Discovery of Anti–Claudin-1 Antibodies as Candidate Therapeutics against Hepatitis C Virus

Mayo Yamashita, Manami Iida, Minoru Tada, Yoshitaka Shirasago, Masayoshi Fukasawa, Shotaro Nagase, Akihiro Watari, Akiko Ishii-Watabe, Kiyohito Yagi, and Masuo Kondoh

Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan (M.Y., M.I., S.N., A.W., K.Y., M.K.); Division of Biological Chemistry and Biologicals, National Institutes of Health Sciences, Tokyo, Japan (M.T., A.I.-W.); Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo, Japan (Y.S., M.F.); and Graduate School of Biological Science, Tokyo University of Science, Chiba, Japan (Y.S.)

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ABSTRACT
Claudin-1 (CLDN1), a known host factor for hepatitis C virus (HCV) entry and cell-to-cell transmission, is a target molecule for inhibiting HCV infection. We previously developed four clones of mouse anti-CLDN1 monoclonal antibody (mAb) that prevented HCV infection in vitro. Two of these mAbs showed the highest antiviral activity. Here, we optimized the anti-CLDN1 mAbs as candidates for therapeutics by protein engineering. Although Fab fragments of the mAbs prevented in vitro HCV infection, their inhibitory effects were much weaker than those of the whole mAbs. In contrast, human chimeric IgG1 mAbs generated by grafting the variable domains of the mouse mAb light and heavy chains inhibited in vitro HCV infection as efficiently as the parental mouse mAbs. However, the chimeric IgG1 mAbs activated Fcγ receptor, suggesting that cytotoxicity against mAb-bound CLDN1-expressing cells occurred through the induction of antibody-dependent cellular cytotoxicity (ADCC). To avoid ADCC-induced side effects, we prepared human chimeric IgG4 mAbs. The chimeric IgG4 mAbs did not activate Fcγ receptor or induce ADCC, but they prevented in vitro HCV infection as efficiently as did the parental mouse mAbs. These findings indicate that the IgG4 form of human chimeric anti-CLDN1 mAb may be a candidate molecule for clinically applicable HCV therapy.

Introduction
One hundred and seventy million people worldwide are chronically infected with hepatitis C virus (HCV). HCV is a leading cause of liver cirrhosis and hepatocellular carcinoma, and overcoming HCV is an important healthcare issue (Murray and Rice, 2011; El-Serag, 2012). Anti-HCV agents are classified into direct-acting antiviral agents (DAAs) and host-targeting agents (HTAs). Some DAAs, such as protease inhibitors and RNA polymerase inhibitors, have been approved and are used clinically (Scheel and Rice, 2013). However, the poor proofreading ability of HCV RNA polymerase leads to the appearance of viruses resistant to DAAs in patients (Sarrazin et al., 2012). In contrast, HTAs are expected to have high genetic barriers and the potential to overcome the development of DAA-resistant viruses (Flisiak et al., 2009; Li et al., 2011; Scheel and Rice, 2013). Host factors involved in HCV genome replication and the entry of HCV into hepatocytes are potent targets for HTAs. For example, cyclophilin and miR-122 are host factors that are involved in the replication of the HCV genome in hepatocytes. Cyclosporine A and miraviren, inhibitors of cyclophilin and miR-122, respectively, prevent in vitro and in vivo HCV replication (Paeshuyse et al., 2006; Hopkins et al., 2010; Lanford et al., 2010). However, a clinical study of cyclosporine A revealed acute pancreatitis in some participants, and the injection of miraviren into chimpanzees led to reductions in serum cholesterol (Flisiak et al., 2009; Lanford et al., 2010). Thus, HTAs with patient safety and high genetic barriers to drug-resistance remain to be developed.

A host factor involved in HCV entry is an attractive target for a novel HTA. A series of HCV studies revealed that heparan sulfate (Barth et al., 2006), CD81 (Pileri et al., 1998), scavenger receptor class B type I (SR-BI) (Scarselli et al., 2002), claudin-1 (CLDN1) (Evans et al., 2007), ocludin (Ploss et al., 2009), and Niemann-Pick C1-like 1 cholesterol-absorption receptor (Sainz et al., 2012) are responsible for HCV entry. Of these factors, CLDN1 is considered to be the most potent target because...
CLDN1 is involved in both HCV entry into host cells via interaction with CD81 and cell-to-cell HCV transmission (Timpe et al., 2008; Harris et al., 2010). Moreover, CLDN1-targeted HTA showed pan-genotypic inhibition of HCV (genotypes 1–6) entry (Fofana et al., 2010), indicating that CLDN1 may offer a high genetic barrier.

