum HCV-RNA levels in chimeric mice infected with HCV genotype 1a (A) or genotype 1b (C) treated with vehicle or several doses of NA808 (closed circles: 5 mg/kg/d; open circles: 3 mg/kg/d; closed triangles: 1.5 mg/kg/d; open triangles: vehicle). (B, D) HCV RNA levels in the livers of chimeric mice infected with HCV genotype 1a (B) or genotype 1b (D) at the end of treatment. Error bars = SD (*P < .05). (E) Time course of serum HCV-RNA levels in the sera of genotype 1a (closed circles), 1b (open circles), 2a (closed triangles), 3a (open triangle), and 4a (closed squares) after intravenous administration of 5 mg/kg/d NA808. Error bars = SD.
Figure 3-2. Time-course studies in chimeric mice inoculated with human HCV-positive sera of several HCV genotypes
HCV RNA levels in chimeric mouse serum after inoculation with HCV genotypes 1a (A), 1b (B), 2a (C), and 4a (D). Error bars indicate S.D.
Figure 3-3. **Antiviral effect of combination treatment of NA808 with PEG-IFN on HCV-infected chimeric mice.**

(A) Time course of serum HCV-RNA levels in chimeric mice infected with genotype 1a, 2a, or 4a and treated with a combination of NA808 (5 mg/kg/d, intravenously) and PEG-IFN (30 μg/kg/twice weekly, subcutaneously). HCV-RNA levels one day before administration are shown by black bars. (B) HCV-RNA levels in the liver 14 days after the initiation of combination therapy with NA808 and PEG-IFN. Error bars = SD. *P < .05; **P < .01.
Figure 3-4. Combination treatment of NA808 with PEG-IFN or direct-acting antiviral agents exhibits robust anti-HCV activity in humanized liver chimeric mice. (A-D) Median change from baseline of serum HCV RNA over the 14-day monotherapy or combination therapy of NA808 (5 mg/kg/d, intravenously) with PEG-IFN (A: 30 μg/kg/twice weekly, subcutaneously), RO-9187 (B: 1000 mg/kg/d, orally), HCV-796 (C: 100 mg/kg/d, orally), or telaprevir (400 mg/kg/d, orally) and/or HCV-796 (D). (E) HCV-RNA levels in livers of HCG9-infected chimeric mice at the end of each treatment. Error bars = SD. (*P < .05).
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Administration of reagents was started at day zero. Time point for collection of blood sample or sacrifice of livers is depicted as B or L in bold letters. Vehicles used in this study were as follows; 10% 2-hydroxypropyl-beta-cyclodextrin/10% polyethylene glycol 300 for NA808, saline for PEG-IFN, and 10% DMSO/10% Cremophor for RO-9187, HCV-796, and telaprevir. These three DAAs were used at suboptimal doses to demonstrate synergy when administered in combination therapies. NA808 (N) was intravenously injected at 5 mg/kg, PEG-IFN (I) was subcutaneously injected at 30 μg/kg, RO-9187 (R), telaprevir (T) and HCV-796 (H) were orally administered at 1000 mg/kg, 400 mg/kg, and 100 mg/kg, respectively. Telaprevir or NA808+telaprevir treatment was continued for up to 28 days, and then mice were sacrificed.
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**Primer Sequence**

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- HCG9-2434-R20: 5'-TCACGATGGTTCTGGTGGAG-3'
- HCG9-325-S22: 5'-CTCGTAGACCCTGACCATGAG-3'
- HCG9-2012-R24: 5'-TGAGTTTACATCCAGTGACACCGA-3'
- HCG9-143-S16: 5'-AGGCCGCACCCCTGCT-3'
- HCG9-3558-R21: 5'-AGGTTGCCAGGAAGGTCTGGG-3'
- HCG9-1153-S20: 5'-TCTGCTCGGCCCTCTATGTA-3'
- HCG9-3548-R19: 5'-GAAGGCTCTGGGCAGCAGTT-3'
- HCG9-2088-S20: 5'-ACTGACTGTITTCGGCAAGCA-3'
- HCG9-4705-R20: 5'-TTGCAGCTATCCACCCGACT-3'
- HCG9-2156-S20: 5'-CAGGTGCCTGTCCACTATCT-3'
- HCG9-4640-R19: 5'-GACAACATCGCCACTGTT-3'
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- HCG9-8832-R20: 5'-TGCTGGGAGGATGACCTA-3'
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Conclusion

In this study, I attempt to estimate the clinical performance of two drug candidates, an anti-IL-6R mAb for Castleman’s disease and a SPT inhibitor for chronic hepatitis C, from preclinical evaluations. Although the preclinical profiles of drug candidates must be clarified to estimate their clinical potential, the applicability of preclinical research to the clinical profiles varies a great deal depending on the availability of suitable experimental models. It is considered difficult to estimate the clinical potential of these two drug candidates from preclinical research by standard techniques, because of species specificity and the lack of appropriate models.

