were effective not only against HCV genotype 2a but also HCV genotype 1b and that the compounds inhibited a HCV replication step. The growth of the replicon cells was suppressed by treatment with 10 μM of clomifene. Clomifene at concentrations less than 10 μM and tamoxifen, raloxifene and ICI 182,780 at 10 μM concentration or lower did not inhibit cell growth (Fig. 3c).

3.4. SERMs inhibited entry of HCVpp but not VSVpp

To further examine the inhibition of early viral processes by the SERMs, we used infectious HCV pseudo-particles (HCVpp). Because HCVpp enter into cell dependent on HCV envelope protein but replicate dependent on retroviral system in the cell, we can exclude other effects of the drug except effect on HCV entry system. Pseudo-particles with the viral envelope glycoprotein mimic the entry of the parental virus, and this system has been used for investigation of HCV entry [7,8,18,20,21]. The infectious titer is determined by luciferase activity. We added tamoxifen to HCVpp- or VSVpp-containing medium and incubated Huh 7.5.1 cells with this medium for 3 h. After washing the cells, fresh medium was added, and the cells were incubated for 3 days. Treatment with tamoxifen reduced the luciferase activity of the cells that were infected with HCVpp in a dose-dependent fashion. In contrast, the luciferase activity caused by VSVpp was not reduced by the same concentrations of tamoxifen (Fig. 4a). We also examined the effect of other SERMs, such as clomifene, raloxifene, ICI 182,780, ZK164015, and MPP, on HCVpp infection. All of these SERMs inhibited the luciferase activity caused by HCVpp but not the activity caused by VSVpp (Fig. 4b). ICI 182,780 showed a weaker effect compared to tamoxifen, clomifene and raloxifene. Next, we examined the effects of these drugs on various genotypes of HCVpp. Although the extent of inhibition was varied, the compounds inhibited all of the genotypes that were examined (Fig. 4c). At a concentration of 10 μM, ICI 182,780 inhibited all of the genotypes of HCVpp other than genotype 2a. These results suggested that the SERMs inhibit entry of all genotypes of HCV.