We previously developed mouse anti-CLDN1 monoclonal antibodies (mAbs) by using a novel method for immunization and screening of antibodies. Several of the resulting clones prevented in vivo HCV infection without unwanted side effects, such as hepatotoxicity, loss of body weight, and apparent abnormality (Nagase et al., 2013; Iida et al., 2014; Kondoh et al., 2014; Fukasawa et al., 2015). We thus provided proof-of-concept for the use of anti-CLDN1 mAbs as CLDN1-targeted HTAs. In the current study, to improve the clinical applicability of anti-CLDN1 mAbs, we used them to create Fab fragments, human chimeric IgG1 mAbs, and human chimeric IgG4 mAbs, and we investigated the effects of these products on HCV infection.

Materials and Methods

Cells. Human CLDN-expressing HT1080 cells (Li et al., 2014) were cultured in Dulbecco's modified Eagle's medium that contained 10% fetal bovine serum (referred to as serum-containing medium). Human embryonic kidney 293T cells, human hepatic Huh7.5.1-8 cells, and S7-A cells (Huh7.5.1-8-derived CLDN1-deficient cells) were cultured in serum-containing medium supplemented with 0.1 M nonessential amino acids, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate (referred to as normal medium). Suspension-adapted FreeStyle CHO-S cells (Life Technologies, Carlsbad, CA) were cultured in FreeStyle CHO Expression Medium (Life Technologies). Human peripheral blood mononuclear cells were obtained from Cell Technology (Cleveland, OH).

Mouse Anti-CLDN1 mAbs and Fab Fragments. Mouse anti-CLDN1 mAbs were purified from ascitic fluid produced in BALB/c mice that had been inoculated intraperitoneally with hybridomas by using immobilized protein G columns. The purified mAbs were dialyzed against phosphate-buffered saline, and the protein concentration was determined by measuring absorbance at 280 nm. The purity of the mAbs was confirmed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue. Mouse IgG (Beckman Coulter, Brea, CA) was used as a control IgG.

Purified mouse anti-CLDN1 mAbs were digested with papain by using the Fab Preparation kit (Pierce Chemical, Rockford, IL) according to the manufacturer’s protocol. The purity of the Fab fragments was checked by using SDS-PAGE with Coomassie Brilliant Blue staining. Normal mouse IgG Fab fragment (Alpha Diagnostic Intl Inc., San Antonio, TX) was used as a control Fab.

Flow Cytometric Analysis. For analysis of mAbs and Fab fragments binding to CLDN-expressing HT1080 cells, Huh7.5.1-8 cells and S7-A cells, cells were incubated in the presence or absence of mAbs or Fab fragments (5 μg/ml), followed by treatment with fluorescein-conjugated goat anti-mouse IgG (for mouse mAbs), goat anti-mouse IgG (Fab) (for Fab fragments of mouse mAb), or goat anti-human IgG (for human chimeric IgG1 and IgG4). The bound cells were analyzed by using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Inhibition Assay for In Vitro Cell Culture-Derived HCV Infection. Cell culture-derived HCV (HCVcc)-JFH1 was prepared from the conditioned medium of Huh7.5.1-8 cells as described previously (Murakami et al., 2009). Huh7.5.1-8 cells (5 × 10^4 cells/well) were seeded in 48-well plates and cultured for 24 hours at 37°C. The cells were treated with mAbs or Fab fragments at the various concentrations for 30 minutes at room temperature, and then HCVcc was added to the wells. After an additional 2 hour culture at room temperature, the cells were washed with fresh normal medium and cultured in normal medium in the presence or absence of each mAb or Fab fragment for 4 days at 37°C. Total RNA was extracted from the cells and culture supernatant. To measure HCV RNA in the total RNA fractions, real-time quantitative reverse-transcription polymerase chain reaction (PCR) assays for HCV were performed by using RNA-direct Realtime PCR Master Mix (Toyobo Co. Ltd., Osaka, Japan) as described previously (Murakami et al., 2009).