Castleman’s disease is a syndrome associated with lymph node hyperplasia, plasmacytosis, fever, anemia, and hypergammaglobulinemia, with an abnormal augmentation of IL-6 [37]. There were no suitable animal models to research Castleman’s disease before IL-6 tgm were established. IL-6 tgm develop various disorders, including massive IgG1 plasmacytosis, mesangial proliferative glomerulonephritis, thrombocytosis, leukocytosis, anemia, and muscle atrophy with dysregulated IL-6 overproduction [15]. Most of these disorders are identical with those in Castleman’s disease. These findings suggest that IL-6 tgm is a suitable Castleman’s disease model with matched pathogenesis and identical symptoms.
It has been reported that blocking the IL-6 signal transduction by administering anti-IL-6 or -IL-6R mAb to neutralize the IL-6 function results in beneficial effects in patients with Castleman’s disease [5, 23]. As described in Chapter 1, I attempt to confirm the clinical benefits of IL-6 signal-blocking agents in preclinical research by administering anti-IL-6R mAb to IL-6 tgm. For this purpose, I prepared a rat anti-mouse IL-6R mAb to block the IL-6 signal in IL-6 tgm, since the anti-IL-6R mAb used in clinic is specific to humans. Administering rat anti-mouse IL-6R mAb with potent IL-6 signal-neutralizing activity completely eliminates the various disorders in IL-6 tgm that are identical to those in Castleman’s disease. These findings are consistent with the previous report by Nishimoto et al. that indicates that administering anti-human IL-6R mAb improves various symptoms, such as anemia, hypoalbuminemia, hypergammaglobulinemia, and lymphadenopathy, in patients with Castleman’s disease [23]. This means I could confirm the effects of IL-6 signal-blocking agents in patients with Castleman’s disease in a preclinical experiment. These findings suggest that even in the difficult-to-estimate diseases the clinical performance of drug candidates could be estimated in preclinical evaluation when using appropriate models that are clinically relevant.

In Chapters 2 and 3, I describe the preclinical evaluation of NA808, a novel identified SPT inhibitor, as an anti-HCV agent. Development of new anti-HCV drugs has been significantly impeded by the lack of suitable infectious models both in vitro and in vivo. This obstacle has been
partially overcome by the development of replicon cells, which can be used for evaluating the *in vitro* anti-HCV effect of drug candidates. However, this system could reflect only RNA replication in the HCV life-cycle and has an adaptive mutation into the replicon genome that permits particularly efficient replication in cultured hepatoma cell lines [4]. Therefore, evaluating HCV drugs by the replicon system alone is considered insufficient for applying the data to patients. The only animal species readily infected with HCV is the chimpanzee, which is difficult to use because of critical ethical problems, the necessity of labor-intensive work, and the high cost. The chimeric mouse with human hepatocytes has recently been developed as a practical small animal model that can be infected with HCV [22]. As described in Chapter 3, I use improved humanized liver chimeric mice as developed by Tateno et al, which have a higher substitution rate of human hepatocytes than the initially developed chimeric mice by Mercer et al [33]. These mice infected with HCV genotypes 1a, 1b, 2a, 3a, and 4a could support long-term HCV infections at clinically relevant titers; hence, these mice are considered to bridge the gap between the replicon system and naive HCV replication in the human liver and to be a suitable model for evaluating the anti-HCV activity of NA808.

The IFN-based current standard therapies for HCV infection have limited efficacy and significant adverse effects even when combined with the recently approved NS3/4 protease inhibitors [7, 11, 17, 20, 28]. Combination therapies of HCV enzyme-targeting agents without the use of IFN are being tested in clinical trials; however, the emergence of resistance mutations limits the
efficacy of these therapies [26, 28]. In addition, the antiviral activity of these agents is reduced for certain HCV genotypes [9]. Therefore, antiviral agents with high barriers to resistance and potent antiviral activity against a wide variety of HCV genotypes are considered necessary to establish robust and effective anti-HCV therapies. The infection and replication process of HCV can use not only HCV proteins but also several known and unknown host protein factors. Drugs that target host protein factors could provide therapies against HCV with a high barrier to resistance and efficacy against a wide spectrum of HCV genotypes. Based on the mechanism of host enzyme-targeted viral inhibition, NA808 is hypothesized to have potential for a high barrier to resistance and for antiviral activity across different HCV genotypes. These expected profiles of NA808 have been observed in replicon cells and well confirmed in humanized liver chimeric mice infected with HCV. Besides, NA808 indicates synergistic antiviral activity with various classes of anti-HCV agents in HCV-infected humanized liver chimeric mice, regardless of their inhibition mechanism. These findings strongly suggest that NA808 is considered a promising drug candidate in the treatment of HCV infection with a high barrier to developing drug resistance, activity against a wide spectrum of HCV genotypes, and synergy with other anti-HCV drugs.

In conclusion, the results in the Castleman’s disease model treated with anti-IL-6R mAb suggest that preclinical assessment might be capable of predicting the clinical performance, when using appropriate models with clinical relevance. In HCV-infected humanized liver chimeric mice,
viral replication occurred in human hepatocytes, and long-term infection could be supported at a clinically relevant titer; hence, these mice are considered a suitable model with clinical relevance for evaluating anti-HCV drug candidates. Therefore, beneficial profiles of NA808 as an anti-HCV drug in HCV-infected humanized liver chimeric mice could reflect the drug’s clinical potential and support the development of NA808 as a treatment for HCV infection or for use in combination with PEG-IFN or HCV polymerase or with protease inhibitors.
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