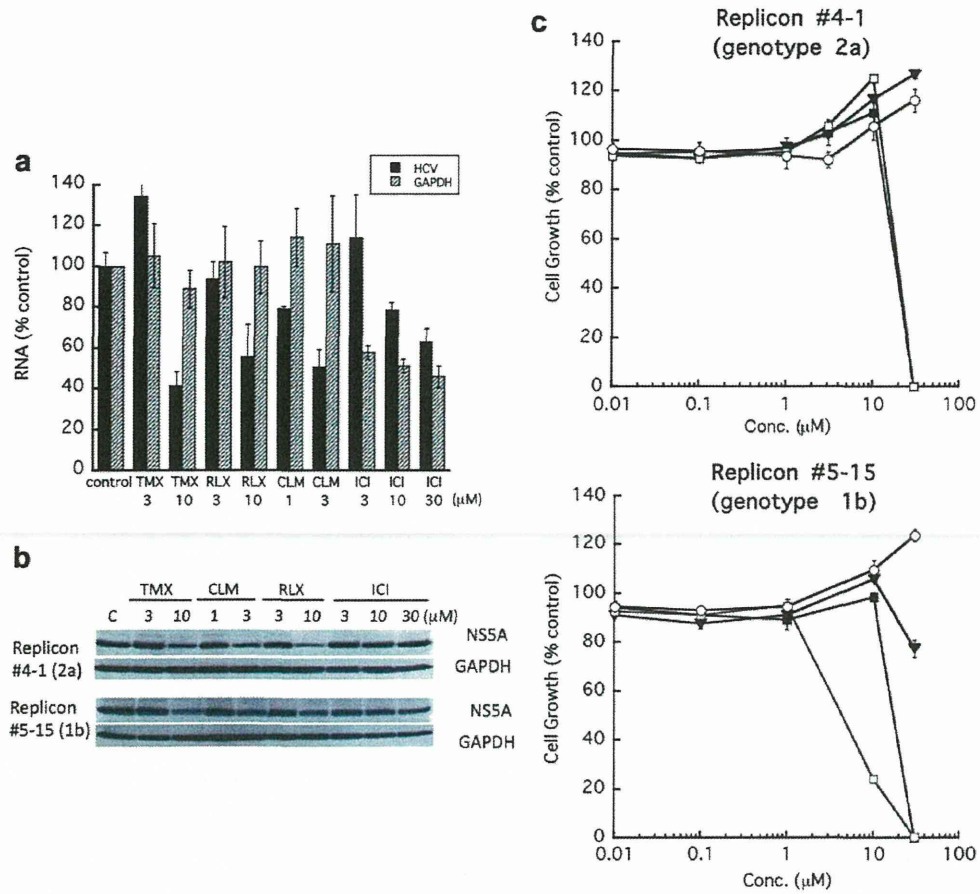

Fig. 3. The effect of SERMs on cells that harbored a subgenomic replicon. A subgenomic-replicon-harboring cell line clone #4-1 (genotype 2a) was treated with SERMs for 48 h. The total RNA was extracted from the cells, and amount of HCV RNA genome was measured. As an internal control, relative amount of GAPDH RNA was measured and indicated as percentage of control cells without drug (a). Another subgenomic-replicon-harboring cell line, clone #5-15 (genotype 1b) was treated with SERMs for 3 days. Cell lysates were subjected to western blotting with an anti-NS5A antibody or an anti-GAPDH antibody (b). Cells that were grown for 3 days in the presence of tamoxifen (closed rectangles), clomifene (open rectangles), raloxifene (closed triangles), or ICI 182,780 (open circles) were measured using the MTT assay. Cell growth is expressed as a percentage of control cells without drug (c). The values are the average of triplicate and the error bars represent the standard deviation of the mean. One representative experiment of two independent experiments is shown.

