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## Amphipathic DNA Polymers Inhibit Hepatitis C Virus Infection by Blocking Viral Entry

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### Abstract

**BACKGROUND & AIMS**—Hepatitis C virus (HCV) gains entry into susceptible cells by interacting with cell surface receptor(s). Viral entry is an attractive target for antiviral development because of the highly conserved mechanism.

**METHODS**—HCV culture systems were used to study the effects of phosphorothioate oligonucleotides (PS-ONs), as amphipathic DNA polymers (APs), on HCV infection. The in vivo effects of APs were tested in urokinase plasminogen activator (uPA)/severe combined immunodeficient (SCID) mice engrafted with human hepatocytes.

**RESULTS**—We show the sequence-independent inhibitory effects of APs on HCV infection. APs were shown to potently inhibit HCV infection at submicromolar concentrations. APs exhibited a size-dependent antiviral activity and were equally active against HCV pseudoparticles of various genotypes. Control phosphodiester oligonucleotide (PO-ON) polymer without the amphipathic structure was inactive. APs had no effect on viral replication in the HCV replicon system or binding of HCV to cells but inhibited viral internalization, indicating that the target of inhibition is at the postbinding, cell entry step. In uPA/SCID mice engrafted with human hepatocytes, APs efficiently blocked de novo HCV infection.

**CONCLUSIONS**—Our results demonstrate that APs are a novel class of antiviral compounds that hold promise as a drug to inhibit HCV entry.

Hepatitis C virus (HCV) infects approximately 200 million people worldwide.<sup>1</sup> The majority of HCV-infected patients fails to clear the virus, and many develop chronic liver disease including cirrhosis with a risk of hepatocellular carcinoma. Treatment of chronic hepatitis C

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Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: 10.1053/j.gastro.2009.04.048.

Conflicts of interest

The authors disclose the following: J.-M.J. and A.V. are employees of REPLICor, Inc. The remaining authors disclose no conflicts.

is currently based on peginterferon-alfa and ribavirin, which is accompanied by substantial adverse effects and is only effective in approximately half of the patients.<sup>2,3</sup> In addition to other viral targets, viral entry is an attractive target for antiviral development because of the potentially conserved mechanism of viral entry.<sup>4</sup> Although several candidate receptors for HCV have been identified,<sup>5–10</sup> the mechanism of HCV entry still remains largely unknown. Previous reports have indicated a pH dependency for entry of HCV pseudoparticles (HCVpp) as well as cell culture-generated HCV (HCVcc), suggesting that HCV enters cells by receptor-mediated endocytosis.<sup>7,11,12</sup> Antiviral compounds targeting the entry step of viral infection have been successfully developed in other viral infections.<sup>13</sup> Recent studies have shown that phosphorothioate oligonucleotides (PS-ONs), as amphipathic DNA polymers (APs), have a sequence-independent anti-viral activity against human immunodeficiency virus type 1 (HIV-1) as entry inhibitors.<sup>14</sup> The antiviral effect of APs appears to be specific to the phosphorothioate backbone, which confers an amphipathic structure, because the phosphodiester oligonucleotides (PO-ONs) as nonamphipathic polymers are ineffective.<sup>14</sup>

## Materials and Methods

### Cell Culture and Oligonucleotide Synthesis

Huh7.5 (provided by Charles Rice), Huh7.5.1 (provided by Francis Chisari), Huh7, and Hep3B cells were maintained at 37°C, 5% CO<sub>2</sub> in Dulbecco's modified Eagle medium, containing 10% fetal bovine serum. All PS-ONs and PO-ONs were synthesized as described previously.<sup>14</sup> Oligonucleotides lacking the phosphorothioate modification (PO-ONs) were synthesized with the addition of 2'-O-methyl ribose modification, which stabilizes oligonucleotides from nuclease degradation.<sup>14</sup> Compounds used in the in vivo experiment were synthesized under good manufacturing practice (GMP) conditions to yield high-purity sodium salts.

### HCV Infection and Replication Assays

The production of cell culture-generated HCV JFH-1 (HCVcc) and HCV pseudovirus (HCVpp) has been reported previously<sup>5,15</sup> and is described in detail in the Supporting Document. HCVpp harboring E1/E2 glyco-proteins from genotypes 1a, 1b, 2a, 3a, 4a, 5a, and 6a were described previously.<sup>16</sup> For viral internalization assay, Hep3B cells were incubated for 1 hour at 4°C to allow binding of HCVpp (pHCV7a) to cells, washed repeatedly with phosphate-buffered saline to remove unbound virus, and treated with concanamycin A (Sigma–Aldrich, St. Louis, MO) (25 nmol/L), Anti-E2 AP33 antibody<sup>17</sup> (25 µg/mL), PS-ON (100 nmol/L), or PO-ON (100 nmol/L) overnight at 37°C for viral entry. The efficiency of infection was measured by luciferase assay 24 hours later. Transient assay of genotypes 1b (Con-1) and 2a (JFH-1) subgenomic reporter replicons have been reported previously<sup>18,19</sup> and are described in detail in the Supporting Document.

### HCV Binding and Fusion Assays

The HCV-like particle (LP) binding assay was performed at 4°C for 1 hour in 100 µL of TNC (50 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, 1 mmol/L CaCl<sub>2</sub>) buffer containing 1% bovine serum albumin as reported previously<sup>20</sup> and is described in detail in the Supporting Document. Both Hep3B and Huh7.5 cells were tested. Direct binding of PS-ON or PO-ON to HCV-LP was measured by a plate-binding assay and is described in the Supporting Document. For viral fusion assay, HCVpp/lipo-some lipid mixing assays with rhodamine-labelled liposomes were performed as previously reported<sup>21</sup> and are described in the Supporting Document.

