were added to Huh 7.5.1 cells. Five days later, the quantity of HCV RNA in the culture supernatant was measured using quantitative real-time RT-PCR [2]. We found that tamoxifen reduced the levels of JFH-1 RNA in the culture supernatant. We also examined the effects of other SERMs and agonists and antagonists of ERα. As shown in Fig. 1, tamoxifen, clomifene, and hydroxytamoxifen, which have a triphenylethylene backbone, exhibited intense inhibitory effects (EC_{50}: approximately 0.1 μM). Triphenylethylene showed reduced inhibitory activity (data not shown). Raloxifene also inhibited viral RNA production at a similar concentration. (EC_{50}: approximately 0.1 μM) (Fig. 1a). Tamoxifen and raloxifene display both ERα antagonist and agonist properties in a dose- and tissue-dependent manner [9]. In contrast, ICI 182,780 (fulvestrant), ZK164015, and MPP (methyl-piperidino-pyrazole) are exclusively antagonistic [10–12]. These ERα antagonists also showed inhibitory activity against JFH-1, but their EC_{50} values were approximately 1 μM (Fig. 1b). As the 50% toxic concentrations (TC_{50}) for these compounds were observed to be greater than 10 μM (Fig. 1a and b), these specific indexes are over 100. In contrast, the ERα agonists 17β-estradiol, diethylstilbestrol, and PPT (1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrozole) did not inhibit HCV (Fig. 1c). As expected, the SERMs that were observed to effectively inhibit HCV RNA production also reduced the core protein levels intracellularly (Fig. 1d).

3.2. SERMs inhibited more than one step of the JFH-1 life cycle

To determine which step of the JFH-1 life cycle was inhibited by the SERMs studied, we performed time-of-addition experiments. As described previously [2], JFH-1 appears to complete one infectious life cycle in approximately 48 h. Huh 7.5.1 cells were inoculated with JFH-1-containing medium (moi 0.1) with or without drug and were then incubated for 2 h. After the medium was removed, fresh medium with or without drug was added. The cells were then incubated for another 46 h. Treatment with 10 μM tamoxifen for 48 h reduced the amount of viral RNA in the medium to 1.7% of levels observed in the control. Treatment with tamoxifen for the first 2 h after infection (0–2 h) reduced viral RNA to 2.3% of the levels observed in the control. The addition of tamoxifen to the fresh medium just after the removal of the virus (2–48 h) resulted in a reduction in the amount of viral RNA to 10.7% of the levels observed in the control. The addition of tamoxifen 24 h after viral inoculation (24–48 h) resulted in a decrease in the amount of viral RNA to 60% of the levels observed in the control (Fig. 2a). This result suggests that tamoxifen inhibits mainly viral entry and some steps during replication. 10 μM of raloxifene exhibited a similar inhibitory pattern but less inhibited by the treatment after the entry step (Fig. 2b). A pure ERα antagonist, ICI 182,780 (30 μM), also exhibited inhibition of both viral entry and the replication steps, but the inhibition of the entry step was not so marked (Fig. 2c).

To further investigate effect on HCV post replication, we infected HCV in the presence of the drugs for 72 h (moi 0.1) and examined their effects on intracellular and extracellular HCV RNA levels. Brefeldin A, an inhibitor of protein transport [13], was used as a positive control of post replication inhibition. In this experimental setting, brefeldin A showed intracellular HCV RNA accumulation suggesting post replication inhibition (Fig. 2d). SERMs generally reduced HCV RNA in cell as well as HCV RNA in medium, although the extent of reduction was different (Fig. 2d). Lower concentration of SERMs reduced extracellular HCV RNA more robustly than intracellular HCV RNA. At a concentration of 0.1 μM, tamoxifen exclusively inhibited HCV RNA in the culture supernatant but not intracellular HCV RNA levels, in a manner similar to that of brefeldin A (Fig. 2d). The results suggest that SERMs inhibit post replication step(s) such as assembly or release. Because low concentrations of tamoxifen failed to inhibit intracellular HCV RNA, SERMs potentially target post replication step(s) more efficiently than replication step. In this condition, higher concentrations (1 and 3 μM) of tamoxifen seemed to inhibit intracellular HCV RNA rather than extracellular HCV RNA, although the reason is not clear.

To determine the effect of these drugs on chronic infection, we used pre-infected Huh 7.5.1 cells. We infected the cell with HCVcc at a moi of 0.01 and incubated for 3 days. Three days after infection, the drugs were added, and the cells were further incubated for 48 h. At the time of drug addition, the cells were persistently infected, and HCVcc was continuously produced and released into the culture supernatant, which is similar condition to chronic infection. HCV RNA was extracted from the culture supernatant and the cells after 48 h and measured copy number of HCV RNA. Both HCV RNA in the culture supernatant and that in the cell were reduced by treatment with the SERMs, but the intracellular HCV RNA levels were less reduced (Fig. 2e). This suggested that the SERMs caused preferential reduction in extracellular HCV RNA through interference with some post replication step(s), such as assembly or release. Brefeldin A accumulated intracellular HCV RNA, and reduced HCV RNA level in the culture supernatant (Fig. 2e).

These data suggested that the SERMs inhibit multiple steps in the HCV life cycle: entry, viral RNA replication and some post replication step(s).

3.3. SERMs inhibited copies and NS5A protein expression in replicon cells

To confirm the effect of these drugs on viral replication, we used two subgenomic replicon cells. The subgenomic replicon cells, derived from Huh7 cells, harbor HCV viral RNA that replicates autonomously, and they express viral proteins. We treated cells that harbored a subgenomic replicon (#4-1, genotype 2a) [3,4] with the SERMs for 48 h and measured the amount of cellular replicon RNA by quantitative RT-PCR. Treatment with 10 μM of tamoxifen, raloxifene, or 3 μM of clomifene, inhibited HCV RNA compare to GAPDH RNA, although statistical significance was shown in only the inhibition of 10 μM of tamoxifen. ICI 182,780 did not show specific inhibition of HCV RNA (Fig. 3a).

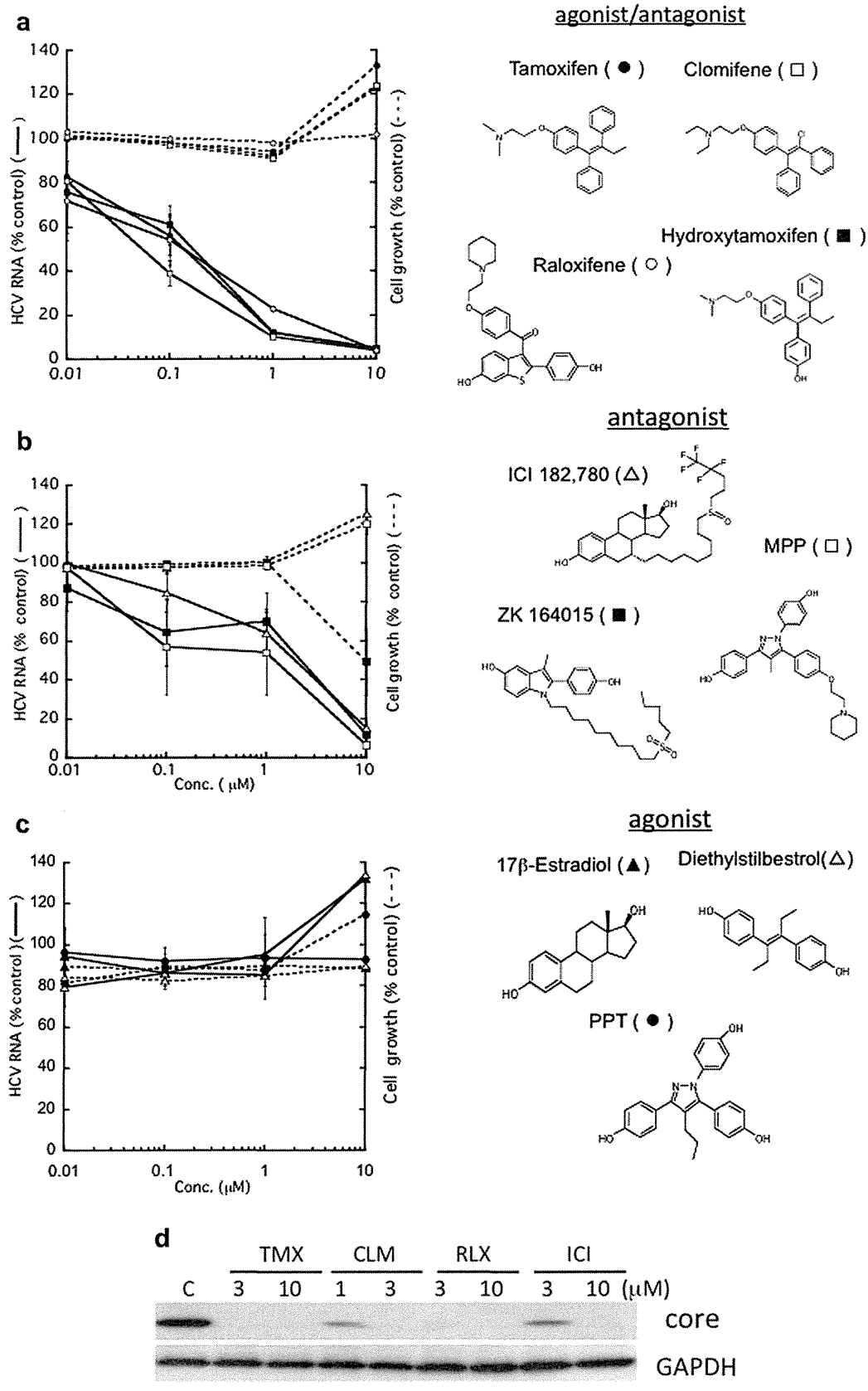

Fig. 1. Effects of SERMs on JFH-1 HCV RNA levels. a) Effects of tamoxifen, clomifene, and raloxifene. Huh 7.5.1 cells were infected with HCV JFH-1 (moi 0.01) in the presence of drugs and were incubated for 5 days. Drugs were added just before viral inoculation. HCV RNA in the medium was measured by tube-capture-RT-PCR [2]. Parallel cultures of cells without virus were analyzed using the MTT assay to detect the inhibition of cell growth due to drug exposure. Tamoxifen (closed circles), clomifene (open rectangles), hydroxytamoxifen (closed rectangles), and raloxifene (open circles). The percentages to control HCV RNA and