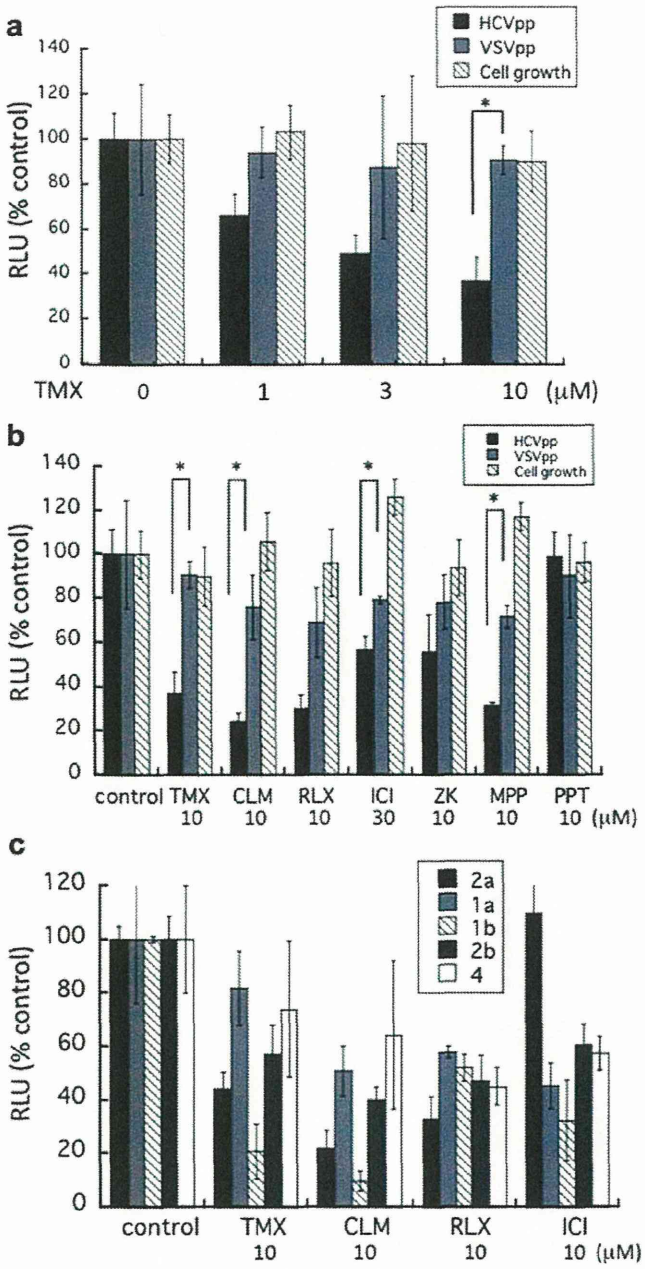

Fig. 4. Effect of SERMs on HCV pseudo-particle (HCVpp) infection. a) Huh 7.5.1 cells were incubated with pseudo-particles (HCVpp or VSVpp) in the presence or absence of tamoxifen for 3 h. The supernatants were removed, medium was added back to the cell cultures, and the cells were incubated for another 3 days. The VSVpp preparation was diluted 600 times so it was infected at similar RLU activity levels compared to HCVpp (approximately 5000 RLU). A parallel culture without pseudo-particles was analyzed using the MTT assay to evaluate the effect of the drugs on cell growth. b) Effects of various SERMs on HCVpp and VSVpp infection. c) Effects of SERMs on the various genotypes of HCVpp infection. The control luciferase activities were approximately 5000 RLU (genotype 2a), 3000 RLU (genotype 1a), 2400 RLU (genotype 1b), 3900 RLU (genotype 2b), and 860 RLU (genotype 4). The values are expressed as the percentage of control cells without drug. The data are the averages of three wells and the error bars are the standard deviation of the mean. *P-value < 0.05. One representative experiment of three independent experiments is shown.

3.5. Effect of tamoxifen on the attachment and entry steps

To better understand how tamoxifen blocks HCV entry, we performed an experiment to discriminate between the inhibition of HCV attachment to cells and the inhibition of post-binding entry events. HCV attaches to several cellular receptors via its E1 and E2 envelope proteins and enters via clathrin-mediated endocytosis [14–16]. We used HCVpp because infection with HCVpp is thought to simulate HCV entry [7,17,18] and the entry is independent of HCV replication. HCVpp binding to the cellular receptors was performed at 4 °C for 1.5 h. Under these conditions, HCVpp bind to the cells but entry is not efficient. The inoculum was removed, and fresh medium was added to the cells. The cells were subsequently incubated at 37 °C. In protocol I, the drug was administered during the binding step at 4 °C. After the shift to 37 °C, treatment with the drug was performed during first hour (protocol II) or after 1 h at 37 °C (protocol III) to distinguish between the inhibition of early and late post-binding events (Fig. 5a). The inoculum was removed after treatment, and fresh medium was added to the cells. We used chloroquine, a lysosome-tropic agent, as a control inhibitor for early entry (protocol II) [19]. We also used an anti-CD81 antibody that specifically inhibits HCV entry through the inhibition of the HCV cellular receptor protein CD81 at early entry [20,21]. As expected, chloroquine inhibited luciferase activity when the cells were treated during the early post-binding step (protocol II). This result suggested that endocytosis occurred primarily during the first post-binding period (protocol II). Anti-CD81 markedly inhibited luciferase activity during protocol II as reported [18,19]. Tamoxifen treatment did not result in clear differences between the protocols and the compound displayed similar activity regardless of the treatment period (Fig. 5b left). As a control, the same experiment was performed using VSVpp. Chloroquine inhibited the early entry step of VSVpp, but anti-CD81 and tamoxifen did not show any inhibition (Fig. 5b right).

Tamoxifen is a lipophilic weak base and inhibits acidification intracellularly [22]. Therefore, we examined whether the inhibition of the endocytosis of HCVpp by tamoxifen was dependent on its function as a weak base. Chloroquine is a weak base and inhibits endosome acidification. The pH sensitivity is considered a good indication of clathrin-dependent endocytosis. Previous reports have indicated that chloroquine inhibited HCVcc and HCVpp infection [14,19]. We adjusted the medium to pH 5.5 and incubated the cells in this acidic medium in the presence or absence of tamoxifen for 2 h post-binding. The acidification of the medium did not affect either the entry of HCVpp or the cell growth (Fig. 5c). Treatment with tamoxifen in the medium with a normal pH (pH 7.1) reduced HCVpp entry, and treatment with the drug in the acidic medium also reduced entry to a similar extent. In contrast, chloroquine treatment in regular medium reduced HCVpp entry, but entry was restored in the acidic medium (Fig. 5c). These results indicate that the inhibitory effect of tamoxifen was not dependent on the function of this compound as a base, unlike the effects of chloroquine.