Inhibition Assay for In Vitro HCV Pseudoparticle Infection. HCV pseudoparticles (HCVpp) were generated as described previously (Murakami et al., 2013). Briefly, a Gag/Pol packaging construct (Gag/Pol 5349), a transfer vector construct (Luc 126), and a glycoprotein-expressing construct (HCV E1 and E2; JFH1, genotype 2a; TH, genotype 1b) (Murakami et al., 2009) were transduced into 293T cells (Logvinoff et al., 2004; Wakita et al., 2005). The medium from the transfected cell cultures was collected and used as the HCVpp stock. For the infection assay, Huh7.5.1-8 cells (5 × 10^4 cells/well) were seeded in 48-well plates and cultured for 24 hours at 37°C. Cells were treated with mAbs or Fab fragments at the various concentrations for 30 minutes at room temperature, and then HCVpp was added to the wells. The cells were cultured for an additional 6 hours at 37°C. The cells were then washed with fresh normal medium and cultured in the presence or absence of each mAb or Fab fragment for 2 days. The cells were lysed, and the luciferase activity of the resulting cellular lysates was measured by using a commercially available luciferase reporter assay kit (PicaGene, Toyo Ink Manufacturing, Tokyo, Japan) according to the manufacturer’s instructions.

Activation of Fc Receptor. To measure the activation of FcγRIIa by antigen-bound mAbs, Jurkat/FcγRIIa/nuclear factor of activated T cells/Luc cells were used as the effector cell as described previously (Tada et al., 2014). CLDN1-expressing HT1080 cells (1 × 10^4 cells/well) were seeded in 96-well plates and cultured for 24 hours at 37°C. Then, Jurkat/FcγRIIa/nuclear factor of activated T cells/Luc cells (1 × 10^5 cells/well) were added to the wells in the presence or absence of mAbs at the indicated concentrations. After 5 hours of incubation at 37°C, luciferase activities were measured by using an EnSpire Multimode Plate Reader (PerkinElmer, Downers Grove, IL) and the ONE-Glo Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s protocol.

Human Chimeric IgG mAbs. cDNA encoding the heavy- and light-chain variable domains of CLDN1 mAb clones 2C1 and 3A2 (Nagase et al., 2013; Iida et al., 2014; Kondoh et al., 2014; Fukasawa et al., 2015) were amplified by PCR, and the PCR products were

![Fig. 1. Claudin specificity of Fab fragment. Huh7.5.1-8 cells (CLDN1-expressing) or S7-A cells (CLDN1-deficient cells derived from Huh7.5.1-8 cells) were incubated with Fab fragments (5 μg/ml), and then treated with fluorescein isothiocyanate–conjugated goat anti-mouse IgG (VH + VL). Solid and open histograms represent vehicle- and Fab-treated cells, respectively.](image-url)
subcloned into pFUSE-CHIg-hG1 (for IgG1), pFUSE-CHIg-hG4 (for IgG4), and pFUSE2-CLIG-hK vectors (InVivoGen, San Diego, CA). To prepare a human chimeric IgG4 mutant, we substituted the codon of serine at position 228 [EU numbering scheme (Edelman et al., 1969)] in pFUSE-CHIg-hG4 with proline, resulting in pFUSE-CHIg-hG4 mutant.

cDNA encoding the heavy-chain region of clones 2C1 and 3A2 was subcloned into the pFUSE-CHIg-hG4 mutant.

Human chimeric mAbs were prepared by using the FreeStyle MAX CHO Expression System (Life Technologies). Briefly, CHO-S cells were cotransfected with pFUSE-CHIgs and pFUSE-CLIg of 2C1 or 3A2 by using the FreeStyle MAX Reagent, and then the transfected cells were cultured for 6 days in FreeStyle CHO Expression Medium. The conditioned medium was recovered and applied to a protein G column. The column was washed with 20 mM sodium phosphate buffer (pH 6.8), and the mAbs were eluted with 0.1 M glycine-HCl (pH 3.0). The eluted fraction containing mAbs was neutralized with 1 M Tris-HCl (pH 8.0), followed by desalting using a PD-10 column (GE Healthcare, Cleveland, OH) and phosphate-buffered saline as the exchange solvent. The concentration of the purified antibody was determined by measuring the absorbance at 280 nm.