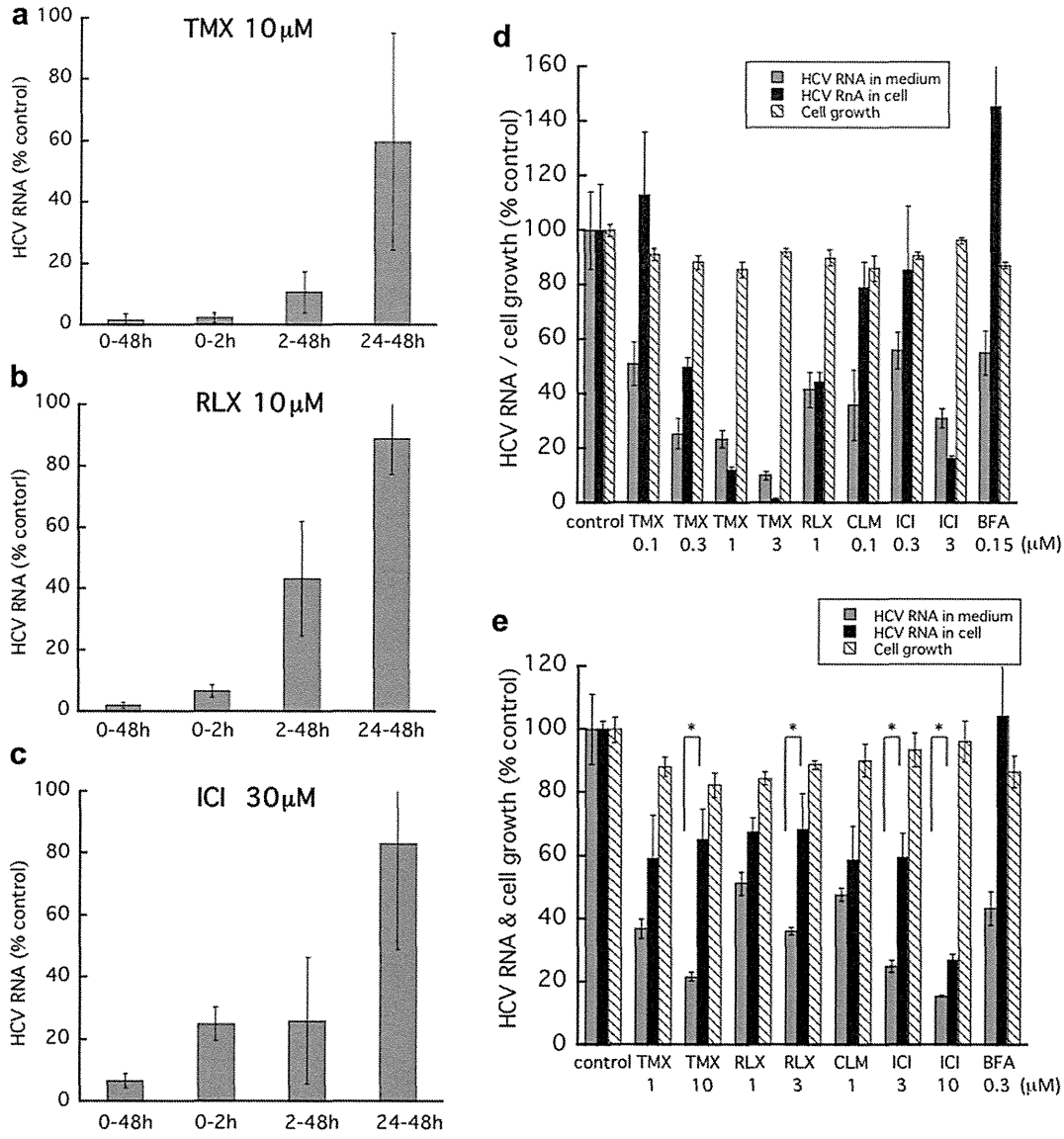

Fig. 2. Time-of-addition experiments (a–c) and the effect of SERMs on extracellular and intracellular HCV RNA in simultaneous infection (d) and in persistent infection (e). Huh 7.5.1 cells were treated with tamoxifen (TMX) (10 μM, a), raloxifene (RLX) (10 μM, b), or ICI 182,780 (ICI) (30 μM, c) during the following time periods: 0–2 h, 2–48 h, or 24–48 h after JFH-1 infection (moi 0.1). Forty-eight hours after infection, the culture supernatant was harvested, and HCV RNA was extracted and subjected to quantitative real-time RT-PCR to determine the number of copies of the JFH-1 genome. The data are the averages of three independent experiments and the standard deviation. d) Effect of treatment with SERMs for 3 days on extra- and intra-cellular HCV RNA levels. Huh 7.5.1 cells were infected with JFH-1 (moi 0.1) just after addition of the SERMs. Three days later, RNA was extracted from the cells and from the culture supernatant. The amount of HCV RNA was measured by quantitative real-time RT-PCR. Brefeldin A (BFA) was used as a positive control. e) Huh 7.5.1 cells were infected at a moi of 0.01, 3 days before addition of drugs. The infected cells were treated with SERMs for 48 h. RNA was subsequently extracted from the cells and the culture supernatant to determine the viral genome copy number. The results are presented as the percentage of control cells without drug. The data are the averages of triplicates and the error bars represent standard deviation. *P-value < 0.05. One representative experiment of two independent experiments is shown.

Next we treated this (#4-1, genotype 2a) and another replicon (#5-15, genotype 1b) [5] with the SERMs for 3 days and examined the effect of the compounds on the HCV NS5A protein levels by western blotting. As shown in Fig. 3b, the SERMs except ICI 182,780 reduced the level of NS5A in accordance with the results in Fig. 3a. ICI 182,780 seemed to slightly reduce NS5A protein in #5-15 replicon cell. The SERMs did not reduce the protein levels of GAPDH in the subgenomic replicon cells (Fig. 3b). These results indicated that SERMs, at least tamoxifen, raloxifene and clomifene,

control cell growth are indicated by solid lines and dotted lines, respectively. b) Effect of the following ERα antagonists: ICI 182,780 (closed triangles), ZK164015 (closed rectangles), and MPP (open rectangles). c) Effect of the following ERα agonists: 17β-estradiol (closed triangles), diethylstilbestrol (open rectangles), and PPT (closed circles). The results are presented as percentages of the control cells that were not treated with drugs. Values are the averages of triplicates, and the error bars represent the standard deviation of the mean. One representative experiment of three independent experiments is shown. d) Huh 7.5.1 cells were infected (moi 0.01) in the presence of tamoxifen (TMX), clomifene (CLM), raloxifene (RLX), or ICI 182,780 (ICI) and incubated for 5 days. Cell lysates were blotted with anti-core and anti-GAPDH antibodies as described in the Section Materials and methods.

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.

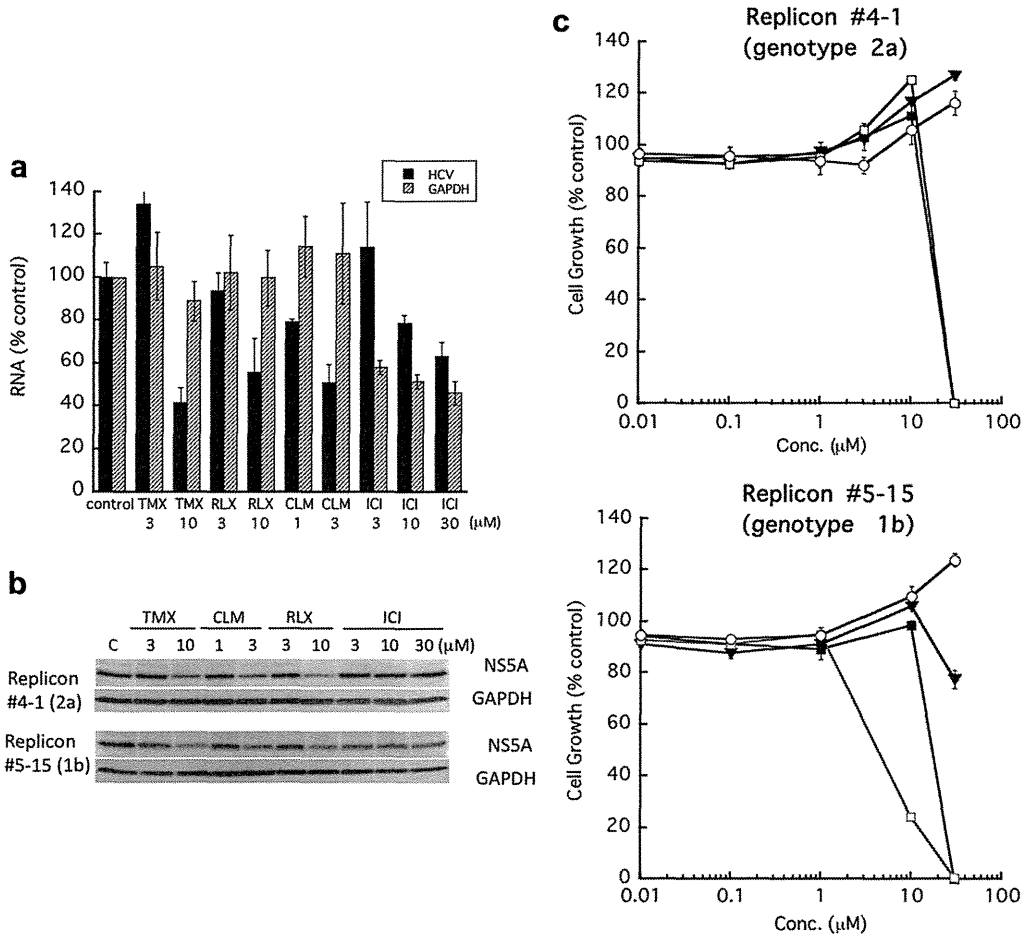

Fig. 3. The effect of SERMs on cells that harbored a subgenomic replicon. A subgenomic-replicon-harboring cell line clone #4-1 (genotype 2a) was treated with SERMs for 48 h. The total RNA was extracted from the cells, and amount of HCV RNA genome was measured. As an internal control, relative amount 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.

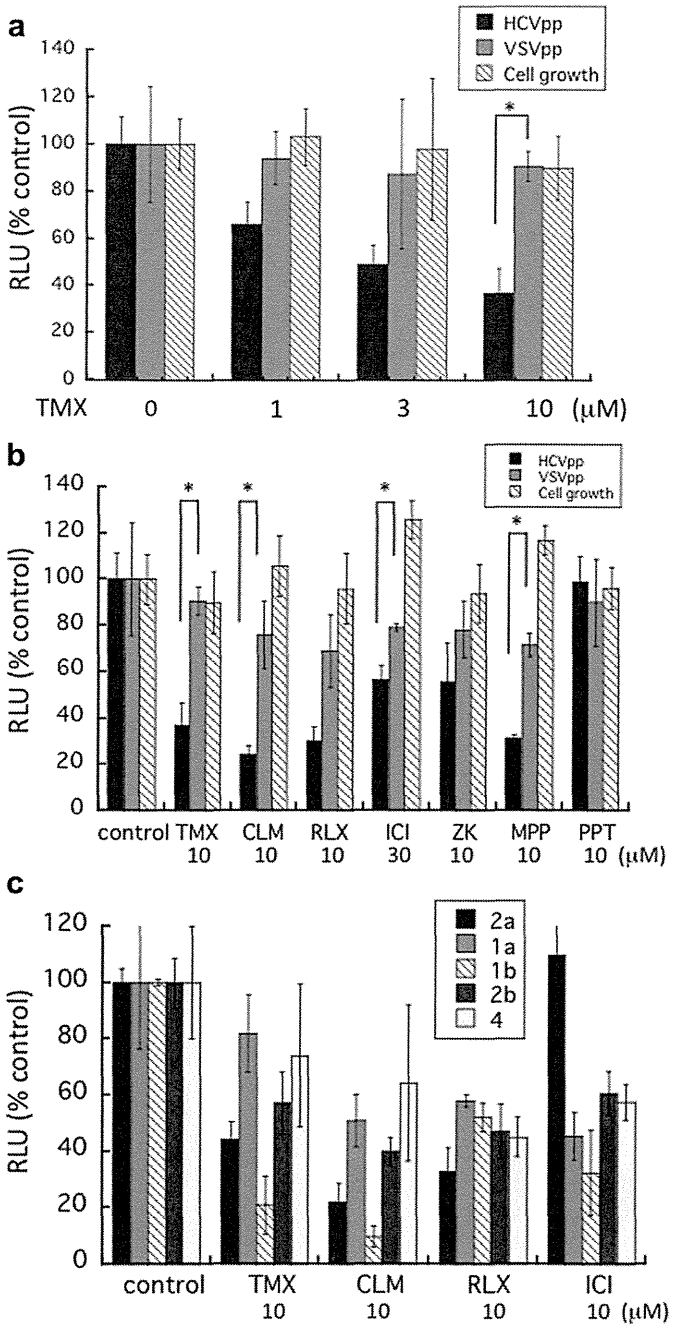

Fig. 4. Effect of SERMs on HCV pseudo-particle (HCVpp) infection. a) Huh 7.5.1 cells were incubated with pseudo-particles (HCVpp or VSVpp) in the presence or absence of tamoxifen for 3 h. The supernatants were removed, medium was added back to the cell cultures, and the cells were incubated for another 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.