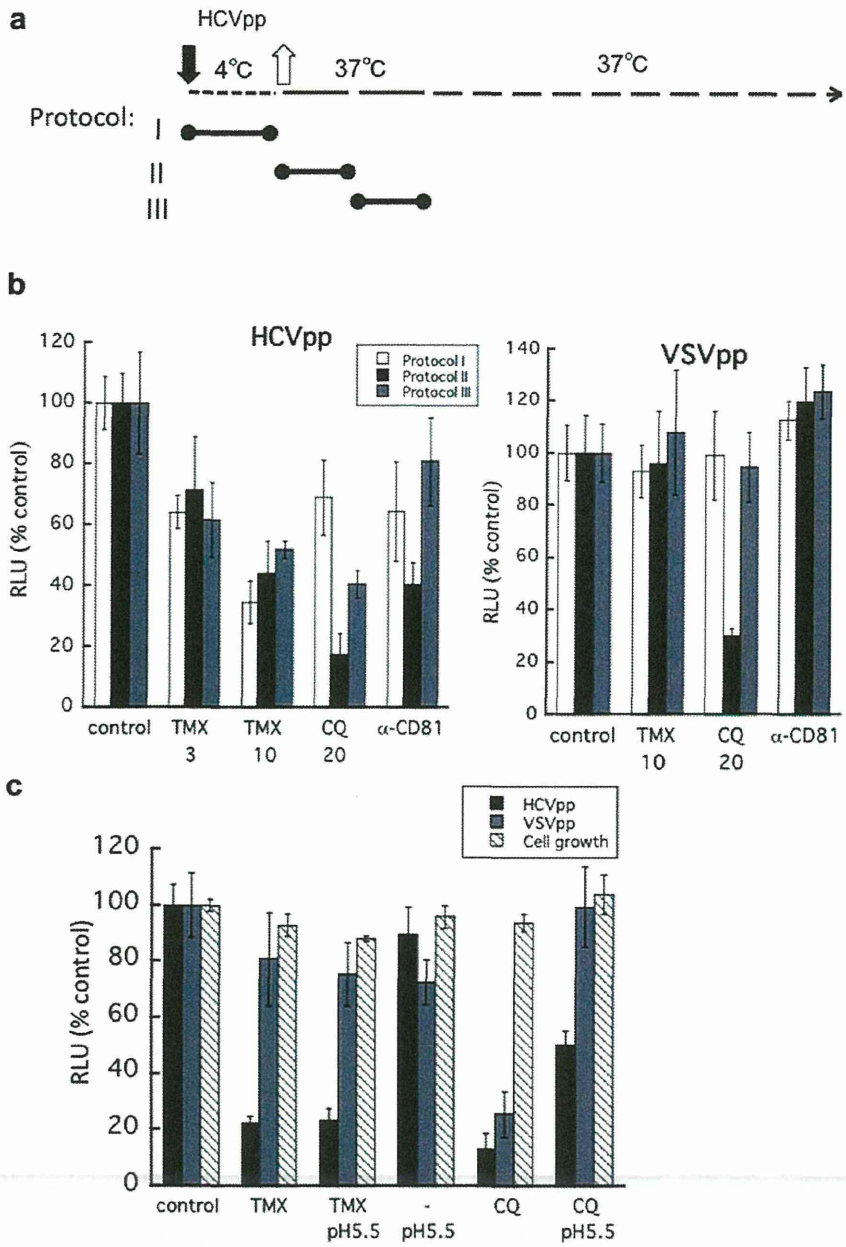

Fig. 5. Effect of tamoxifen on the attachment and endocytosis of HCVpp. a) Experimental design. HCVpp attachment to cells was performed at 4 °C for 1.5 h in the presence or absence of drug. Under these conditions, HCVpp bind to the cells but do not efficiently enter the cells. The inoculum was then removed, and fresh medium was added to the cells. The cells were subsequently incubated at 37 °C. The cells were treated with drug during the binding period at 4 °C (protocol I), during first hour after the shift to 37 °C (protocol II), or 1 h after the shift to 37 °C (protocol III). The drug-containing medium was removed for every treatment, and new medium was supplied to the cells. b) Effects of tamoxifen (TMX) (3 and 10 μM), chloroquine (CQ) (20 μM) and anti-CD81 antibody (20 μg/ml) on HCVpp attachment (protocol I) and post-binding events (protocol II and III) (left). Effects of tamoxifen, chloroquine and anti-CD81 on VSVpp (right). c) Effects of exposure to low pH on the inhibition of HCVpp entry by tamoxifen and chloroquine. The cells were incubated with HCVpp at 4 °C for 1.5 h in the absence of drug. After removing the inoculum, regular (pH 7.1) or acidic medium that was adjusted with HCl to pH 5.5, either with or without drug (tamoxifen, 10 μM, chloroquine, 20 μM), was added to the cells. The cells were subsequently incubated at 37 °C. The drug-containing medium was removed after 2 h of incubation, and the cells were incubated for an additional 3 days with fresh, regular medium. The values are expressed as the percentage of control cells without drug. The data are the averages of three wells and the error bars represent the standard deviation of the mean. One representative experiment of three independent experiments is shown.