### HCV Infection in Chimeric Mice

Human hepatocyte-transplanted mice generated in severe combined immunodeficient (SCID)/urokinase plasminogen activator (uPA) mice were purchased from PhenixBio (Hiroshima,

Japan).<sup>22</sup> These uPA/SCID mice stably transplanted with human hepatocytes were treated intraperitoneally with 10 mg/kg of poly C PS-ON or poly AC PS-ON (40mer) on days -1, 0, 1, 3, 5, and 7. Control poly C PO-ON (40mer stabilized by 2'-O-methyl ribose modification) was also tested. A fourth group of mice did not receive any compounds (only normal saline administration). Approximately 5–15 mice were included in each group. The mice were intravenously inoculated on day 0 with HCV patient serum containing  $3.9 \times 10^3$  copies of HCV genotype 1b. Serum samples were obtained on days 0 (prior to HCV inoculation), 7, 14, 21, 28, and 35 for HCV RNA, HCV core antigen, and human albumin determination. Human albumin in the blood of the chimeric mice was measured with the Alb-II Kit (Eiken Chemical, Tokyo, Japan).

### Statistical Analysis

Data from at least triplicate experiments were averaged and expressed as means  $\pm$  standard deviations. Statistical analysis was performed using the Student *t* test or Welch *t* test. *P* values of less than .05 were considered statistically significant.

## Results

### APs Inhibit HCV Infection in a Sequence-Independent Manner

To assess whether APs can inhibit HCV infection, fully degenerate 40mer oligonucleotides that were either phosphorothioated (PS-ON) resulting in a stable amphipathic DNA polymer or that had a 2'-O-methyl modification on the ribose moiety (PO-ON) conferring stability but not altering the polyanionic nature of DNA<sup>14,23</sup> were tested. Huh7.5 cells were infected with HCVcc in the presence of either PS-ON or PO-ON. At 72 hours postinfection, HCV-infected cells were assessed by immunofluorescence assay (Figure 1A) and intracellular HCV RNA quantification (Figure 1B). HCV infection was significantly inhibited by PS-ON and not PO-ON ( $P < .05$ ). The inhibitory effect of PS-ON was also confirmed by reduced HCV core antigen and HCV RNA levels in the culture supernatant, as compared with those of the PO-ON-treated cells ( $P < .05$ ) (Figure 1C and D). To evaluate further the efficacy of PS-ON against viral entry, HCVpp harboring genotype 1b was used to infect Hep3B. The PS-ON blocked infection of HCVpp in a similar dose-dependent manner (Figure 1E). The PO-ON exhibited some inhibitory effect at high concentration, which could be attributed to noncytotoxic inhibition of cellular adherence by the polyanion nature of PO-ON. To assess whether the PS-ON inhibitory effect is specific for HCV, retroviral pseudovirus carrying the vesicular stomatitis virus G protein (VSVGpp) was tested in the presence of PS-ON or PO-ON. Neither PS-ON nor PO-ON had any effect on VSVGpp infection (Figure 1F). Furthermore, adenoviral infection was not inhibited by PS-ON (Supplementary Figure 1).

A series of homo- and heteropolymeric APs including poly G, A, T, C, TG AC, TC, and AG PS-ONs were tested for their inhibitory activities on HCV infection in both HCVcc and the HCVpp systems. These APs had similar inhibitory activities as the degenerate PS-ON with random sequence in the HCVcc system except for poly G and poly A (Figure 2A). Similar effects were also observed on HCV core antigen and HCV RNA levels in the culture supernatant (Figure 2B and C). In the HCVpp system, these PS-ONs also had similar inhibitory effects (Figure 2D).

### AP Inhibition of HCV Infection Is Dependent on Size and Amphipathicity

Different sizes of degenerate PS-ONs (6-, 10-, 20-, 30-, 40-, 50- and 80mer) were tested for their inhibitory activities in the HCVcc and HCVpp systems. Only PS-ONs with lengths of 40mer or greater potentially inhibited HCV infection (Figure 2E). This result was confirmed with the poly C PS-ONs (Supplementary Figure 2). To determine the requirement of amphipathicity for antiviral activity of these compounds, additional oligonucleotide analogs that had

diminished hydrophilic character were prepared and include degenerate PS-ON analogs with either the base and/or the sugar removed (Supplementary Figure 3). An additional degenerate PS-ON analog containing the 2'-O-methyl ribose modification that does not affect the amphipathicity was tested. These analogs were tested for their inhibitory activities in the HCVcc and HCVpp systems. Only analogs that retained the amphipathic properties inhibited HCV infection (Figure 2F). These observations suggest that the amphipathic nature of these PS-ONs is necessary for inhibiting HCV infection.

### APs Inhibit Infection of Various Genotypes of HCV Without Affecting Replication and Cell Attachment

To study the effects of APs on various HCV genotypes, HCVpp harboring E1/E2 glycoproteins from genotypes 1a, 1b, 2a, 3a, 4a, 5a, and 6a were tested.<sup>16</sup> Infections by all genotypes were equally blocked by the degenerate PS-ON, whereas the degenerate PO-ON had no effect (Figure 3A). Similar observation was obtained with the poly C compounds (Supplementary Figure 2D).

The degenerate PS-ON compound was tested for its effect on viral replication in the HCV replicon system, which supports viral replication without the viral entry step. Genotype 1b and 2a subgenomic replicons were tested. Subgenomic replicon RNAs containing luciferase reporter were transfected into Huh7.5 cells, and the replication efficiency was determined in the presence of the PS-ON or PO-ON control. Neither PS-ON nor PO-ON displayed any antiviral activities in both subgenomic replicon systems (Figure 3B). To eliminate the possibility that PS-ON may induce an antiviral state with increasing time of exposure to cells, the HCV replicon assay was performed after exposure to either PS-ON or PO-ON for 24–48 hours, and no difference in replication was observed (data not shown). Furthermore, Huh7.5 cells treated with PS-ON or PO-ON did not produce any detectable levels of type I interferons.