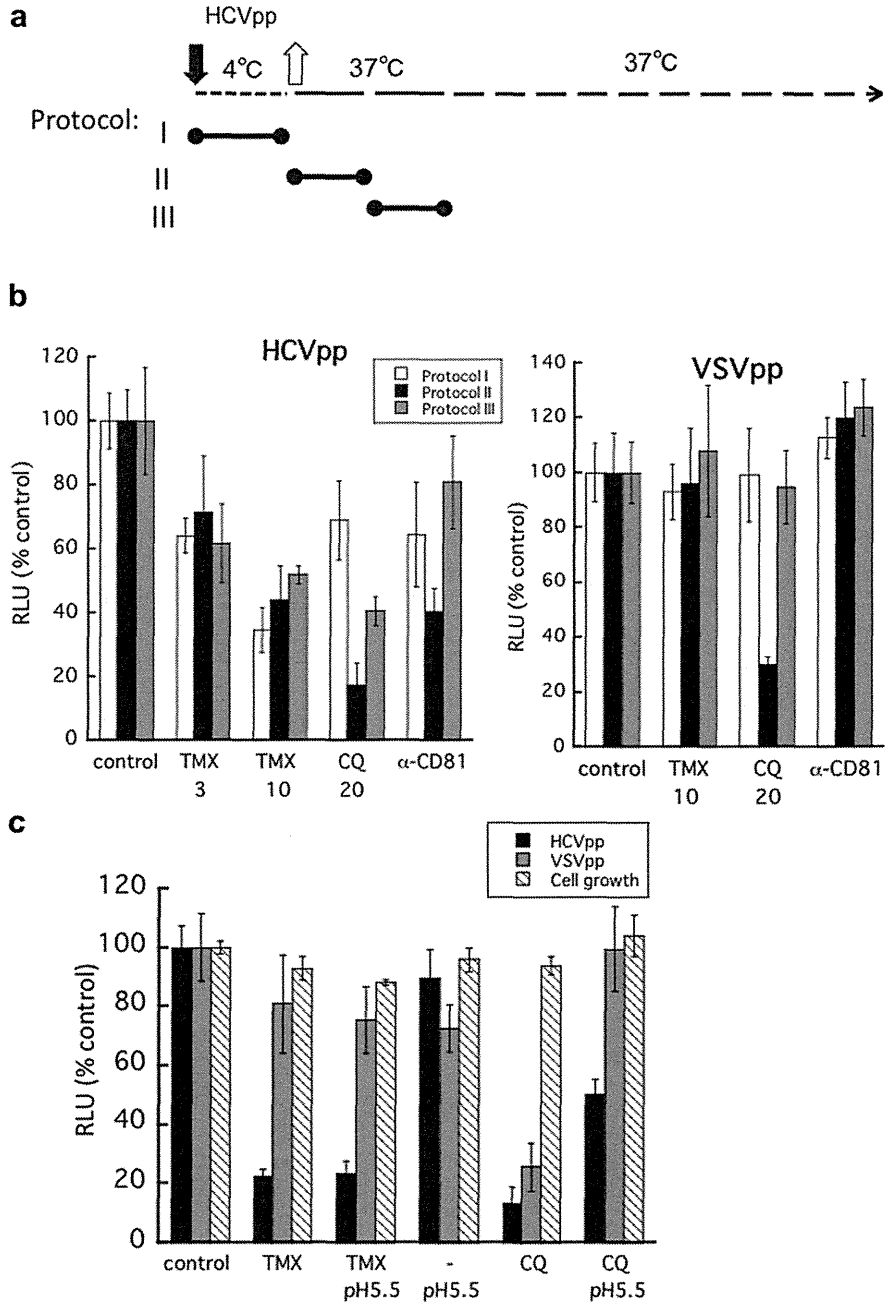

Fig. 5. Effect of tamoxifen on the attachment and endocytosis of HCVpp. a) Experimental design. HCVpp attachment to cells was performed at 4 °C for 1.5 h in the presence or absence of drug. Under these conditions, HCVpp bind to the cells but do not efficiently enter the cells. The inoculum was then removed, 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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References

[1] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H.G. Krausslich, M. Mizokami, R. Bartenschlager, T.J. Liang, Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (2005) 791–796.

[2] Y. Murakami, K. Noguchi, S. Yamagoe, T. Suzuki, T. Wakita, H. Fukazawa, Identification of bisindolylmaleimides and indolocarbazoles as inhibitors of HCV replication by tube-capture-RT-PCR, Antivir. Res. 83 (2009) 112–117.

[3] T. Kato, T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, T. Wakita, Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon, Gastroenterology 125 (2003) 1808–1817.

[4] T. Date, T. Kato, M. Miyamoto, Z. Zhao, K. Yasui, M. Mizokami, T. Wakita, Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells, J. Biol. Chem. 279 (2004) 22371–22376.

[5] V. Lohmann, F. Korner, J. Koch, U. Herian, L. Theilmann, R. Bartenschlager, Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line, Science 285 (1999) 110–113.

[6] H. Fukazawa, S. Mizuno, Y. Uehara, A microplate assay for quantitation of anchorage-independent growth of transformed cells, Anal. Biochem. 228 (1995) 83–90.

[7] B. Bartosch, J. Dubuisson, F.L. Cosset, Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes, J. Exp. Med. 197 (2003) 633–642.

[8] D. Lavillette, A.W. Tarr, C. Voisset, P. Donot, B. Bartosch, C. Bain, A.H. Patel, J. Dubuisson, J.K. Ball, F.L. Cosset, Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus, Hepatology 41 (2005) 265–274.

[9] C.K. Osborne, H. Zhao, S.A. Fuqua, Selective estrogen receptor modulators: structure, function, and clinical use, J. Clin. Oncol. 18 (2000) 3172–3186.

[10] A.E. Wakeling, M. Dukes, J. Bowler, A potent specific pure antiestrogen with clinical potential, Cancer Res. 51 (1991) 3867–3873.

[11] C. Biberger, E. von Angerer, 2-Phenylindoles with sulfur containing side chains. Estrogen receptor affinity, antiestrogenic potency, and antitumor activity, J. Steroid Biochem. Mol. Biol. 58 (1996) 31–43.

[12] J. Sun, Y.R. Huang, W.R. Harrington, S. Sheng, J.A. Katzenellenbogen, B.S. Katzenellenbogen, Antagonists selective for estrogen receptor alpha, Endocrinology 143 (2002) 941–947.

[13] N. Sciaky, J. Presley, C. Smith, K.J. Zaal, N. Cole, J.E. Moreira, M. Terasaki, E. Siggia, J. Lippincott-Schwartz, Golgi tubule traffic and the effects of brefeldin A visualized in living cells, J. Cell Biol. 139 (1997) 1137–1155.

[14] E. Blanchard, S. Belouzard, L. Goueslain, T. Wakita, J. Dubuisson, C. Wychowski, Y. Rouille, Hepatitis C virus entry depends on clathrin-mediated endocytosis, J. Virol. 80 (2006) 6964–6972.

[15] L. Meertens, C. Bertaux, T. Dragic, Hepatitis C virus entry requires a critical postinternalization step and delivery to early endosomes via clathrin-coated vesicles, J. Virol. 80 (2006) 11571–11578.

[16] D.M. Tscherne, C.T. Jones, M.J. Evans, B.D. Lindenbach, J.A. McKeating, C.M. Rice, Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry, J. Virol. 80 (2006) 1734–1741.

[17] A. Op De Beeck, C. Voisset, B. Bartosch, Y. Ciczora, L. Cocquerel, Z. Keck, S. Foung, F.L. Cosset, J. Dubuisson, Characterization of functional hepatitis C virus envelope glycoproteins, J. Virol. 78 (2004) 2994–3002.

[18] B. Bartosch, F.L. Cosset, Cell entry of hepatitis C virus, Virology 348 (2006) 1–12.

[19] E.G. Cormier, R.J. Durso, F. Tsamis, L. Boussemart, C. Manix, W.C. Olson, J.P. Gardner, T. Dragic, L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 14067–14072.

[20] E.G. Cormier, F. Tsamis, F. Kajumo, R.J. Durso, J.P. Gardner, T. Dragic, CD81 is an entry coreceptor for hepatitis C virus, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 7270–7274.

[21] G. Koutsoudakis, A. Kaul, E. Steinmann, S. Kallis, V. Lohmann, T. Pietschmann, R. Bartenschlager, Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses, J. Virol. 80 (2006) 5308–5320.

[22] N. Altan, Y. Chen, M. Schindler, S.M. Simon, Tamoxifen inhibits acidification in cells independent of the estrogen receptor, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 4432–4437.

[23] K. Watashi, D. Inoue, M. Hijikata, K. Goto, H.H. Aly, K. Shimotohno, Anti-hepatitis C virus activity of tamoxifen reveals the functional association of estrogen receptor with viral RNA polymerase NS5B, J. Biol. Chem. 282 (2007) 32765–32772.

[24] P. Gastaminza, C. Whitten-Bauer, F.V. Chisari, Unbiased probing of the entire hepatitis C virus life cycle identifies clinical compounds that target multiple aspects of the infection, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 291–296.
[25] K. Chockalingam, R.L. Simeon, C.M. Rice, Z. Chen, A cell protection screen reveals potent inhibitors of multiple stages of the hepatitis C virus life cycle, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 3764–3769.
[26] P. de Medina, G. Favre, M. Poirot, Multiple targeting by the antitumor drug tamoxifen: a structure-activity study, Curr. Med. Chem. Anticancer Agents 4 (2004) 491–508.
[27] E.R. Prossnitz, J.B. Arterburn, L.A. Sklar, GPR30: a G protein-coupled receptor for estrogen, Mol. Cell. Endocrinol. 265–266 (2007) 138–142.
[28] P. Thomas, Y. Pang, E.J. Filardo, J. Dong, Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells, Endocrinology 146 (2005) 624–632.
[29] M.K. Dennis, R. Burai, C. Ramesh, W.K. Petrie, S.N. Alcon, T.K. Nayak, C.G. Bologa, A. Leitao, E. Brailoiu, E. Deliu, N.J. Dun, L.A. Sklar, H.J. Hathaway, J.B. Arterburn, T.I. Oprea, E.R. Prossnitz, In vivo effects of a GPR30 antagonist, Nat. Chem. Biol. 5 (2009) 421–427.