4. Discussion

We screened for HCV inhibitors using the JFH-1-Huh 7.5.1 cell culture system and found that tamoxifen and ERα antagonists, but not ERα agonists, inhibited HCV JFH-1 infection. Although there are some reports about the HCV inhibitory effects of tamoxifen and other SERMs, we presented further information about the inhibitory effects of these substances. The time-of-addition experiments (Fig. 2a–c) suggested that these SERMs inhibit the entry and replication

steps in the HCV life cycle. These SERMs, except ICI 182,780, reduced level of HCV genome (genotype 2a) and NS5A (genotypes 1b and 2a) in the subgenomic replicon cells (Fig. 3), which supports the hypothesis that the inhibitory effect of the SERMs occurred during the replication steps. Further we observed that SERMs preferentially reduced extracellular HCV RNA compare to intracellular HCV RNA in the newly (Fig. 2d) and persistently infected cells (Fig. 2e). It suggests that the SERMs also target post replication step(s) in the viral life cycle, such as assembly and release. A low concentration of tamoxifen (0.1 μM) accumulated intracellular HCV RNA (Fig. 2d), which suggests that SERMs target post replication step(s) more efficiently than replication steps. Additionally, these compounds inhibited HCVpp infection (Fig. 4), which supports an inhibitory effect during the entry step. The inhibition of entry was mediated through the inhibition of viral binding to cells and through the inhibition of a post-binding event (Fig. 5b). Taken together, SERMs seemed to target multiple steps of the HCV viral life cycle.

Among the SERMs, ICI 182,780 did not exhibit distinct inhibition of replication in the replicon cells (Fig. 3a and b), although the compound seemed to inhibit entry and replication steps according to the result of time-of-addition experiment (Fig. 2c). The replicon cells are derived from Huh 7 cell. Although viral sensitivity to the SERMs might be dependent on the cell that HCV infect, it remains unclear whether ICI 182,780 inhibits replication step or not. However, the compound affected post replication step in a similar manner to other SERMs (Fig. 2d and e). It is unlikely that ICI 182,780 is an inhibitor with different mechanisms.

The inhibitory effect of tamoxifen on HCV replication has been previously reported by Watashi et al. [23]. They also demonstrated that ICI 182,780 inhibited HCV replication. This effect was discovered using a cell line that harbored a subgenomic replicon (genotype 1b). Additionally, these researchers reported that RNA interference-mediated knockdown of ERα, not ERβ, reduced HCV replication, but the reduction was not related to ERE-mediated transactivation activity. They suggested that ERα interacts with the HCV viral polymerase NS5B and that ERα promotes the participation of NS5B with the HCV replication complex. Using the Huh 7.5.1-JFH-1 screening system, Gastaminza et al. and Chockalingam et al. found that toremifene and raloxifene, respectively, function as HCV inhibitors. Gastaminza et al. [24] reported that toremifene inhibited HCV infection by inhibiting both the entry and release steps of the viral life cycle. Chockalingam et al. [25] determined that raloxifene inhibited the entry and replication steps, as we also observed. Our results are in accordance with these previous reports and other information about the inhibitory effects of SERMs.

Pseudo-particle experiments confirmed that SERMs affected the entry step of HCV viral life cycle (Fig. 4b), although the inhibitory effects were lower compare to those against HCVcc (Fig. 2a–c). The reason for the difference in sensitivity may account for some difference in the two entry systems. Otherwise, in the treatment with the drugs for the first 2 h of HCVcc infection, some amount of the drugs might enter the cell and remain affecting the other steps. The SERMs affected not only genotype 2a but also other genotypes of HCVpp that were examined, suggests that these chemicals have effects on various genotypes of HCV. Although the SERMs appeared to inhibit multiple steps of the HCV life cycle, the primary target step in the viral life cycle might be the entry step. In the time-of-addition experiments, treatment with tamoxifen or raloxifene during the first 2 h was more effective than treatment during the subsequent 2–48 h (Fig. 2a). These SERMs are thought to primarily prevent viral entry and to inhibit post replication step and replication at higher concentrations.

As shown in Fig. 5, an experiment that could discriminate an effect on viral attachment from an effect at the post-binding processes indicated that tamoxifen inhibited both steps. The inhibition of endocytosis by tamoxifen was not rescued by exposure to a low pH. This suggests that the observed inhibition is the result of a mechanism that is independent of the compound's function as a base. HCV entry is a highly complicated process that involves numerous viral and cellular factors. Tamoxifen is thought to target multiple steps that are involved in the attachment and entry steps of the HCV life cycle, which results in high levels of inhibition.