To dissect further the effect of PS-ON on viral entry, we applied the HCV-LP binding assay, which has been developed as a surrogate system to assess HCV binding to cells.<sup>24–26</sup> HCV-LP were incubated in the presence of PS-ON and PO-ON for 1 hour at 4°C with Huh7.5 or Hep3B cells. Under this condition, virus attaches to the cells but does not enter. HCV patients' serum containing high-level of anti-E1/E2 antibodies was included as a control. The binding was detected with FITC-labeled mouse monoclonal anti-E2 antibodies (Figure 3C). The results showed that the anti-HCV antibodies inhibited the HCV-LP binding to the cells, whereas the PS-ON and PO-ON-treated HCV-LP did not inhibit HCV-LP binding. To validate the HCV-LP binding assay, HCVcc binding to cells was performed in the presence of PS-ON, PO-ON, or HCV serum. HCV RNA bound to the cells was quantified to determine the percentage of binding. As shown in Figure 3D, HCV antibody significantly inhibited HCVcc binding to cells (~80%), whereas PS-ON and PO-ON had minor effects (<20%). These results suggest that the target of inhibition by APs is at the postbinding, cell entry step.

To address the question of whether PS-ON binds to HCV directly to inhibit HCV infection, HCV binding assays were performed. First, in an immunoassay format using HCV-LP as a capture antigen, neither PS-ON nor PO-ON showed any significant binding to HCV-LP (Table 1). Second, sedimentation density gradient analysis did not show a preferential cosedimentation of HCVcc with PS-ON or PO-ON in comparison with the control preparation (Figure 3E), indicating that neither PS-ON nor PO-ON binds to HCVcc to any significant extent. The amount of PS-ON in the HCVcc or the control fraction was higher than that of PO-ON, probably reflecting the different physical properties of PS-ON and PO-ON. However, it is possible that low-affinity binding of HCV and PS-ON could be present and required for subsequent inhibitory action but not detected by the currently applied assays.

### APs Inhibit Viral Internalization

To determine which entry step that APs targets, the HCVpp assay was performed in the presence of concanamycin A (25 nmol/L), degenerate PS-ON (100 nmol/L), degenerate PO-ON (100 nmol/L), or AP33+ALP98 monoclonal anti-E2 antibodies (25  $\mu$ g/mL total concentration) at 37°C. Hep3B cells were first incubated with HCVpp at 4°C to allow binding and then at 37°C with various compounds after the inoculating HCVpp was removed. Concanamycin A is known to inhibit HCV entry by preventing acidification of endosome.<sup>12</sup> As shown in Figure 4A, AP33+ALP98 anti-E2 antibodies blocked HCV binding to the cells but had no effect on HCV entry. On the other hand, both concanamycin A and the degenerate PS-ON inhibited HCV entry.

To demonstrate that APs may inhibit HCV internalization at the fusion step, a viral fusion assay was performed with HCVpp or VSVpp as control.<sup>21</sup> Degenerate sequence and poly C PS-ONs and the control PO-ONs were tested. Both PS-ON compounds showed significant inhibition of HCVpp fusion over their control PO-ON, whereas VSVGpp fusion was largely unaffected by either PS-ON or PO-ON (Figure 4B and Supplementary Figure 4). The inhibitory effect of PS-ON on fusion was evident on both the rate and maximum of fusion in the assay.

### APs Inhibit HCV Infection In Vivo

To test the efficiency of APs in vivo, sodium salts of amphipathic polymers (40mers) of poly C and poly AC and their respective PO-ON controls were prepared. Degenerate oligonucleotides were avoided because they might potentially contain CpG motifs, which could induce endogenous interferons, although in vitro testing did not reveal such a possibility. Human hepatocyte-transplanted uPA/SCID mice were treated with these compounds as described and inoculated with infectious HCV genotype 1b patient serum. In this model, the production of human albumin in serum was monitored for the engraftment index of human hepatocytes. All mice showed robust and comparable human albumin concentrations that did not change significantly during the experimental period (Figure 5). Only 1 animal in the poly C PS-ON-treated group (n = 7) and 2 in the poly AC PS-ON-treated group (n = 5) were HCV positive. The remaining mice in both groups of mice were persistently negative. All 7 mice in the poly C PO-ON-treated mice (100%) and 14 of 15 untreated mice (normal saline placebo) were HCV positive (93%). The *P* value was statistically significance between the PS-ON- and PO-ON-treated groups (*P* < .05). To rule out the possibility that these protected mice were not intrinsically resistant to HCV infection despite robust human hepatocytes engraftment, some of them were rechallenged with infectious HCV inoculum several weeks later. They all became infected, supporting the specific inhibitory effect of APs on de novo HCV infection in this in vivo model.

### Discussion

Current therapy for hepatitis C is based on peginterferon and ribavirin. However, the therapy is only effective in approximately half of the patients, and there is little option to those who fail current therapy. Recent advances in the development of small molecule inhibitors targeting the viral-encoded enzymes showed promise,<sup>27</sup> but viral resistance to these drugs is a major clinical issue because HCV is highly variable with rapid viral proliferation and low-fidelity replication. Phosphorothiate modification of oligonucleotides was initially designed to reduce enzymatic degradation. This modification also increases the hydrophobicity of the phosphodiester backbone and thus imparts an amphipathic character to the oligonucleotide polymer.<sup>28</sup> Recent studies showed that the amphipathic PS-ONs have a sequence-independent antiviral activity against HIV-1 and other viruses,<sup>14,29</sup> suggesting that these compounds may exhibit a broad-spectrum antiviral activity.