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Inhibition of autophagy potentiates the antitumor effect of the multikinase inhibitor sorafenib in hepatocellular carcinoma

Satoshi Shimizu[1]*, Tetsuo Takehara[1]*, Hayato Hikita[1], Takahiro Kodama[1], Hinako Tsunematsu[1], Takuya Miyagi[1], Atsushi Hosui[1], Hisashi Ishida[1], Tomohide Tatsumi[1], Tatsuya Kanto[1], Naoki Hiramatsu[1], Naonobu Fujita[2], Tamotsu Yoshimori[2] and Norio Hayashi[3]

[1] Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[2] Department of Genetics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[3] Kansai-Rosai Hospital, Amagasaki, Hyogo, Japan

Multikinase inhibitor sorafenib inhibits proliferation and angiogenesis of tumors by suppressing the Raf/MEK/ERK signaling pathway and VEGF receptor tyrosine kinase. It significantly prolongs median survival of patients with advanced hepatocellular carcinoma (HCC) but the response is disease-stabilizing and cytostatic rather than one of tumor regression. To examine the mechanisms underlying the relative resistance in HCC, we investigated the role of autophagy, an evolutionarily conserved self-digestion pathway, in hepatoma cells *in vitro* and *in vivo*. Sorafenib treatment led to accumulation of autophagosomes as evidenced by conversion from LC3-I to LC3-II observed by immunoblot in Huh7, HLF and PLC/PRF/5 cells. This induction was due to activation of autophagic flux, as there was further increase in LC3-II expression upon treatment with lysosomal inhibitors, clear decline of the autophagy substrate p62, and an mRFP-GFP-LC3 fluorescence change in sorafenib-treated hepatoma cells. Sorafenib inhibited the mammalian target of rapamycin complex 1 and its inhibition led to accumulation of LC3-II. Pharmacological inhibition of autophagic flux by chloroquine increased apoptosis and decreased cell viability in hepatoma cells. siRNA-mediated knockdown of the ATG7 gene also sensitized hepatoma cells to sorafenib. Finally, sorafenib induced autophagy in Huh7 xenograft tumors in nude mice and coadministration with chloroquine significantly suppressed tumor growth compared with sorafenib alone. In conclusion, sorafenib administration induced autophagosome formation and enhanced autophagic activity, which conferred a survival advantage to hepatoma cells. Concomitant inhibition of autophagy may be an attractive strategy for unlocking the antitumor potential of sorafenib in HCC.

Sorafenib is an orally available multikinase inhibitor recently approved as the first molecular targeting compound for hepatocellular carcinoma (HCC).[1] Sorafenib inhibits Raf kinases, including Raf-1 and B-Raf, which are members of the Raf/MEK/ERK signaling pathway, and inhibits a number of receptor tyrosine kinases involved in neo-angiogenesis and tumor progression, such as vascular endothelial growth factor receptor (VEGFR) 2, platelet-derived growth factor receptor β and c-Kit. Two randomized, placebo-controlled trials revealed that sorafenib significantly prolongs the median survival of patients with advanced HCC but the response is disease-stabilizing and cytostatic rather than one of tumor regression.[2,3] Therefore, a more detailed understanding of the mechanisms underlying both the antitumor effect and the primary resistance to this compound may provide insights that can help to improve the therapeutic outcome in HCC.

Macroautophagy (hereafter referred to as autophagy) is an evolutionally conserved catabolic process that transports cellular macromolecules and organelles to a lysosomal degradation pathway.[4] It is regulated by autophagy-related (*atg*) genes that control the formation and maturation of a double-membrane vesicle, autophagosome, which sequestrates cellular proteins and organelles. Autophagosomes then fuse with lysosomes to form autolysosomes, in which lysosomal enzymes digest the sequestered content and inner membrane. Autophagy is typically induced under starvation, initially considered to be a survival strategy that recycles cellular components to meet energy requirements. Autophagy also occurs at low basal levels in virtually all cells to perform homeostatic functions such as turnover of long-lived or damaged proteins and organelles. On the other hand, autophagy can mediate cell death under certain conditions probably through overactivation of self-digestion, which is considered to be Type II programmed cell death.[5] Therefore, autophagy can promote both cell survival and death depending on the cellular context and/or initiating stimulus.

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*S.S. and T.T. contributed equally to this work and share first authorship.
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Correspondence to: Tetsuo Takehara, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan, Tel.: +81-6-6879-3621, Fax: +81-6-6879-3629, E-mail: takehara@gh.med.osaka-u.ac.jp

Autophagy has been shown to be involved in cancer development and progression in a variety of ways.[6] Genetic evidence supports a tumor suppressive role of autophagy in cancer development. The *Beclin 1* autophagy gene is monoallelically deleted in a subset of human sporadic breast, ovarian and prostate cancer. Heterozygous disruption of *Beclin 1* increases the frequency of spontaneous malignancies in mice.[7] On the other hand, tumor cells display autophagy or autophagic cell death under a variety of stress-inducing conditions as well as anticancer therapies.[8] Therefore, autophagy promotes or inhibits tumor progression which is also dependent on the cell types and stimuli. Recently, sorafenib has been reported to induce autophagosome accumulation, as evidenced by GFP-LC3 markers, in tumor cells.[9–11] However, its biological and clinical significance has not yet been addressed. In the present study, we examined autophagy of hepatoma cells treated with sorafenib and demonstrate that sorafenib not only induces autophagosome formation but also activates autophagic flux which is an adaptive response to this compound, and that concomitant inhibition of autophagy may be therapeutically useful for improving the anti-HCC effect.

Material and Methods

Cell lines

Hepatoma cell lines Huh7, HLF and PLC/PRF/5 were cultured with Dulbecco's modified Eagle medium (DMEM). Huh7 and HLF were obtained from the JCRB/HSRRB cell bank (Osaka, Japan) and PLC/PRF/5 was obtained from ATCC (Manassas, VA). All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2.

Western immunoblot

Cells or tissues were lysed and immunoblotted as previously described.[12] For immunodetection, the following antibodies were used: anti-microtubule-associated protein 1 light chain (LC3) polyclonal antibody (Ab) (MBL, Nagoya, Japan); anti-ATG7 polyclonal Ab (MBL); anti-Beclin1 polyclonal Ab (CST, Danvers, MA); anti-p62 polyclonal Ab (MBL); anti-phospho-ERK polyclonal Ab (CST); anti-phospho-S6K polyclonal Ab (CST); anti-phospho-4E-BP1 polyclonal Ab (CST); anti-phospho-Akt polyclonal Ab (CST).

Transfection with fluorescent LC3 plasmid

Cells were transfected with monomeric red fluorescence protein (mRFP)-GFP tandem fluorescent-tagged LC3 expression plasmid (ptfLC3)[13] using Fugene6 (Roche Applied Science, Hague Road, IN) according to the manufacturer's instructions. At 48 hr after transfection, the medium was changed to DMEM containing sorafenib or DMSO, and the cells were further cultured and examined under a BZ8100 fluorescent microscope (Keyence, Osaka, Japan).

In vitro treatment with sorafenib

Hepatoma cells were transfected with 5 nM Silencer Select siRNAs (Ambion, Austin, TX) either of *ATG7* or negative control using RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Forty-eight hours after transfection, the medium was changed to DMEM containing sorafenib or DMSO. Cells were further cultured and assayed for cell viability by WST assay using the cell count reagent SF (Nacalai Tesque, Kyoto, Japan) and analyzed for apoptosis using Annexin V-FITC apoptosis detection kit (Biovision, Mountain View, CA). We defined apoptotic cells as Annexin V-FITC positive and propidium iodide (PI) negative cells. PI negative cells were gated and the positive cell rate of Annexin V-FITC was determined. The supernatant of the cultured cells was assayed for caspase-3/7 activity using Caspase-Glo 3/7 assay (Promega, Madison, WI) as previously reported.[12] For the treatment with a pharmacological inhibitor of autophagy, cells were cultured with DMEM containing chloroquine (Sigma-Aldrich, St. Louis, MO) or bafilomycin A1 (Sigma-Aldrich) with sorafenib or DMSO and assayed for cell viability and caspase-3/7 activity in the same manner.

Electron microscopy

Samples were fixed with 2.5% glutaraldehyde solution buffered at pH 7.4 with 0.1 M Millonig's phosphate at 4°C for 2 hr, postfixed in 1% osmium tetroxide solution at 4°C for 1 hr, dehydrated in graded concentrations of ethanol and embedded in Nissin EM Quetol 812 epoxy resin. Ultrathin sections (80 nm) cut on a Reichert ultramicrotome (Ultracut E) were stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7650 electron microscope at 80 kV.

Xenograft experiments

To produce a xenograft tumor, 3–5 $\times$ 10^6 Huh7 cells were subcutaneously injected to Balb/c nude mice. Sorafenib tablets were crushed and orally administered daily with water containing 12.5% cremophor EL (Sigma-Aldrich) and 12.5% ethanol, as previously described.[14] Chloroquine was dissolved in PBS and intraperitoneally administered daily. We estimated the volume of the xenograft tumor using the following formula: tumor volume $= \pi/6 \times$ (major axis) $\times$ (minor axis)^2. Mice were maintained in a specific pathogen-free facility and treated with humane care with approval from the Animal Care and Use Committee of Osaka University Medical School.

Statistical analysis

Data are presented as mean $\pm$ SD. Comparisons between two groups were performed by unpaired t test. Multiple comparisons were performed by ANOVA with Scheffe post-hoc test. $p < 0.05$ was considered statistically significant.

Results

In vitro treatment with sorafenib induces accumulation of autophagosomes in hepatoma cell lines

To examine the effect of sorafenib on autophagy in human HCC, we treated the hepatoma cell line Huh7 with sorafenib *in vitro*. First, we assessed the expression of LC3, a