At present, the mechanism of the entry inhibition by SERMs is not clear. It is possible that tamoxifen targets viral molecules, but we have no evidence to support this hypothesis. ERα might be a target molecule because all of the antagonists of ERα that were examined had an inhibitory effect. Watashi et al. indicated that ERα is involved in HCV replication [23]. ERα is thought to be present in the cytoplasm, which is where HCV replicates. However, it is doubtful that ERα is present on the cell surface where viral entry occurs. The addition of 17β-estradiol with tamoxifen did not prevent the inhibitory effect of tamoxifen in the HCVpp experiment (data not shown). This result suggests that tamoxifen does not compete with 17β-estradiol for the target molecules involved in HCV entry. Additionally, a pure ERα antagonist, ICI 182,780, was a less effective inhibitor of the entry step. Based on these results, it is thought that the molecule responsible for HCV entry that is targeted by SERMs is not ERα.

Tamoxifen has various targets other than ERα, such as P-glycoprotein (GPR30), calmodulin, and protein kinase C [26]. GPR30 (G protein-coupled receptor protein 30) is a membrane-associated estrogen receptor that is distinct from the classical ER [27]. Tamoxifen and ICI 182,780 are agonists of GPR30 [28]. We examined the effect of a specific GPR30 agonist, G-1, and a GPR30 antagonist, G-15, on HCVpp infection [29]. G-1 and G-15 did not inhibit HCVpp infection. Conversely, HCVpp infection was observed to increase upon addition of these compounds (data not shown). This result suggested that GPR30 is not involved in the inhibition of HCV entry.

We previously reported that a typical PKC inhibitor, bisindolylmaleimide I (BIM I), inhibited HCV replication [2]. BIM I (10 μM) inhibited both HCVpp and VSVpp infection in a similar manner by approximately 50% (data not shown). This suggests that BIM I has a different mechanism for the

inhibition of entry compared to tamoxifen. PKC is not thought to be involved in the HCV-specific inhibition of entry by SERMs.

There were few reports of HCV entry inhibitors until the development of the cell-culture JFH-1 infection system. It has recently been reported that fluphenazine, trifluoperazine and related chemicals exhibit a strong, dose-dependent inhibition of HCV entry without significantly affecting the entry of VSVpp [24,25]. These compounds are structurally similar to chlorpromazine, which is an inhibitor of the clathrin-coated pit formation that is required for HCV entry [14]. Interestingly, these compounds and the SERMs have a common structural characteristic: planar, multiple aromatic rings with a tertiary amine side chain. Tamoxifen, raloxifene and ERα antagonists all have this structure, but the ERα agonists do not have these structures. Fluphenazine and related chemicals may inhibit HCV entry through a mechanism that is similar to tamoxifen.

In summary, we observed a significant HCV inhibitory effect of various SERMs using the Huh 7.5.1 cell-JFH-1 infection system. Additionally, we demonstrated that SERMs could be useful for the treatment of HCV. Because it takes a great deal of time and money to develop a new drug from a novel chemical compound, it may be easier to use previously developed drugs that can be used for new applications. Tamoxifen, toremifene, and raloxifene are all drugs that have been in use for an extended period of time. In our present *in vitro* study, the effective concentrations for the HCV inhibitory effects of the SERMs were approximately 0.1–10 μM. In the case of tamoxifen, 20 mg per day, administered for 8 weeks resulted in plasma concentrations of approximately 0.5 μM. These concentrations could be sufficient to exert an anti-HCV effect. SERMs should be investigated to determine their efficacy for treating HCV clinically. Further examination of the mechanism of the entry inhibition mediated by SERMs would produce significant new data relevant to the understanding of HCV entry.

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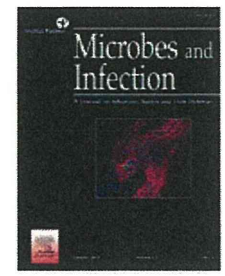

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other approaches that use replicon cells and the recently described Huh 7.5.1-JFH-1 (genotype 2a)-

antibodies against the core antigen (2H9), NS5A (Austral Biologicals, San Raman, CA, USA), or GAPDH (Santa Cruz Biotech. Inc., Santa Cruz, USA). After incubation with horseradish peroxidase-conjugated secondary antibodies, the protein bands on the PVDF membranes were detected using an ECL system (GE Healthcare UK Ltd., Amersham Place, UK).