Our data showed that PS-ON blocked HCV infection in the HCVcc and HCVpp systems in a similarly dose-dependent manner, with 50% inhibitory concentration in the nanomolar range. PS-ON had no effect on infection of VSVGpp (an enveloped RNA virus with mechanism of viral entry distinct from type I and II fusion) or adeno-virus (a nonenveloped DNA virus). The amphipathic nature of PS-ON is crucial for its anti-HCV property because PS-ON analogs lacking the amphipathicity are inactive. Polynucleotides are polyanions, a class of compounds that have been shown to interfere with a variety of viral infections.<sup>30,31</sup> However, our data showed clearly that the polyanionic nature is not relevant to the PS-ON inhibitory activity because the control PO-ON is not active in HCV inhibition. Furthermore, the inhibitory effect of PS-ON could not be explained by the increased stability of the phosphorothioation because the control PO-ON has the 2'-O-methyl modification that also stabilizes the oligonucleotides.

The inhibitory activity of APs is sequence independent but length dependent. The degenerate APs were equally effective as the homo- and heteropolymeric sequences, with the exception of poly A and G, which can form unique polypurine quartet structures in solution.<sup>32</sup> The minimal length of PS-ON required for potent inhibitory activities is 40mer, which appears to be the same for all active PS-ON compounds. This length-specific requirement may indicate a critical structural feature of the HCV entry process that is susceptible to these compounds. Although the degenerate PS-ON may contain CpG motif, the other hetero- and homopolymer PS-ONs tested herein are devoid of CpG motifs. The comparable anti-viral activity of these compounds to the degenerate PS-ON demonstrates that the antiviral activity is not mediated by the potential CpG-mediated induction of interferon. Furthermore, Huh7.5 cells express very little or none of the cell surface toll-like receptors involved in recognition of nucleic acid-based motifs,<sup>33</sup> and we did not observe any production of endogenous type I inter-ferons in cells exposed to either PS-ON or PO-ON.

The inhibitory activity of PS-ON appears to target the postbinding and prereplication stage and possibly at the fusion step of HCV infection. The fusion process appears to be structurally conserved among many enveloped viruses and can be classified into types I and II.<sup>34</sup> The type I membrane fusion is exemplified by the influenza and HIV-1 via hemagglutinin and gp41, respectively. The type II fusion includes the alpha-viruses and flaviviruses.<sup>34–36</sup> It has been proposed that HCV uses a type II fusion process because of its similarity to flaviviruses.<sup>37</sup> Our recent study suggests that HCV and flaviviruses are indeed structurally similar.<sup>38</sup> It is conceivable that the fusion process of HCV may be susceptible to inhibition by the amphipathic structure of PS-ON, but further confirmation is necessary. HCV entry has been shown to occur via receptor-mediated endocytosis and is sensitive to lysosomotropic agents and inhibitors of vacuolar ATPases.<sup>39</sup> The finding that PS-ON acts at the postbinding step like concanamycin A and bafilomycin A1, which are potent inhibitors of the vacuolar ATPases, supports this hypothesis. Furthermore, all HCV genotypes appeared to be susceptible to the APs equally, suggesting that the process involved is highly conserved.

HCV entry involves multiple cellular factors, such as CD81, SR-B1, Claudin-1, heparin sulfate, DC-SIGN, and L-SIGN, and possibly LDL receptor.<sup>5–10,31</sup> CD81 and Claudin-1 have been postulated to act on the postbinding step.<sup>6,40</sup> SR-B1 is likely involved in an early viral entry step to the cells. Its interaction with apolipoproteins and cholesterol transfer property appear to be important for viral entry,<sup>41</sup> possibly at the level of membrane fusion.<sup>42</sup> The overall mechanism of HCV entry is complex and involves multiple factors and steps. The APs likely interact with 1 of these essential steps to abort HCV entry. The unique inhibitory effect of the APs on HCV infection makes it a valuable reagent to study the molecular pathway of HCV entry. The APs can also be developed as a molecular probe to image and dissect biochemically this complex process.

Our study demonstrates that APs are potent inhibitors of HCV infection. APs are equally effective against all HCV genotypes and can inhibit de novo HCV infection in the human hepatocyte-transplanted uPA/SCID mouse model. This approach has the advantage of a novel and highly conserved target mechanism that is distinct from the small molecule inhibitors being developed clinically as well as the well-established pharmacology of antisense-based nucleic acid molecules in clinical trials. The effectiveness of this class of compounds in blocking de novo HCV infection supports its value in liver transplantation to prevent reinfection, which occurs invariably and presents a major problem for the management of these patients.<sup>43</sup> So far, prophylactic reagents based on neutralizing antibodies have been disappointing in clinical trials of liver transplantation.<sup>44</sup> Our studies illustrate the promise of this class of compounds as a potent antiviral against HCV and support its further development in the therapy of hepatitis C.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations used in this paper

APs	amphipathic DNA polymers
HCV	hepatitis C virus
HCVcc	cell culture-generated HCV
HCVpp	HCV pseudoparticles
VSVGpp	vesicular stomatitis virus G protein pseudo-particle
PO-ON	phosphodiester oligonucleotide
PS-ONs	phosphorothioate oligonucleotides