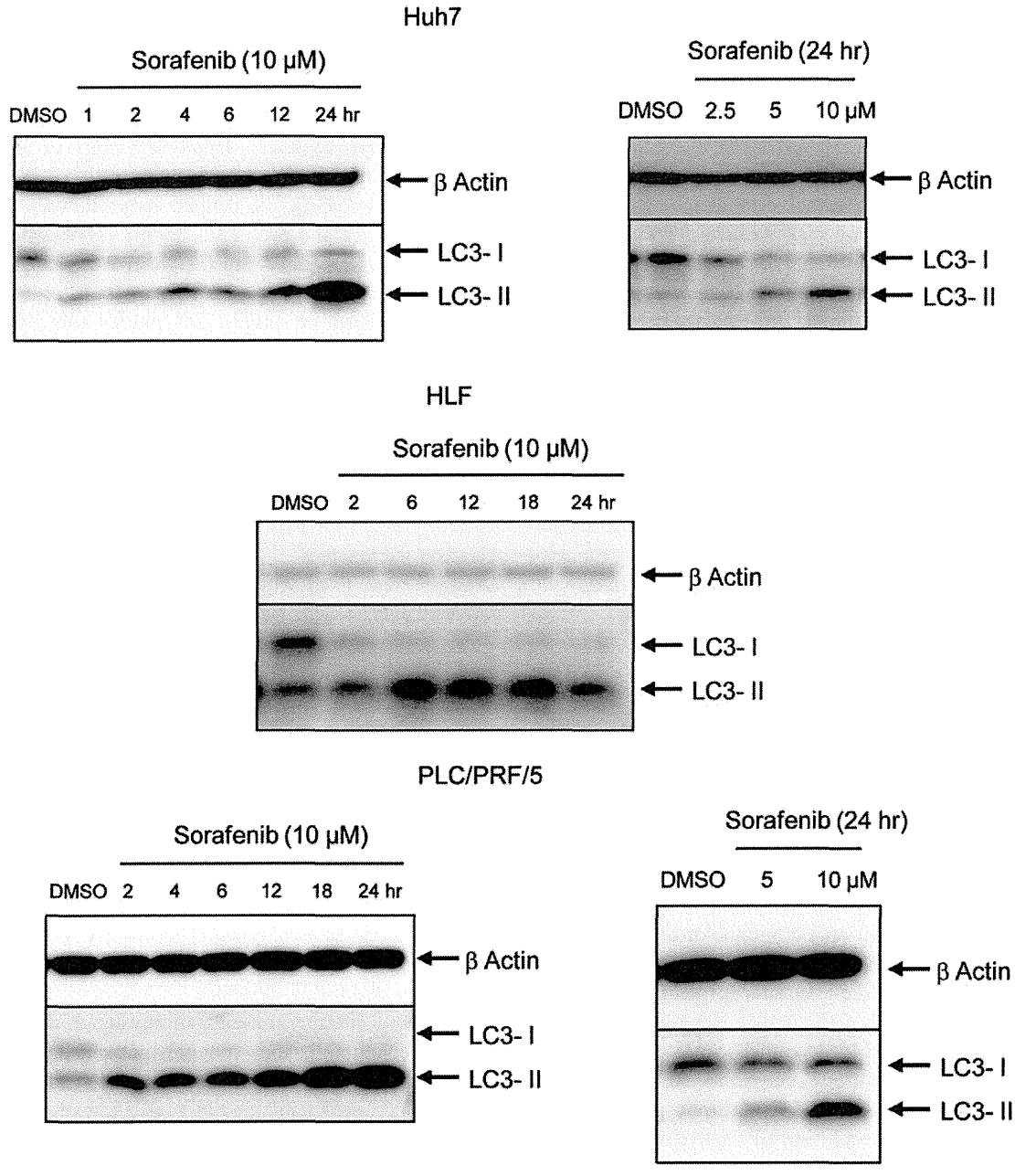

Figure 1. Sorafenib induces accumulation of autophagosomes in hepatoma cells. Western blot showing an increase in LC3-II in Huh7, HLF and PLC/PRF/5 hepatoma cells after treatment with sorafenib. Hepatoma cells were treated with 2.5, 5 or 10 μM sorafenib for the indicated times and analyzed for LC3 expression by western blot. Hepatoma cells treated with DMSO-containing media for 24 hr are shown as the control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mammalian homolog of yeast *atg8*, by immunoblot. During the progress of autophagy, the cytoplasmic form LC3-I is converted to the membrane-bound lipidated form LC3-II which is detected by a mobility shift on electrophoresis.[15] When Huh7 cells were treated with 10 μM sorafenib, LC3 conversion was observed as early as 1 hr after the treatment and gradually increased at later time points (Fig. 1). We examined the dose-dependency of this response in Huh7 cells as well. Under 2.5 μM sorafenib treatment, the amount of LC3-II did not show an obvious increase, however, the amount of LC3-I decreased which indicates modest activation of autophagosome formation. Under 5 and 10 μM sorafenib treatment, the amount of LC3-II clearly increased. Next, we investigated the effect of sorafenib on other hepatoma cell lines, HLF and PLC/PRF/5. Under sorafenib treatment, LC3 conversion was observed at 2 hr after the initiation of treatment and gradually increased until 24 hr in HLF cells and PLC/PRF/5 cells in the same manner as in Huh7 cells.

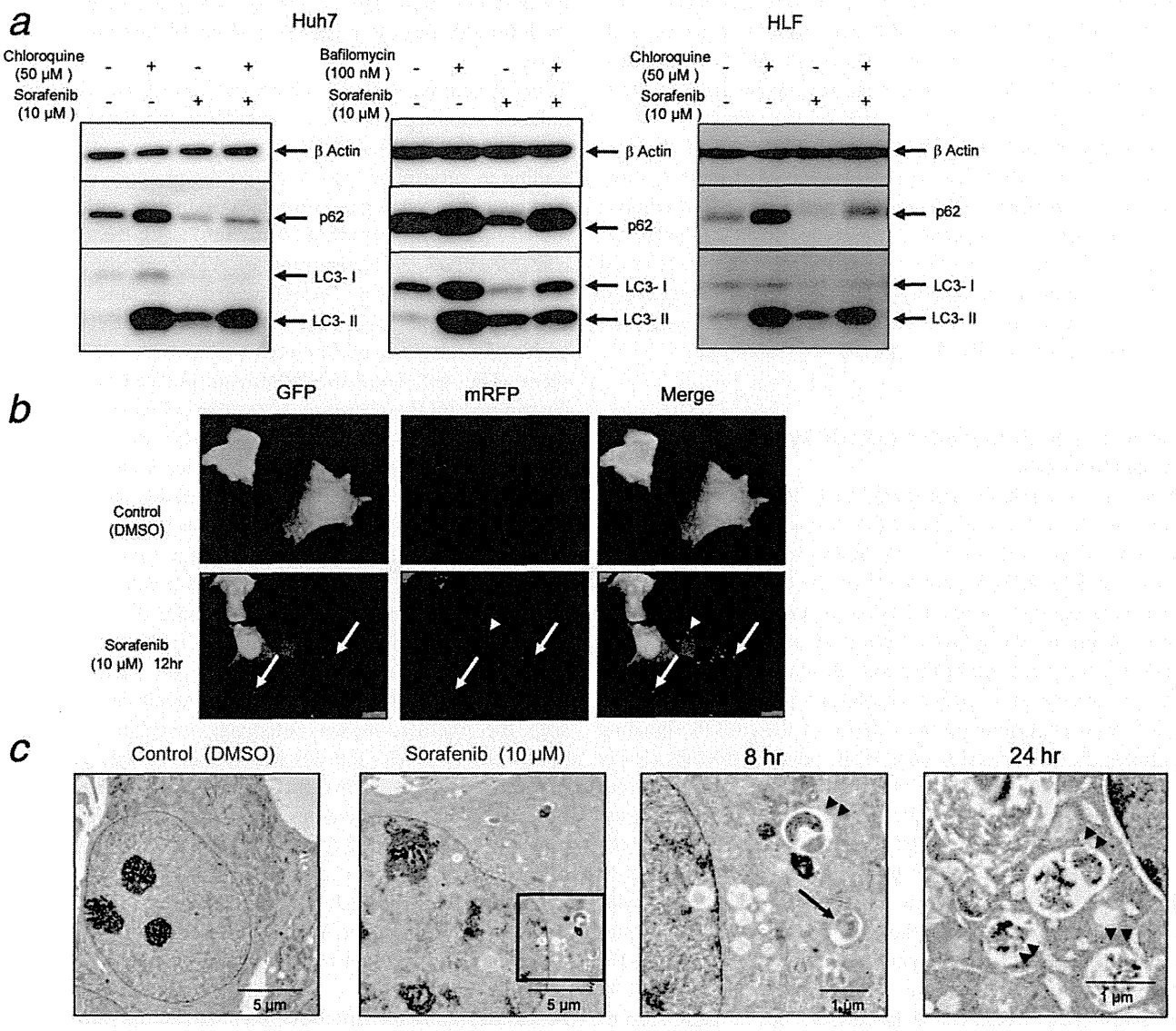

Figure 2. Sorafenib activates autophagic flux in hepatoma cells. (*a*). Western blot showing p62 degradation and LC3 lipidation in Huh7 cells and HLF cells treated with sorafenib and/or lysosomal inhibitors. Huh7 cells or HLF cells were treated with or without 10 μM sorafenib in the presence or absence of 50 μM chloroquine or 100 nM bafilomycin A1 for 12 hr. (*b*). Photographs of fluorescence microscopy of punctate fluorescence of a transfected mRFP-GFP-LC3 construct in Huh7 cells after 12-hr treatment with 10 μM sorafenib. Arrows indicate a typical example of colocalized particles of GFP and mRFP signal, while the arrowhead points to a typical example of a particle with an mRFP signal but without a GFP signal. *C.* Photographs from transmission electron microscopy showing autophagic vacuoles including autophagosomes (arrow) and probably autolysosomes (arrowhead) in Huh7 cells treated with 10 μM sorafenib.

Sorafenib activates autophagic flux in hepatoma cells

To clarify whether the accumulation of autophagosomes induced by sorafenib is a result of induction of autophagosome formation or inhibition of autophagosome degradation, we first measured the amount of p62, a selective substrate of autophagy, by immunoblot. Activation of the autophagic flux leads to a decline in p62 expression, and *vice versa*.[16] When Huh7 cells or HLF cells were treated with sorafenib, the amount of p62 decreased despite the accumulation of LC3-II implying that this accumulation of LC3-II is associated with autophagosome degradation (Fig. 2*a*). In addition, when cells were treated with both sorafenib and chloroquine, accumulation of LC3-II was further enhanced compared to the sorafenib-treated group, while the levels of p62 expression increased. We also used bafilomycin A1, which inhibits fusion of autophagosome and lysosome, and obtained similar results. Our findings indicate that the LC3-II accumulation induced by sorafenib results from activation of autophagosome formation but not from just inhibition of the autophagosome degradation steps. Second, we examined the color

change of mRFP-GFP tandem fluorescent-tagged LC3 (mRFP-GFP-LC3). When Huh7 cells were transfected with the mRFP-GFP-LC3 expression plasmid ptfLC3 and then treated with sorafenib, some punctate signals showed both GFP and mRFP signals but part of the punctate signals exhibited only mRFP signals (Fig. 2*b*). Because GFP fluorescence but not mRFP fluorescence is attenuated under lysosomal acidic condition,[13] this observation supports that autophagy induced by sorafenib proceeds to the lysosomal degradation phase. Finally, electron microscopy revealed abundant autophagic vacuoles such as autophagosomes and probably autolysosomes in sorafenib-treated Huh7 cells, but scarcely in control cells (Fig. 2*c*).

Sorafenib selectively inhibits the activity of TORC1 in hepatoma cells

Sorafenib was initially developed as a Raf kinase inhibitor, however, it can also inhibit other tyrosine kinases such as VEGR-2, Flt-3 and c-Kit.[17] The inhibitory effect of sorafenib on the Raf/MEK/ERK pathway[18] or the STAT3 pathway[19] is widely recognized in several types of cancer, but the effect of sorafenib on the PI3K/Akt pathway and the mTOR pathway has not been established yet. Because the mTOR pathway is known as a major regulatory pathway of autophagy,[20] we next examined the activity of the mTOR signaling pathway in Huh7 cells and HLF cells. Sorafenib clearly inhibited the activity of the mammalian target of rapamycin complex 1 (mTORC1), which is measured by the dephosphorylation of S6K and 4E-BP1 in Huh7 cells and HLF cells (Fig. 3*a*). 4E-BP1 is initially phosphorylated at threonine 37 and threonine 46, which promotes subsequent phosphorylation and decreases electrophoretic mobility.[21] With sorafenib administration, the upper band of phosphorylated 4E-BP1 gradually decreased and shifted to the lower band. At 24 hours after treatment initiation, the lower band diminished as well, indicating further dephosphorylation of 4E-BP1 at threonine 37 and 46. On the other hand, sorafenib treatment increased the phosphorylation of Akt at threonine 308 and serine 473 in these cells. The phosphorylation at threonine 308 suggests the activation of upstream PI3K while the phosphorylation at serine 473 suggests the activation of mTORC2.[22] Therefore, sorafenib can be presumed to possess a selective inhibitory effect on the activity of mTORC1 independent of PI3K and Akt. Administration of sorafenib clearly inhibited the phosphorylation of ERK as early as 2 hours after treatment, which is consistent with a previous report.[18] The expression of ATG7 and Beclin 1, autophagy-related gene products, did not change under sorafenib treatment. Next, we treated Huh7 cells with rapamycin or Torin1[23] to determine the impact of mTORC1 activity on autophagy induction. As expected, the levels of LC3-II increased upon rapamycin treatment in Huh7 cells (Fig. 3*b*). A similar result was obtained using another mTOR inhibitor, Torin1.

