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Identification of bisindolylmaleimides and indolocarbazoles as inhibitors of HCV replication by tube-capture-RT-PCR

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ABSTRACT

We devised a screening method for hepatitis C virus (HCV) inhibitors by exploiting the JFH1 viral culture system. The viral RNA released in the medium was adsorbed onto PCR plates, and real-time RT-PCR was performed by directly adding the one-step RT-PCR reaction mixture to the wells. The "tube-capture-RT-PCR" method obviates the need for labor-intensive RNA isolation and should allow high-throughput screening of HCV inhibitors. To substantiate the validity of the assay for drug screening, a pilot screen of an inhibitor library composed of 95 compounds was performed. In addition to the known inhibitors of HCV replication included in the library, the assay identified the PKC inhibitor bisindolylmaleimide I (BIM I) as an HCV replication inhibitor. BIM I was also effective in reducing the viral protein level in genotype 1b and 2a subgenomic replicon cells, indicating inhibition of HCV replication. Further assays revealed that a broad range of bisindolylmaleimides and indolocarbazoles inhibit HCV, but no correlation was found between the PKC inhibition pattern and anti-HCV activity. These series of compounds represent new classes of inhibitors that may warrant further development.

1. Introduction

Hepatitis C virus, a major cause of chronic liver disease, has infected over 170 million people. The current mainstream anti-HCV therapy is a combination of interferon (IFN) and ribavirin. However, the therapy is not effective in approximately half of HCV-infected patients and has considerable side effects in many patients; thus, there is an urgent need for novel HCV therapies.

Various assays for HCV drug screening have been reported, many of which rely on HCV replicon systems. Although HCV replicon-based systems have greatly facilitated HCV research and drug discovery, these systems do not completely reflect the entire HCV life cycle and are not capable of identifying inhibitors of several important steps such as viral attachment, entry, and release. The recently introduced HCV cell culture systems (Wakita et al., 2005) should overcome these limitations and enable identification of inhibitors that would not be recognized by the replicon-based screens.

Here we describe a simple screening method for discovering anti-HCV drugs using the JFH1 viral culture system. Antiviral activity was determined by RT-PCR measurement of viral RNA released in the medium of infected cells. To increase efficiency, we devised a method that avoids tedious RNA isolation.

As a proof of concept, the method was used to evaluate a compound library and successfully confirmed the anti-HCV activity of cyclosporin A. In addition, a potent and selective PKC inhibitor, BIM I, was also identified as an anti-HCV agent. We found that other bisindolylmaleimides and indolocarbazoles also inhibit HCV, whereas anti-HCV activity was not associated with PKC inhibition. HCV inhibition by bisindolylmaleimides or indolocarbazoles has not been reported, and we expect that our assay will facilitate the identification of previously unrecognized HCV inhibitors. The bisindolylmaleimides and indolocarbazoles are already in clinical trials and may merit attention as HCV drug candidates.

2. Materials and methods

2.1. Cells and virus

Plasmid pJFH1, containing full-length cDNA of the JFH1 isolate, was used to generate HCV production in cell culture, as described elsewhere (Wakita et al., 2005), and the supernatant was passaged in Huh 7.5.1 cells. To prepare virus stock for screening, naïve Huh 7.5.1 cells were infected with the passaged supernatant virus, and the medium was collected 7 days post-infection and stored at

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−80 °C until use. The infectious titers of the viruses were determined by immunofluorescence analysis of the infected Huh 7.5.1 cells using anti-core antibody (2H9). The infectious titers of the stocks were generally about 3×10^5–1×10^6 ffu/ml, corresponding to about 3×10^7–1×10^8 copies of JFH1 RNA/ml. A subgenomic replicon cell, clone 4-1, which harbors the genotype 2a HCV genome (Kato et al., 2003; Date et al., 2004) and clone 5-15, which harbors the genotype 1b HCV genome (Lohmann et al., 1999), were also cultured in Dulbecco's Modified Eagle's medium (DMEM) with fetal bovine serum (FBS).

2.2. Reagents

The SCADS inhibitor kit I was provided by the Screening Committee of Anticancer Drugs supported by a Grant-in-Aid for Scientific Research on the Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science and Technology, Japan. The PKC β isozyme selective inhibitor LY333531 (Ruboxistaurin) was from Alexis Corp. (Lausen, Switzerland). Other chemicals were purchased from Merck Calbiochem (Darmstadt, Germany). Interferon-α (IFN-α) was from PeproTech, Inc. (Princeton Business Park, Princeton, NJ).

2.3. Quantitative real-time RT-PCR

Huh 7.5.1. cells were seeded in 96-well plates at a density of 20,000 cells per well in a volume of 120 μl. The next day, 15 μl of test compounds was added and the cells were infected with 15 μl of virus stock of HCV-JFH1 at a multiplicity of infection (MOI) of 0.01. After 5 days of culture, 100 μl of medium was transferred to a PCR plate, incubated on ice for 30 min, centrifuged at 3500 rpm for 15 min, and then removed. Twenty microliters of One Step SYBR PrimeScript RT-PCR Kit reaction mixture (Takara-Bio Co., Otsu, Japan) was added into the PCR plate wells, and quantitative real-time PCR was performed using an ABI Prism 7000 sequence detector (PE Applied Biosystems, Foster City, CA). The primers used were 5′-GAGTGTCGTACAGCCTCCAG-3′ (nucleotides 97–116), and 5′-AGGCCTTTCGCAACCCA-3′ (nucleotides 280–264) from the noncoding region of HCV-JFH1, at a concentration of 200 nM. Media from the control wells without drug were serially diluted to create a standard curve, which was used to determine the relative amount of HCV RNA in the media of HCV-infected cells treated with the compounds. Cell growth was monitored by MTT assay, as described previously (Fukazawa et al., 1995).