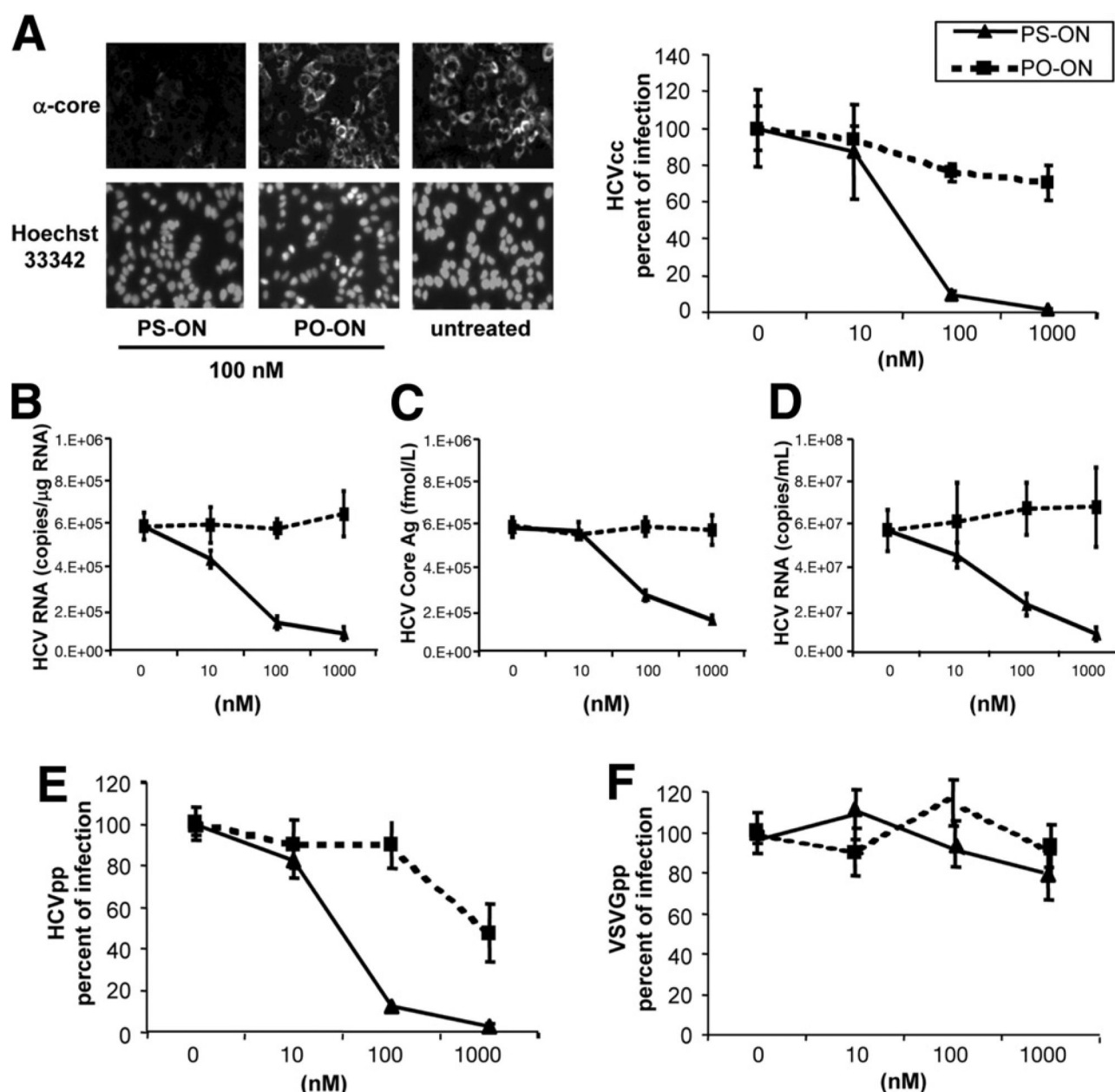
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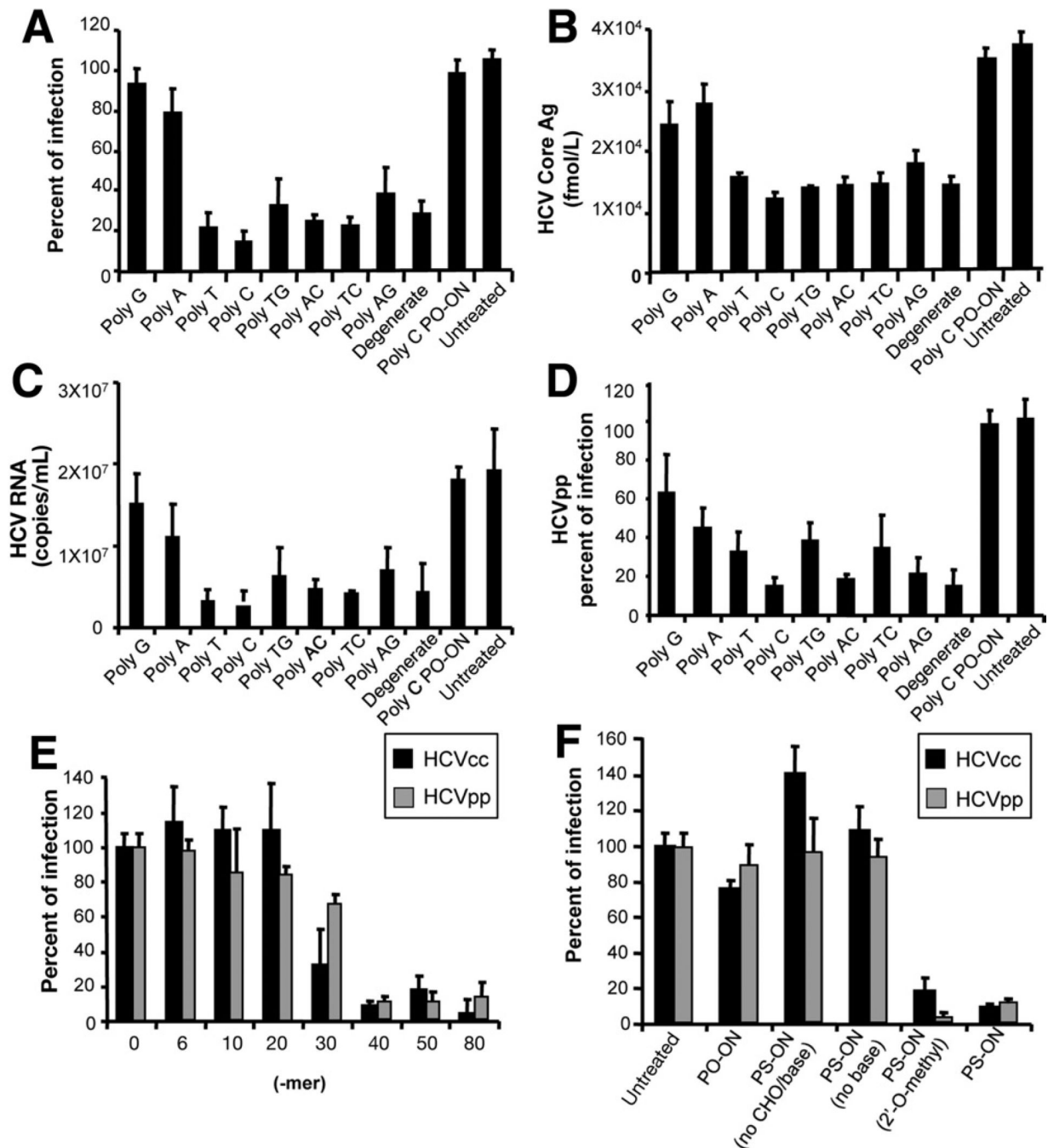