Statistical Analysis. Data were analyzed by the Student’s t test. The significant difference was set at P < 0.05.

Results

Effects of Fab Fragments on HCV Entry. We previously created four clones of mouse anti-CLDN1 mAbs, among which two clones (2C1 and 3A2) showed the highest preventive activity for HCV infection (Nagase et al., 2013; Iida et al., 2014; Kondoh et al., 2014; Fukasawa et al., 2015). In this study, we therefore optimized mAbs 2C1 and 3A2 toward clinical applications. We first digested these mAbs with papain because the resulting Fab fragments have improved tissue penetration and no activation of effector systems (Stockwin and Holmes, 2003).

Like the parental mAbs, the corresponding Fab fragments bound to CLDN1-expressing cells but not to those that expressed CLDN2, 3, 4, 6, 7, or 9 (Supplemental Fig. 1). To confirm the specificity of the Fab fragments, we investigated their interaction with Huh7.5.1-8 cells and the CLDN1-deficient S7-A cell line derived from those cells. The Fab fragments bound to Huh7.5.1-8 cells but not to S7-A cells (Fig. 1). Therefore, Fab fragmentation of the mAbs did not affect their CLDN1-specific recognition.

Although treatment of Huh7.5.1-8 cells with the parental mAb [25 μg/ml (0.17 μM)] completely prevented infection of HCVcc, Fab fragments 2C1 and 3A2 at 25 μg/ml (0.5 μM) attenuated HCVcc infection by only 51% and 75% of control antibody-treated infection levels, respectively (Fig. 2A). To clarify the mode of action of Fabs, we investigated their effect on HCV infection by surrogate HCV entry assay using HCVpp (genotypes 1b and 2a). Treatment of cells with the intact parental mAb [0.17 μM] prevented infection of HCVpp (1b and 2a) to less than 5% of the control level (Fig. 2, B and C). However, Fab fragments of 2C1 (0.5 μM) reduced HCVpp entry to 46% (genotype 1b) and 49% (genotype 2a) of the level observed after control Fab treatments (Fig. 2B). 3A2 Fab fragments (0.5 μM) did not attenuate HCVpp (1b) entry.
The chimeric mAbs (2C1 and 3A2) did not activate complement-dependent cytotoxicity (Supplemental Fig. 5). Activation of Fcγ receptor IIIa through the receptor IIIa was activated by the chimeric mAbs using Jurkat/FcγRIIIa/nuclear factor of activated T cells/Luc cells, in which luciferase expression was associated with activation of Fcγ receptor IIIa (Houot et al., 2011; Tada et al., 2014). The chimeric mAbs activated Fcγ receptor IIIa through the interaction of the chimeric IgG1 mAbs with CLDN1-expressing cells, suggesting induction of ADCC by natural killer cells against CLDN1-expressing cells (Fig. 5).

**Effects of Human–Mouse Chimeric IgG4 on HCV Entry.**

To avoid the adverse effects due to inflammation via ADCC, we selected IgG4 among IgG subclasses because IgG4 is reportedly a poor trigger of complement-dependent cytotoxicity and ADCC (Stockwin and Holmes, 2003). One problem associated with IgG4 is that bispecific antibodies arise frequently in vivo, due to the ease in which monovalent portions containing one heavy chain and one light chain can be exchanged among IgG4 molecules (Aalberse and Schuurman, 2002). Substitution of the serine at position 228 [EU numbering scheme (Edelman et al., 1969)] with proline can avoid the formation of half-monoclonal IgG4 molecules (Aalberse and Schuurman, 2002). We prepared both human chimeric IgG4 and IgG4 mutants (Ser228Pro) of 2C1 and 3A2 by grafting the heavy and light chains of the variable regions.
Denaturing SDS-PAGE analysis showed that IgG half-molecules occurred with the IgG4 constructs but not the IgG4 mutants (Fig. 6A), indicating that the IgG4 mutant is more stable than IgG4. Both the IgG4 and IgG4 mutant versions of 2C1 and 3A2 retained specific binding to CLDN1 (Supplemental Fig. 6, A and B). The IgG4 and IgG4 mutant constructs also bound to Huh7.5.1-8 cells but not to S7-A cells (data not shown) (Fig. 6B). Together, these results indicate that the IgG4 and IgG4 mutant versions of 2C1 and 3A2 retained CLDN-binding specificity.