Inhibition of autophagy by siRNAs or a pharmacological inhibitor enhanced the apoptotic effect of sorafenib *in vitro*

From these results, we considered two possibilities: sorafenib-induced autophagy may be a mechanism of action of the antitumor effect of sorafenib or a stress-responsive phenomenon leading to survival of tumor cells in the presence of sorafenib treatment. To investigate the role of autophagy under sorafenib treatment, we introduced into Huh7 cells, the siRNA specific for *ATG7*. Administration of *ATG7* siRNA suppressed LC3-II expression in DMSO-treated cells and sorafenib-treated cells, indicating that autophagy is clearly suppressed under physiological conditions as well as with sorafenib treatment (Fig. 4*a*). Sorafenib treatment induced apoptosis, as determined by the elevation of caspase-3/7 activity or by the increase of Annexin V positive cells, and decreased the viability of Huh7 cells (Fig. 4*b*). Of importance is the finding that *ATG7* knockdown significantly enhanced the sorafenib-induced apoptosis and decreased cell viability in Huh7 cells. These observations imply that autophagy plays a protective role for hepatoma cells under sorafenib treatment and could be a target for enhancing its antitumor effects. We performed an ATG7 knockdown experiment using HLF cells as well and obtained a similar result (Fig. 4*c*).

Next, we treated Huh7 cells with sorafenib in combination with the pharmacological autophagy inhibitor chloroquine, which clearly blocks the downstream autophagic pathway in hepatoma cells as shown in Figure 2*a*. Chloroquine itself induced a modest activation of caspase-3/7 at a high dose under our experimental conditions (Fig. 5). However, in combination with sorafenib, chloroquine markedly enhanced the apoptotic effect of sorafenib and reduced cell viability in a dose-dependent manner. We investigated the effect of chloroquine on PLC/PRF/5 cells as well, and obtained a similar result.

Autophagy inhibitor chloroquine enhanced the anti-tumor effect of sorafenib in a xenograft model

To examine the significance of autophagy *in vivo*, nude mice were subcutaneously injected with Huh7 cells to generate xenograft tumors. To examine whether sorafenib induces autophagy in the *in vivo* setting, we administered sorafenib or vehicle for 7 days to mice bearing xenograft tumors. As we reported previously,[14] sorafenib treatment significantly suppressed tumor growth compared with the vehicle alone (data not shown). Consistent with the *in vitro* finding, xenograft tumors from sorafenib-administered mice displayed accumulation of LC3-II on immunoblot compared with those from vehicle-treated mice (Fig. 6*a*). To examine the therapeutic significance of autophagy inhibition for sorafenib therapy, mice with Huh7 xenograft were randomly assigned to two groups when the diameter of the subcutaneous tumor reached about 1 centimeter: sorafenib administration group and sorafenib plus chloroquine administration group. Coadministration of chloroquine and sorafenib for 7 days led to significant suppression of tumor growth compared with

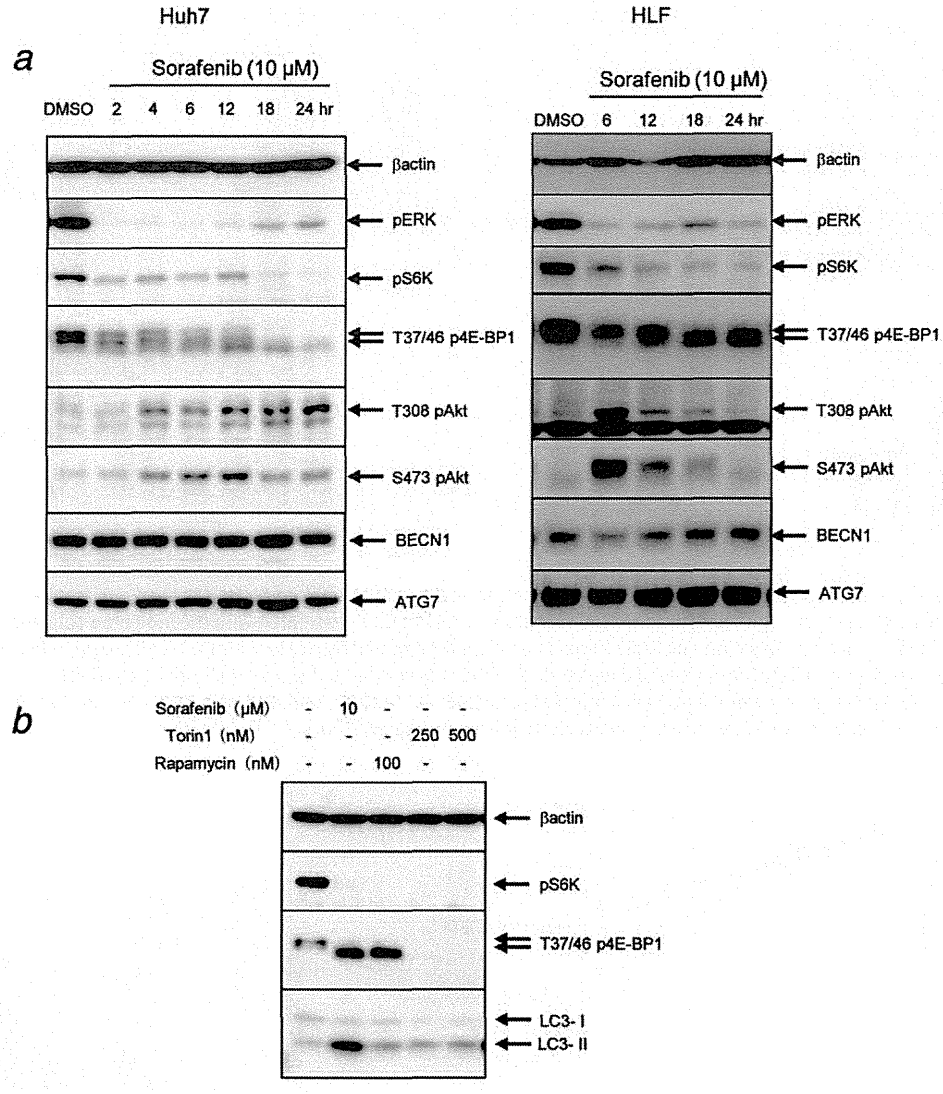

Figure 3. Raf/MEK/ERK and Akt/mTOR/S6K pathways in hepatoma cells treated with sorafenib. (*a*). Western blot showing decrease in ERK, S6K and 4E-BP1 phosphorylation, increase in Akt phosphorylation and stable expression of Beclin 1 and ATG7 in Huh7 cells and HLF cells after treatment with 10 μM sorafenib. (*b*). Western blot showing that rapamycin or Torin1 dephosphorylates both S6K and 4E-BP1 and increases the expression of LC3-II in Huh7 cells. Huh7 cells were treated with 100 nM rapamycin or the indicated concentration of Torin1 for 12 hr. Huh7 treated with sorafenib (10 μM, 12 hr) serves as a positive control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

administration of sorafenib alone (Fig. 6*b*). Administration of chloroquine alone did not affect the growth of the tumor. We performed TUNEL staining and immunohistological staining of cleaved caspase-3 of the xenograft tumor to examine the contribution of apoptosis in this xenograft model. However, nonspecific staining of the xenograft tumors treated with sorafenib interfered with an accurate evaluation of the apoptotic change (data not shown).

Discussion

Accumulating evidence indicates that cancer therapies such as irradiation and administration of cytotoxic drugs and chemicals induce autophagy and autophagic cell death in a variety of tumor cells.[8] Research has shown that autophagy induced by these treatments sometimes protects tumor cells (autophagic resistance) but promotes cell death in other settings (autophagic Type II programmed cell death). For example, temozolomide, a DNA alkylating agent,[24] and ionizing radiation[25] induce autophagy in malignant glioma cells and a variety of epithelial tumors, respectively, and this inhibition enhances antitumor effects. On the other hand, poly(dI:dC) induces endosome-mediated autophagy leading to cell death in melanoma cells.[26] Arsenic trioxide induces autophagic cell death in leukemia cells.[27] In the present study, we demonstrated that sorafenib, a recently approved molecular targeting drug for HCC, induced autophagy which appeared to

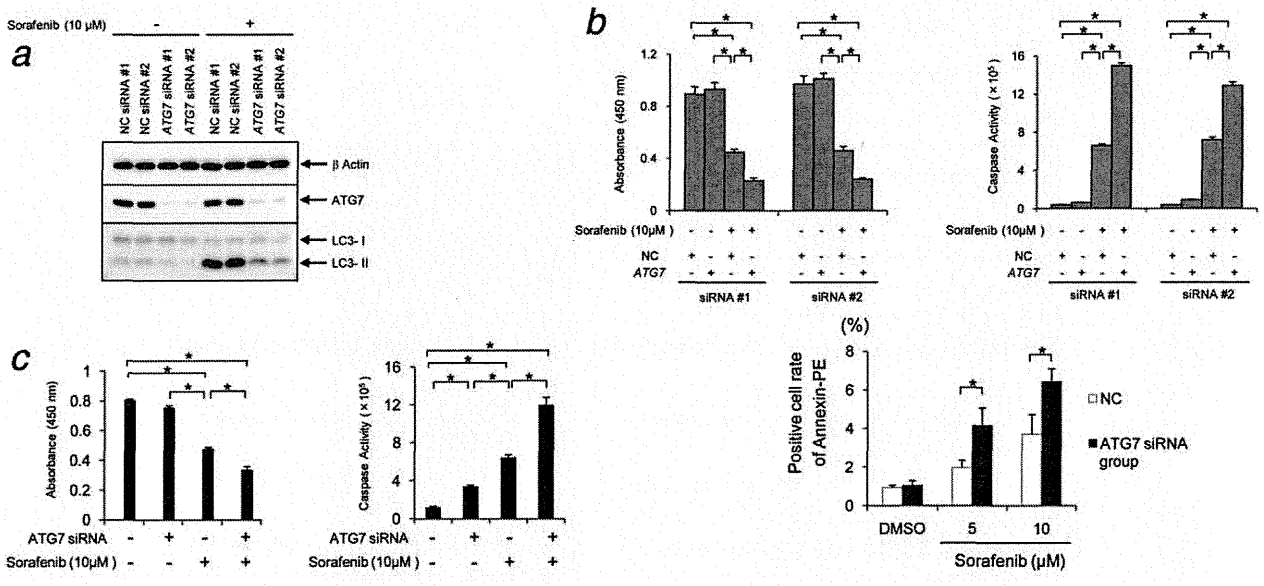

Figure 4. Genetic ablation of autophagy increases sensitivity of hepatoma cells to sorafenib. (*a*,*b*). Huh7 cells were transfected with two different sets of *ATG7* siRNA (no. 1 and 2) or control siRNA (no. 1 and 2) for 48 hr and then treated with the indicated concentration of sorafenib or vehicle for an additional 18 hr. LC3 lipidation and ATG7 expression were determined by western blot (*a*). Cell growth was determined by WST assay, while apoptosis was monitored by the activity of caspase-3/7 in the supernatant or by annexin V positive cell rate ($n = 4$) (*b*). (*c*) HLF cells were transfected with *ATG7* siRNA and examined for cell viability and caspase-3/7 activity in the same manner as Huh7 cells ($n = 4$). *$p < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