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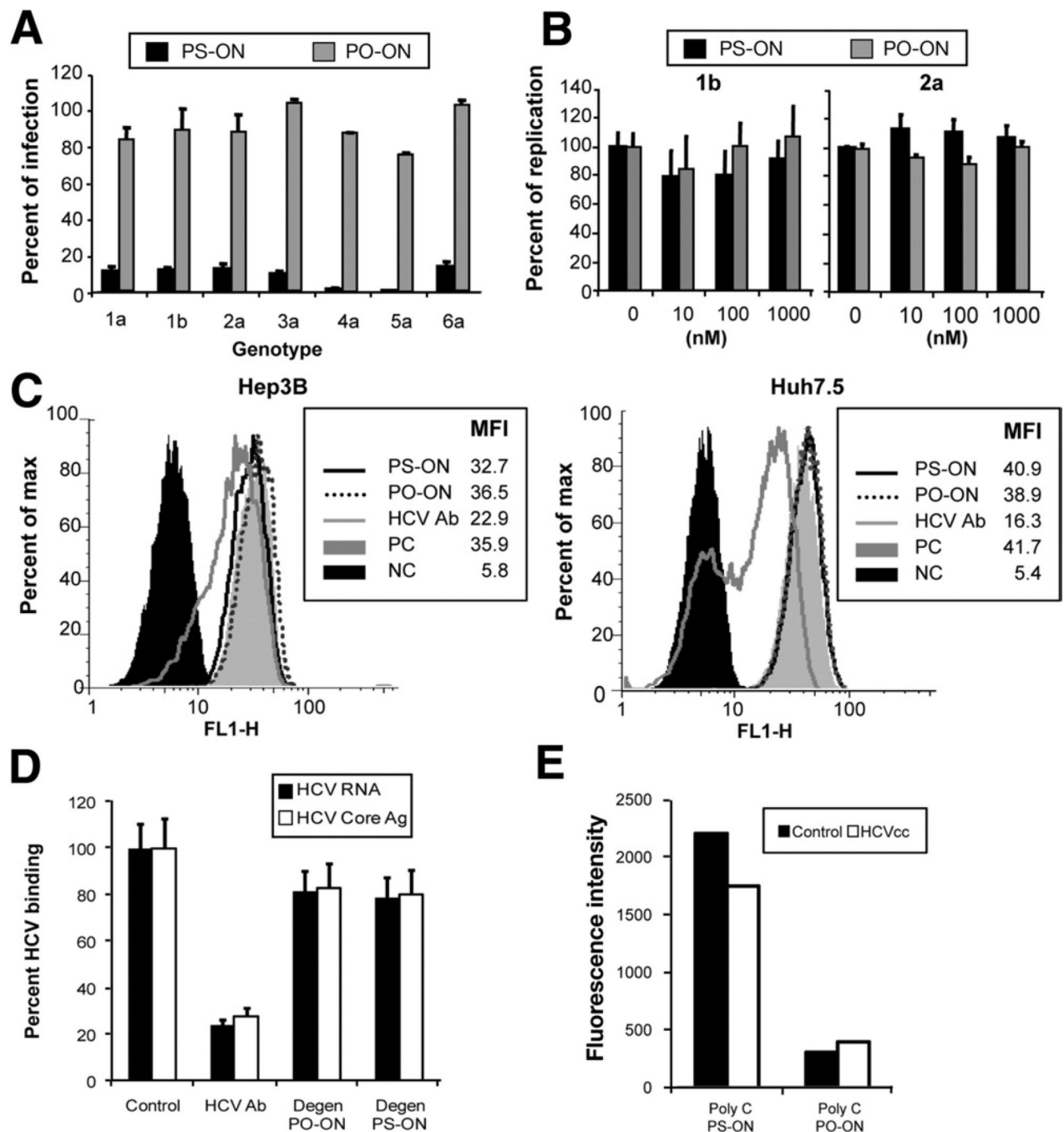
**Figure 1.**