Moreover, IgG4 and mutant IgG4 constructs of 2C1 and 3A2 mAbs dose-dependently decreased HCVcc levels in the cells (Fig. 7A; Supplemental Fig. 7). We also confirmed the effects of the IgG4 and IgG4 mutant mAbs on infection of HCVpp (1b and 2a). As expected in light of the data from the HCVcc analysis, the IgG4 and IgG4 mutant versions of 2C1 and 3A2 effectively prevented HCVpp infection in a dose-dependent manner (data not shown) (Fig. 7B). We further investigated whether the IgG4 and IgG4 mutant mAbs activated Fcγ receptor IIIa, potentially leading to undesirable inflammation. The human chimeric IgG4 and IgG4 mutant versions of 2C1 and 3A2 mAbs dose-dependently decreased HCVcc levels in the cells (Fig. 7A; Supplemental Fig. 7). These findings indicate that conversion of anti-CLDN1 mAbs 2C1 and 3A2 to the IgG4 subclass enables the resulting reagents to retain the inhibitory activity of the parental mouse mAbs on HCV infection and potentially reduces the side effects due to activation of Fcγ receptor IIIa, thereby avoiding ADCC against mAb-bound cells.

**Discussion**

Previously, we developed mouse anti-CLDN1 mAbs that prevented in vitro and in vivo HCV infection (Nagase et al., 2013; Iida et al., 2014; Kondoh et al., 2014; Fukasawa et al., 2015). We used parental mouse mAbs, their Fab fragments, and human chimeric IgG1, human chimeric IgG4, and human chimeric IgG4 mutant constructs as lead compounds to optimize for the development of CLDN1-targeted HTAs. In general, Fab fragments have better tissue penetration than do full-length mAbs (Stockwin and Holmes, 2003), however, Fab fragments (molecular mass ~50 kDa) offer less steric interference of the binding of virus to cellular receptors than do intact IgG molecules (molecular mass ~150 kDa). We found that the Fab fragments of our mouse mAbs had much weaker inhibitory activity against HCV infection than did the parental mAbs. In addition, the affinity to CLDN1 also may be weaker than that of the intact mAbs because Fab fragments are monovalent. Furthermore, in other virus models, Fab fragments neutralized viral infection by a different mechanism from that used by the full-length IgG (McInerney et al., 1997; Edwards and Dimmock, 2000, 2001). A detailed analysis addressing this difference between Fab fragments and the intact mAbs is needed. Taken together, our findings indicate that Fab fragmentation of anti-CLDN1 mAbs is unsuitable for future clinical application.

In contrast to the Fab fragments, the human chimeric IgG1 and IgG4 forms of the anti-CLDN1 mAbs were as effective against HCV infection as were the parental mouse mAbs. Hepatotoxicity is an exacerbating factor for hepatitis C, and therefore the induction of ADCC by therapeutic Abs has to be avoided. The human chimeric IgG1 form of our mAbs activated Fcγ receptor IIIa, indicating the activation of ADCC to the mAb-bound cells. However, the human chimeric IgG1...
form activated ADCC and the human chimeric IgG4 forms did not (Supplemental Fig. 8). One potential drawback of the IgG4 form is that it frequently exchanges IgG half-molecules, resulting in the generation of bispecific IgG4 molecules in plasma (Neergaard et al., 2014). Therefore, we created a mutant human chimeric IgG4 construct by substituting serine at position 228 with proline; this point mutation generates inter-H-chain binding in the hinge region, resulting in the formation of stable divalent IgG4 (Aalberse and Schuurman, 2002). The mutant form of human chimeric IgG4 mAbs retained the full anti-HCV activity of the parental mouse mAbs without activation of Fcγ receptor IIIa and subsequent ADCC against hepatocytes.