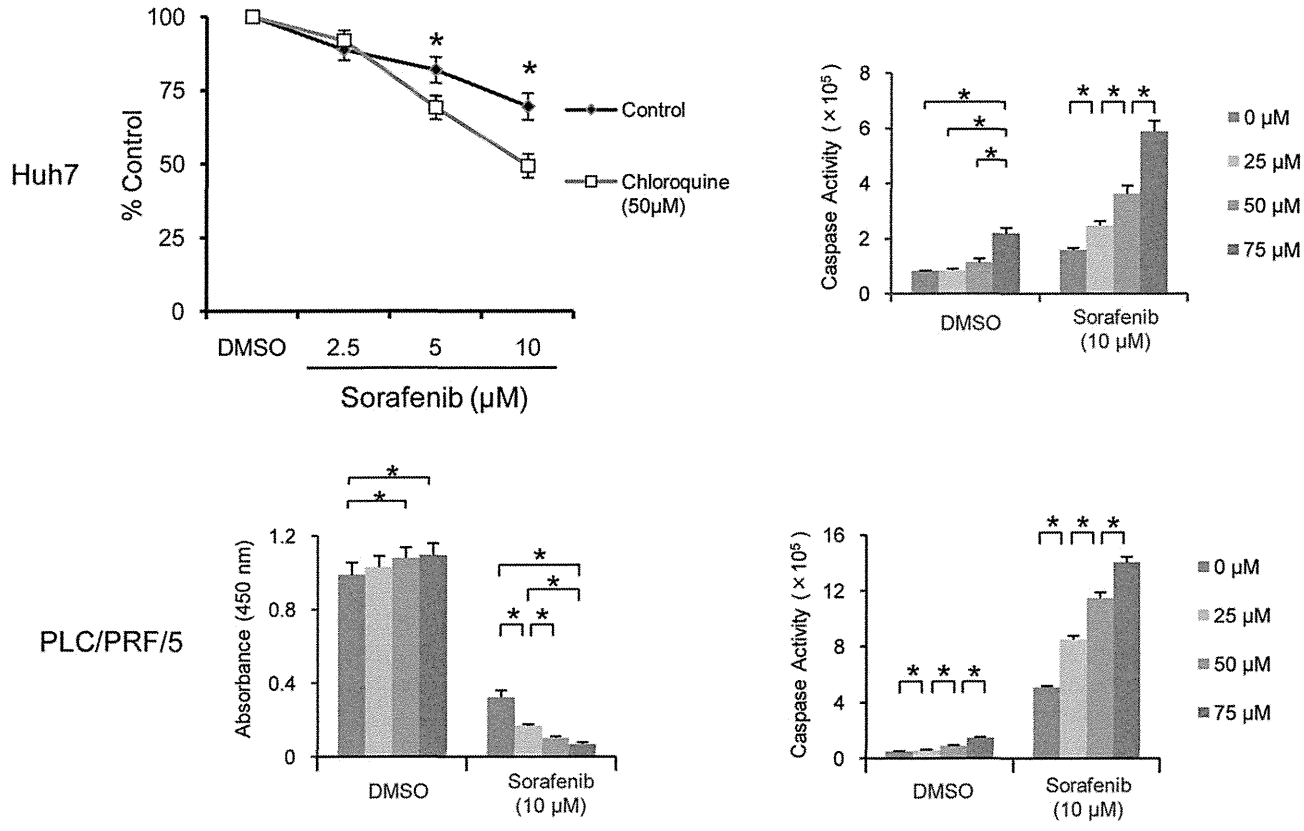

Figure 5. Pharmacological inhibition of autophagy increases sensitivity of hepatoma cells to sorafenib. Huh7 cells or PLC/PRF/5 cells were treated with or without the indicated concentration of sorafenib in the presence or absence of chloroquine for 18 hr. Capsase-3/7 activity was monitored in the supernatant, while cell growth was determined by WST assay ($n = 4$). *$p < 0.05$.

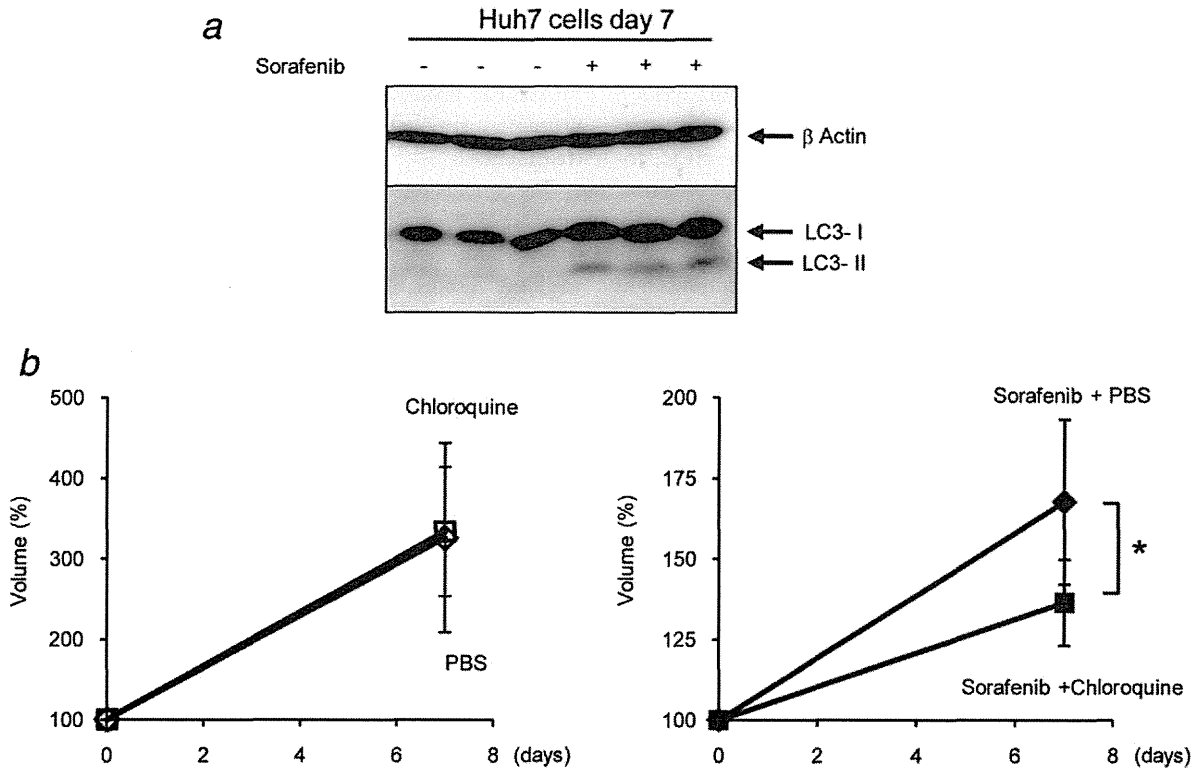

Figure 6. Inhibition of autophagy potentiates sorafenib-induced antitumor effects in Huh7 xenograft. (*a*). Western blot showing increase in LC3-II expression in Huh7 xenograft tumor after sorafenib therapy. Mice bearing xenograft tumor were administered sorafenib (30 mg kg^{-1}) or vehicle for 7 days ($n = 3$/group). (*b*). Chloroquine (60 mg kg^{-1}) itself did not affect the tumor growth of Huh7 xenograft (left panel), ($n = 7$/group), but enhanced the effect of sorafenib (30 mg kg^{-1}) in a synergistic manner (right panel), ($n = 6$/group). Mice bearing xenograft tumor were administered sorafenib and/or chloroquine for 7 days. Tumor volume at 7 days is shown as a percentage of that before initiation of the therapy. *$p < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

promote survival of hepatoma cells and thereby may be a cellular adaptive response related to primary resistance to this compound.

LC3 lipidation and its association with the isolation membranes have been established as useful signs for autophagy detectable by immunoblotting and fluorescence microscopy, facilitating research on autophagy. Previous research has shown that sorafenib induces GFP-LC3 punctate structure and LC3-II conversion in tumor cells.[9–11] However, these techniques should be analyzed more carefully, because positive results clearly indicate increased numbers of autophagosomes but do not always mean upregulation of autophagic flux.[28] For example, treatment with vinblastine or nocodazole leads to LC3 conversion and produces GFP-LC3 punctate structures, resulting from blockade of the fusion of autophagosomes and lysosomes but not from autophagy induction.[29,30] In the present study, we applied several methods including LC3 turnover assay using a lysosomal inhibitor of chloroquine or bafilomycin A1, measurement of the amount of a selective autophagy substrate p62, and observation of the mRFP-GFP color change using a fluorescent-tagged LC3 probe, to obtain evidence showing that sorafenib not only increases the number of autophagosomes but also activates the autophagic flux.

The underlying mechanisms by which sorafenib induces autophagy are not completely clear at present. In addition to the well-known target Raf/MEK/MAPK pathway, sorafenib clearly inhibited the mTORC1 pathway in the present study. Because mTOR inhibition by rapamycin or Torin1 activates autophagosome formation in hepatoma cells, sorafenib-induced inhibition of the mTORC1 pathway might be involved in sorafenib-mediated induction of autophagy. Recently, a putative tumor-suppressor gene *p53* has been shown to transactivate an autophagy-inducing gene, *dram*,[31] and *p53*-dependent induction of autophagy has been documented in response to DNA damage or reexpression of p53 in p53-negative tumor cells.[32] Because the hepatoma cells used in the present study (Huh7, HLF and PLC/PRF/5) possess mutant *p53*, sorafenib-induced adaptive autophagy could occur independently of p53. This finding may be important, because more than half of advanced HCC cases are p53-defective.[33] In such cases, our observations could be applicable and relevant.

Study of rodent carcinogenesis has revealed that autophagic protein degradation is reduced in HCC.[34] In human,

malignant HCC cell lines and HCC tissue with recurrent disease display lower autophagic activity with decreased expression of Beclin 1.[35] The autophagic pathway contributes to the growth-inhibitory effect of TGF-beta in hepatoma cells.[36] Taken together, these findings suggest that defects in autophagy may promote development or progression of HCC, focusing on the tumor suppressive or antitumor effect of autophagy in the liver or HCC. In contrast, the present study clearly showed that autophagy induced by sorafenib protects hepatoma cells from apoptotic cell death, thus shedding light on the tumor-promoting effect of autophagy in HCC. Inhibition of autophagy at both an early step (by *ATG7* knockdown) and a late step (by chloroquine treatment) sensitized hepatoma cells by converting the autophagic process to an apoptotic process. Of importance are the findings that sorafenib induced autophagy in a xenograft model and that coadministration of chloroquine and sorafenib led to better suppression of xenograft tumor than sorafenib alone. Although further study is needed to elucidate the mechanism(s) involved in autophagy-mediated protection of tumor cells, the induced autophagy might degrade the damaged or harmful cellular proteins and organelles to suppress apoptosis and promote survival of hepatoma cells under sorafenib treatment.