Effect of PS-ON on HCV infection. (A) Huh7.5 cells were infected with HCVcc in the presence of various concentrations of 40mer PS-ON or PO-ON (degenerate sequence). Two days after infection, infected cells were detected by immunofluorescence assay using anticore antibodies (*left panel*). Percentage of infection was determined by dividing the number of HCV-expressing cells in treated over the untreated cells (*right panel*). The intracellular HCV RNA levels (B) and HCV core Ag (C) and supernatant HCV RNA (D) levels in the culture medium were determined. Hep3B cells were infected with (E) HCVpp genotype 1b or (F) VSVGpp and treated with various concentrations of PS-ON and PO-ON, and luciferase activities were determined 2 days later. Results are shown as percentages of infection + standard deviations (SD).

**Figure 2.**

Sequence-independent and size- and phosphorothioation-dependent effects of PS-ON on HCV infection. A series of 40mer PS-ONs with specific sequences including poly poly G, A, T, C, TG AC, TC, and AG were tested for their inhibitory effect on HCV infection. (A) HCVcc was inoculated with Huh7.5 cells and treated with 100 nmol/L of these homo- and heteropolymeric PS-ONs. Expression of HCV core was detected by immunofluorescence assay using anticore antibodies. (B) The HCV core Ag titers and (C) HCV RNA levels in the culture medium were determined. (D) Hep3B cells were infected with HCVpp geno-type 1b and treated with these various PS-ONs at 100 nmol/L, and luciferase activities were determined 2 days later. (E) Various sizes of PS-ON (10 – 80mers) at 100 nmol/L were tested in the HCVcc and HCVpp

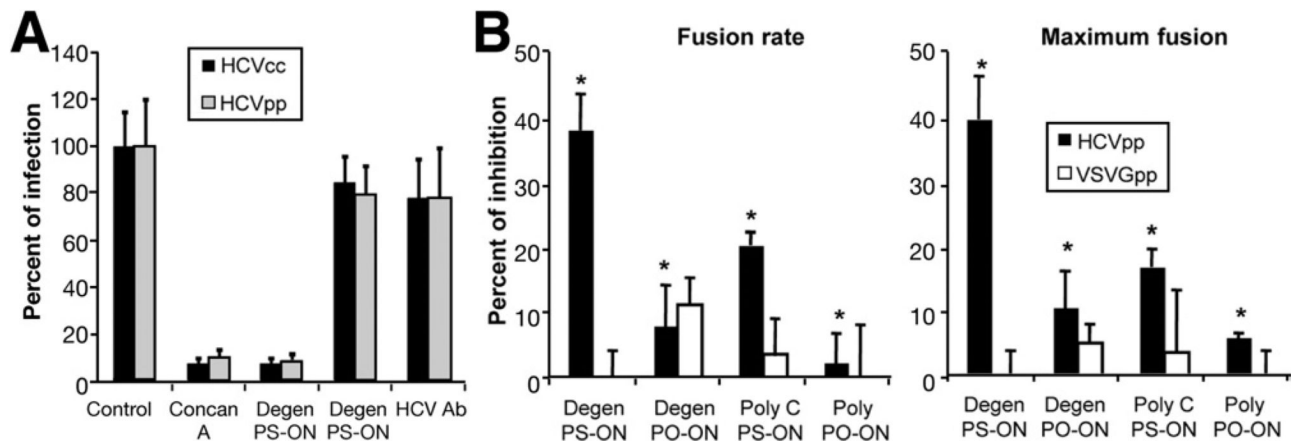
systems. (F) Various structures of oligonucleotides, PS-ON analogue with phosphorothioate backbone but without the sugar or base, and PS-ON analogue with 2'-O-methyl ribose modification, were synthesized. Each 40mer oligonucleotide at 100 nmol/L was tested in the HCVcc and HCVpp systems. All results are shown as percentages of infection + SD.

**Figure 3.**

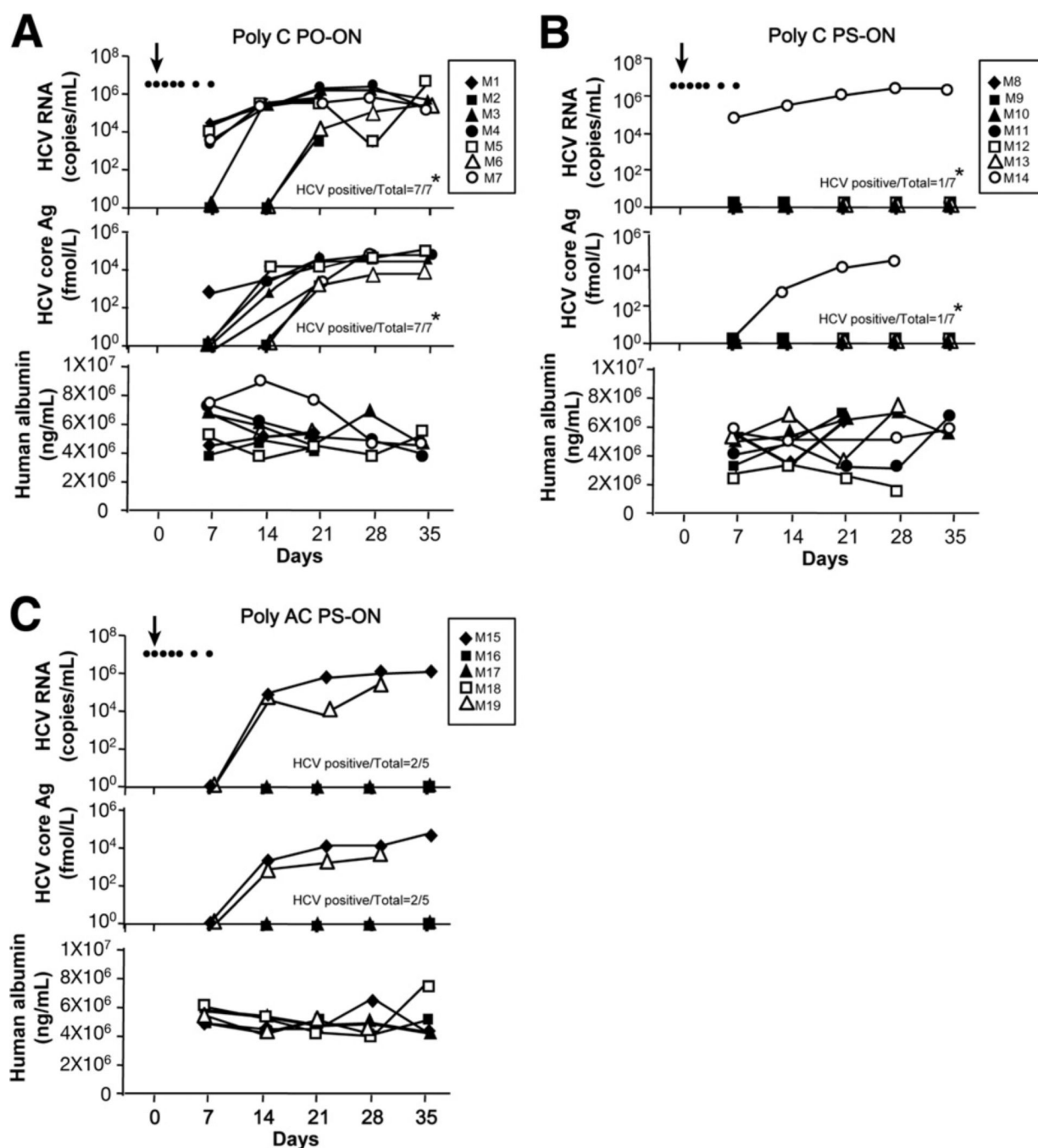
Effects of PS-ON on infection of various HCV genotypes, HCV replication, and cell binding. (A) HCVpp harboring E1/E2 glycoproteins from genotypes 1a, 1b, 2a, 3a, 4a, 5a, and 6a were inoculated into Hep3B cells and simultaneously treated with 100 nmol/L of degenerate PS-ON and PO-ON (40mer). Luciferase activities were determined 2 days later. (B) Subgenomic RNA of genotype 1b Con1 or 2a JFH1 were transfected into Huh7.5 cells. Four hours after transfection, a set of transfected cells was harvested as a control for transfection efficacy, and the remaining cells were treated with 100 nmol/L of PS-ON and PO-ON. Cells were then harvested at 72 hours posttransfection and luciferase activities determined. The replication level was presented as the ratio of the luciferase activity of the sample at 72 hours over that of