The concepts of HTAs have been proven in human-liver-chimeric mice by using antibodies against for CD81, SR-BI, and CLDN1 (Meuleman et al., 2008; Timpe et al., 2008; Iida et al., 2014; Kondoh et al., 2014; Fukasawa et al., 2015). CD81 is a widely expressed cell-surface protein that is involved in signal transduction and cell adhesion in the immune system (Levy et al., 1998). SR-BI is a receptor highly expressed in hepatocytes and steroidogenic tissues and is associated with the metabolism of high-density lipoproteins (Krieger, 2001). Therefore, HCV binds to CD81 or SR-BI, which are localized in the cellular surface but also play biologic functions in the liver. CLDN1 is embedded in the tight junctions (TJs) between adjacent cells and therefore plays a role in the TJ seal, preventing the free movement of solutes between the apical and basolateral sides of epithelial cell sheets (Furuse and Tsukita, 2006). In the liver, CLDN1 is predominantly expressed at the apical bile canalicular membrane in normal liver tissue, consistent with its function in the TJ seal. However, HCV infection perturbs the TJ seal, leading to basolateral translocation of CLDN1 (Reynolds et al., 2008). Therefore, antibodies against CLDN1 may interact with the cell-surface CLDN1 that is involved in HCV entry, without modulation of the CLDN1 embedded in TJ seals. Therefore, CLDN1 is a safe and effective target for the development of HTAs. Indeed, treatment of cells with 2C1 or 3A2 did not perturb TJ integrity. 2C1 and 3A2 also inhibited HCV infection of human liver chimeric mice without adverse effects (Iida et al., 2014; Kondoh et al., 2014; Fukasawa et al., 2015).

In summary, we optimized mouse anti-CLDN1 mAbs toward the clinical anti-HCV application by using antibody engineering to successfully create human chimeric IgG4 mAb constructs. The chimeric IgG4 mutant forms prevented HCV infection as well as did the parental mAbs. Moreover, the chimeric IgG4 mAbs did not induce ADCC against mAb-bound cells. Therefore, the chimeric IgG4 forms may be potential therapeutic Abs for the development of HTAs for HCV therapy.

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Authorship Contributions

Participated in research design: Yamashita, Iida, Tada, Fukasawa, Kondoh.

Conducted experiments: Yamashita, Iida, Shirasago.

Contributed new reagents or analytic tools: Tada, Shirasago, Nagase, Watari, Ishii-Watabe, Fukasawa.


Wrote or contributed to the writing of the manuscript: Yamashita, Tada, Ishii-Watabe, Fukasawa, Kondoh.

References


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Fig. 7. Effects of chimeric IgG4 mutant mAbs on HCV infection. (A) HCVcc assay. Huh7.5.1-8 cells were treated with or without chimeric IgG4 mutant at the indicated concentrations as described in the legend of Fig. 2A. The cellular HCV RNA contents were quantified by real-time quantitative reverse-transcription PCR and expressed as percentages of the control values. Data are presented as mean ± S.D. (*n* = 4). *Significant difference between mouse mAb and chimeric IgG4 mutant (*P* < 0.05). (B) HCVpp assay. Huh7.5.1-8 cells were treated with or without chimeric mAb at the indicated concentrations as described in the legends of Fig. 2B. Cellular luciferase activities were measured and expressed as percentages of the control values. Data are presented as mean ± S.D. (*n* = 4). *Significant difference between mouse mAb and chimeric IgG4 mutant (*P* < 0.05).

Fig. 8. Effects of chimeric Abs on the activation of Fcγ receptor IIIa.

The cellular HTAs for HCV therapy.

In summary, we optimized mouse anti-CLDN1 mAbs toward the clinical anti-HCV application by using antibody engineering to successfully create human chimeric IgG4 mAb constructs. The chimeric IgG4 mutant forms prevented HCV infection as well as did the parental mAbs. Moreover, the chimeric IgG4 mAbs did not induce ADCC against mAb-bound cells. Therefore, the chimeric IgG4 forms may be potential therapeutic Abs for the development of HTAs for HCV therapy.