In conclusion, the present study demonstrates both *in vitro* and *in vivo* that sorafenib induces autophagosome formation and upregulates cellular autophagy in tumor cells, which is an adaptive response to this drug, and raises the important possibility that autophagy may be a novel target for cancer treatment with sorafenib therapy.

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References

1. Finn RS. Drug therapy: sorafenib. *Hepatology* 2010;51:1843–9.
2. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;359:378–90.
3. Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009;10:25–34.
4. Yoshimori T. Autophagy: a regulated bulk degradation process inside cells. *Biochem Biophys Res Commun* 2004;313:453–8.
5. Tsujimoto Y, Shimizu S. Another way to die: autophagic programmed cell death. *Cell Death Differ* 2005;12 (Suppl 2):1528–34.
6. White E, DiPaola RS. The double-edged sword of autophagy modulation in cancer. *Clin Cancer Res* 2009;15:5308–16.
7. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* 2003;112: 1809–20.
8. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. *Nat Rev Cancer* 2005;5: 726–34.
9. Ullén A, Farnebo M, Thyrell L, Mahmoudi S, Kharaziha P, Lennartsson L, Grandér D, Panaretakis T, Nilsson S. Sorafenib induces apoptosis and autophagy in prostate cancer cells in vitro. *Int J Oncol* 2010;37:15–20.
10. Park MA, Zhang G, Martin AP, Hamed H, Mitchell C, Hylemon PB, Graf M, Rahmani M, Ryan K, Liu X, Spiegel S, Norris J, et al. Vorinostat and sorafenib increase ER stress, autophagy and apoptosis via ceramide-dependent CD95 and PERK activation. *Cancer Biol Ther* 2008;7:1648–62.
11. Park MA, Reinehr R, Häussinger D, Voelkel-Johnson C, Ogretmen B, Yacoub A, Grant S, Dent P. Sorafenib activates CD95 and promotes autophagy and cell death via Src family kinases in gastrointestinal tumor cells. *Mol Cancer Ther* 2010;9:2220–31.
12. Shimizu S, Takehara T, Hikita H, Kodama T, Miyagi T, Hosui A, Tatsumi T, Ishida H, Noda T, Nagano H, Doki Y, Mori M, et al. The let-7 family of microRNAs inhibits Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma. *J Hepatol* 2010; 52:698–704.
13. Kimura S, Noda T, Yoshimori T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* 2007;3:452–60.
14. Hikita H, Takehara T, Shimizu S, Kodama T, Shigekawa M, Iwase K, Hosui A, Miyagi T, Tatsumi T, Ishida H, Li W, Kanto T, et al. The Bcl-xL inhibitor, ABT-737, efficiently induces apoptosis and suppresses growth of hepatoma cells in combination with sorafenib. *Hepatology* 2010;52:1310–21.
15. Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. *Autophagy* 2007;3:542–5.
16. Bjørkøy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, Johansen T. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* 2005;171:603–14.
17. Sridhar SS, Hedley D, Siu LL. Raf kinase as a target for anticancer therapeutics. *Mol Cancer Ther* 2005;4:677–85.
18. Liu L, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, Wilhelm S, Lynch M, Carter C. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Res* 2006;66:11851–8.
19. Blechacz BR, Smoot RL, Bronk SF, Werneburg NW, Sirica AE, Gores GJ. Sorafenib inhibits signal transducer and activator of transcription-3 signaling in cholangiocarcinoma cells by activating the phosphatase shatterproof 2. *Hepatology* 2009;50:1861–70.
20. Díaz-Troya S, Pérez-Pérez ME, Florencio FJ, Crespo JL. The role of TOR in autophagy regulation from yeast to plants and mammals. *Autophagy* 2008;4:851–65.
21. Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, Aebersold R, Sonenberg N. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev* 1999;13:1422–37.
22. Foster KG, Fingar DC. Mammalian target of rapamycin (mTOR): conducting the cellular signaling symphony. *J Biol Chem* 2010;285: 14071–7.
23. Thoreen CC, Kang SA, Chang JW, Liu Q, Zhang J, Gao Y, Reichling LJ, Sim T, Sabatini DM, Gray NS. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *J Biol Chem* 2009;284: 8023–32.
24. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Differ* 2004;11:448–57.
25. Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D, Yahalom J. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* 2001;61:439–44.
26. Tormo D, Checińska A, Alonso-Curbelo D, Pérez-Guijarro E, Cañón E, Riveiro-Falkenbach E, Calvo TG, Larribere L, Megías D, Mulero F, Piris

MA, Dash R, et al. Targeted activation of innate immunity for therapeutic induction of autophagy and apoptosis in melanoma cells. *Cancer Cell* 2009;16:103–14.
27. Goussetis DJ, Altman JK, Glaser H, McNeer JL, Tallman MS, Platanias LC. Autophagy is a critical mechanism for the induction of the antileukemic effects of arsenic trioxide. *J Biol Chem* 2010;285: 29989–97.
28. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. *Cell* 2010;140: 313–26.
29. Seglen PO, Brinchmann MF. Purification of autophagosomes from rat hepatocytes. *Autophagy* 2010;6:542–7.
30. Bampton ET, Goemans CG, Niranjan D, Mizushima N, Tolkovsky AM. The dynamics of autophagy visualized in live cells: from autophagosome formation to fusion with endo/lysosomes. *Autophagy* 2005;1:23–36.
31. Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, Gasco M, Garrone O, Crook T, Ryan KM. DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell* 2006;126:121–34.
32. Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, Thomas-Tikhonenko A, Thompson CB. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Invest* 2007;117:326–36.
33. Hussain SP, Schwank J, Staib F, Wang XW, Harris CC. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene* 2007;26: 2166–76.
34. Kisen GO, Tessitore L, Costelli P, Gordon PB, Schwarze PE, Baccino FM, Seglen PO. Reduced autophagic activity in primary rat hepatocellular carcinoma and ascites hepatoma cells. *Carcinogenesis* 1993;14: 2501–5.
35. Ding ZB, Shi YH, Zhou J, Qiu SJ, Xu Y, Dai Z, Shi GM, Wang XY, Ke AW, Wu B, Fan J. Association of autophagy defect with a malignant phenotype and poor prognosis of hepatocellular carcinoma. *Cancer Res* 2008;68: 9167–75.
36. Kiyono K, Suzuki HI, Matsuyama H, Morishita Y, Komuro A, Kano MR, Sugimoto K, Miyazono K. Autophagy is activated by TGF-beta and potentiates TGF-beta-mediated growth inhibition in human hepatocellular carcinoma cells. *Cancer Res* 2009;69:8844–52.

Research Article

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Bak deficiency inhibits liver carcinogenesis: A causal link between apoptosis and carcinogenesis

Hayato Hikita[1], Takahiro Kodama[1], Satoshi Shimizu[1], Wei Li[1], Minoru Shigekawa[1], Satoshi Tanaka[1], Atsushi Hosui[1], Takuya Miyagi[1], Tomohide Tatsumi[1], Tatsuya Kanto[1], Naoki Hiramatsu[1], Eiichi Morii[2], Norio Hayashi[3], Tetsuo Takehara[1,*]

[1]*Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan;*
[2]*Department of Pathology, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan;*
[3]*Kansai-Rosai Hospital, Amagasaki, Hyogo 660-8511, Japan*

Background & Aims: Hepatocyte apoptosis is a key feature of chronic liver disease including viral hepatitis and steatohepatitis. A previous study demonstrated that absence of the Bcl-2 family protein Mcl-1 led to increased hepatocyte apoptosis and development of liver tumors in mice. Since Mcl-1 not only inhibits the mitochondrial pathway of apoptosis but can also inhibit cell cycle progression and promote DNA repair, it remains to be proven whether the tumor suppressive effects of Mcl-1 are mediated by prevention of apoptosis.

Methods: We examined liver tumor development, fibrogenesis, and oxidative stress in livers of hepatocyte-specific knockout (KO) of *Mcl-1* or *Bcl-xL*, another key antagonist of apoptosis in hepatocytes. We also examined the impact of additional KO of *Bak*, a downstream molecule of Mcl-1 towards apoptosis but not the cell cycle or DNA damage pathway, on tumor development, hepatocyte apoptosis, and inflammation.

Results: *Bcl-xL* KO led to a high incidence of liver tumors in 1.5-year-old mice, similar to *Mcl-1* KO. Bcl-xL- or Mcl-1-deficient livers showed higher levels of TNF-α production and oxidative stress than wild-type livers at as early as 6 weeks of age and oxidative DNA damage at 1.5 years. Deletion of Bak significantly inhibited hepatocyte apoptosis in *Mcl-1* KO mice and reduced the incidence of liver cancer, coinciding with reduction of TNF-α production, oxidative stress, and oxidative DNA damage in non-cancerous livers.

Conclusions: Our findings strongly suggest that chronically increased apoptosis in hepatocytes is carcinogenic and offer genetic evidence that inhibition of apoptosis may suppress liver carcinogenesis in chronic liver disease.

Keywords: Bcl-xL; Mcl-1; 8-OHdG.
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* Corresponding author. Address: Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 3621; fax: +81 6 6879 3629.
E-mail address: takehara@gh.med.osaka-u.ac.jp (T. Takehara).
Abbreviations: HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; RT-PCR, reverse-transcription PCR; HO-1, heme oxygenase-1; NQO1, NAD(P)H:quinone oxidoreductase 1; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

Introduction

Apoptosis of epithelial cells, as well as infiltration of inflammatory cells or deposits of fibers, is frequently observed in the chronic diseased liver, which is a high-risk condition for hepatocellular carcinoma (HCC) [1]. For example, Fas-mediated hepatocyte apoptosis is a mechanism of cell death in chronic hepatitis C virus infection and hepatitis B virus infection [2,3]. Hepatocyte apoptosis shows correlation with inflammation and fibrosis in non-alcoholic steatohepatitis [4]. Cytokeratin 18 neoepitope, a well-established marker of caspase activity in serum, is elevated and associated with liver injury in chronic viral hepatitis and non-alcoholic steatohepatitis [5–7]. Although viral factors and overt organ inflammation linked to liver cancer development have been extensively studied [8,9], less information is available on the involvement of hepatocyte apoptosis in liver cancer development.

Bcl-xL and Mcl-1 are among the anti-apoptotic members of the Bcl-2 family, which antagonizes the pro-apoptotic function of Bak and/or Bax at the mitochondrial outer membrane. We previously reported that hepatocyte-specific *Bcl-xL* or *Mcl-1* knockout (KO) mice showed persistent apoptosis of hepatocytes in the adult liver and mild fibrotic responses [10,11]. A very recent study by Weber *et al.* [12] demonstrated that hepatocyte-specific *Mcl-1* KO mice developed liver tumors in old age. This observation raised the important possibility that apoptosis in hepatocytes could lead to the development of liver cancer. However, as Mcl-1 has been reported to possess functions other than anti-apoptosis, such as cell cycle inhibition [13,14] and DNA damage repair [15,16], it is difficult to conclude that the phenotypes observed in *Mcl-1* KO are simply ascribable to apoptosis. Indeed, *Mcl-1* KO mice showed not only increased apoptosis but also increased regeneration in the liver [12]. In the present study, we demonstrated that hepatocyte-specific *Bcl-xL* KO mice also develop liver cancer in old age and that deficiency of Bak, a downstream effector molecule of Mcl-1 towards the