4 hours. Percentages of replication were determined by dividing the replication level of treated over that of untreated samples. (C) Hep3B and Huh7.5 cells were incubated with 20  $\mu\text{g/mL}$  HCV-LP and 100 nmol/L PS-ON or PO-ON at 4°C for 1 hour. The cells were washed and incubated with anti-E2 ALP98 monoclonal antibody for 30 minutes followed by FITC-labeled goat anti-mouse immunoglobulin for 30 minutes at 4°C. HCV-LP binding was analyzed by flow cytometry. The *black filled peaks* are negative controls without the anti-E2 antibody. The *gray filled peaks* are positive controls showing HCV-LP binding without any compounds. The *black solid lines* and *gray dotted lines* represent treatments with PS-ON and PO-ON, respectively. The *gray solid line* represents samples in the presence of HCV serum that has been shown previously to inhibit HCV-LP binding. The mean fluorescence intensity (*MFI*) of each sample is shown. (D) HCVcc was incubated with Huh7.5 cells in the presence of HCV serum PS-ON or PO-ON at 4°C for 1 hour. The unbound virus was washed off, and the bound HCVcc was determined by HCV RNA quantification and HCV core Ag assay. (E) HCVcc was incubated with Cy3-labeled degenerate PS-ON or PO-ON (40mer) and subjected to iodixanol density gradient analysis as described in the online Supporting Document. Control preparation generated the same way was used for comparison. The fluorescence intensity of the fraction where infectious HCV sedimented was determined and shown.

**Figure 4.**

Effects of PS-ON on HCV viral entry. (A) Hep3B cells were incubated with HCVpp at 4°C for 1 hour to bind the virus and washed to remove the unbound virus. Cells were then incubated with fresh culture medium containing 25 nmol/L concanamycin A, 100 nmol/L PS-ON, 100 nmol/L PO-ON, or 25  $\mu$ g/mL (total concentration) AP33+ALP98 monoclonal antibodies at 37°C for 16 hours. The luciferase activities were determined 24 hours later. Results are shown as percentages of infection + SD. (B) Fusion assay was performed with HCVpp or VSVGpp in the presence of PS-ON (degenerate or poly C) or the PO-ON controls. The results are expressed as mean percentages (means + SD) of inhibition of either the fusion rate at the origin of the fusion kinetics (*left panel*) or the maximum fusion of the curve at 500 S (*right panel*) relative to incubation in the absence of the compounds. The fusion curves are shown in Supplementary Figure 4. \* $P < .05$  comparing the PS-ON and the corresponding PO-ON in the HCVpp fusion assay.

**Figure 5.**

Effects of PS-ON on HCV infection in vivo. Human hepatocytes-transplanted uPA/SCID mice were treated intraperitoneally with 10 mg/kg of PS-ON (poly C) (n = 7) or (poly AC) (n = 5) on days -1, 0, 1, 3, 5, and 7 (indicated by dots). The corresponding control PO-ON (poly C) was also tested (n = 7). A fourth group of mice did not receive any compounds (n = 15). The mice were intravenously inoculated on day 0 with HCV patient serum containing  $3.9 \times 10^3$  copies of HCV genotype 1b (indicated by arrow). Serum samples were obtained on days 0 (prior to HCV inoculation), 7, 14, 21, 28, and 35 for HCV RNA and human albumin determination. HCV core antigen was also measured and showed the same results as the HCV RNA determination.

Table 1

Lack of Binding of PS-ON to HCV-LP

	PS-ON (nmol/L) <sup>a</sup>				PO-ON (nmol/L) <sup>a</sup>				AP33 <sup>b</sup> 1 µg/mL
	0	10	100	1000	0	10	100	1000	
HCV-LP	155 + 14	105 + 9	162 + 13	116 + 10	137 + 12	15 + 10	123 + 12	157 + 13	1475 + 150
Control	147 + 13	117 + 10	120 + 11	117 + 9	111 + 10	33 + 14	100 + 8	103 + 9	153 + 16

<sup>a</sup> Cy3 labeled degenerate PS-ON and PO-ON (40mer).

<sup>b</sup> AP33 binding to HCV-LP was detected with Cy3-labeled goat anti-mouse IgG antibody.