For further analysis of the drug effect and determination of the copy number of HCV RNA in medium and cells, HCV RNA was extracted from 140 μl medium with the QIAamp Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany), and eluted with 60 μl of elution buffer. Eight microliters of the viral RNA eluate was subjected to quantitative real-time PCR using Taqman EZ RT-PCR Core reagents (PE Applied Biosystems). The primers were 5′-CGGGAGAGCCATAGTGG (nucleotides 129–145) and 5′-AGTACCACAAGGCCTTTCG (nucleotides 289–271) at a concentration of 200 nM, and the Taqman probe was FAM-5′-CTGCGGAACCGGTGAGTACAC-3′-TAMRA (nucleotides 147–167) at a concentration of 300 nM (Takeuchi et al., 1999). Standard JFH1 RNA for measurement of copy number was transcribed from plasmid pSRG-JFH1-Luci, which was derived from pSRG-JFH1 (Kato et al., 2003), using the AmpliScribe T7 High Yield Transcription Kit (Epicentre Biotechnologies, Madison, WI). The transcribed RNA was purified and diluted with ribonuclease-free water containing yeast tRNA and 0.2% DTT, as previously described (Suzuki et al., 2005).

2.4. Western blotting

Cells were lysed with Radio-ImmunoPrecipitation Assay (RIPA) buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 25 μg/ml of each of antipain, pepstatin, and leupeptin, and centrifuged. The amount of protein in the supernatant was then measured. Cell lysates containing equal amounts of protein were separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes, and probed with antibodies against core (2H9), NS5A (Austral Biologicals, San Raman, CA), α-tubulin (Merck Calbiochem), and GAPDH (Santa Cruz Biotech. Inc., Santa Cruz, CA). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and specific proteins were visualized by chemiluminescence.

3. Results

3.1. Assay development

To establish an efficient RT-PCR-based screen for anti-HCV agents, we searched for methods that could be carried out without labor-intensive RNA isolation. We tested whether tube-trapping methods used to obtain plant viral RNAs for RT-PCR (Rowhani et al., 1995; James, 1999; Suehiro et al., 2005) could be applied to HCV. A JFH1 stock solution (3×10^5 ffu/ml, 3×10^7 copies/ml) was serially diluted fourfold, put into the wells of the PCR plate, incubated on ice for 30 min, and then centrifuged at 3500 rpm for 15 min. The supernatant was removed and quantitative RT-PCR was performed by direct addition of the one-step RT-PCR reaction mixture. We found that HCV, like plant viruses, are adsorbed onto the well wall during incubation. As shown in Fig. 1a, RNA adsorption appeared to be linear over a broad range of viral concentrations. HCV RNA could still be detected after seven fourfold dilutions, indicating that the "tube-capture" is a quantitative method that can detect less than 200 copies of HCV RNA. We compared the CT values from this "tube-capture method" with those from the conventional method of RNA extraction. The efficiency of RNA recovery by "tube-capture" was calculated to be about 9% of the conventional method. However, as shown in Fig. 1a, there was a close correlation between the CT values obtained from the two methods ($R = 0.988$), demonstrating the usefulness of this method.

3.2. Identification of BIM I as an inhibitor of HCV infection

To explore the possibility of tube-capture-RT-PCR as a simple screen for discovering anti-HCV compounds, we first tested whether the method would detect the antiviral activity of IFN-α. Huh7.5.1 cells were seeded in a 96-well plate and infected with HCV-JFH1 at an MOI of 0.01. After 5 days, HCV RNA released in the medium was assayed by the tube-capture method. Under these conditions, the CT from the control medium was usually about 18–20. As shown in Fig. 1b, a substantial reduction in the amount of HCV RNA was observed when the cells were infected in the presence of IFN-α.

For further validation of the ability of the assay to identify HCV inhibitors, we performed a pilot screen using an inhibitor kit provided by the Screening Committee of Anticancer Drugs (SCADS inhibitor kit I). This kit contains 95 inhibitors including cyclosporin A, a compound reported to inhibit HCV replication.

Cyclosporin A was identified (Fig. 1c), providing a proof of concept for screening for anti-HCV drug candidates. In addition, our assay also identified the PKC inhibitor bisindolylmaleimide I (BIM I) (Fig. 2a, black columns). The IC_{50} was about 0.1 μM, which is comparable to that of cyclosporin A and about 200-fold lower than the IC_{50} for cell growth (Fig. 2b). In addition, BIM I inhibited the cytopathic effect of HCV JFH1. Infection with HCV resulted in about a 20% reduction of cell growth. BIM I at 1 μM enhanced the growth of

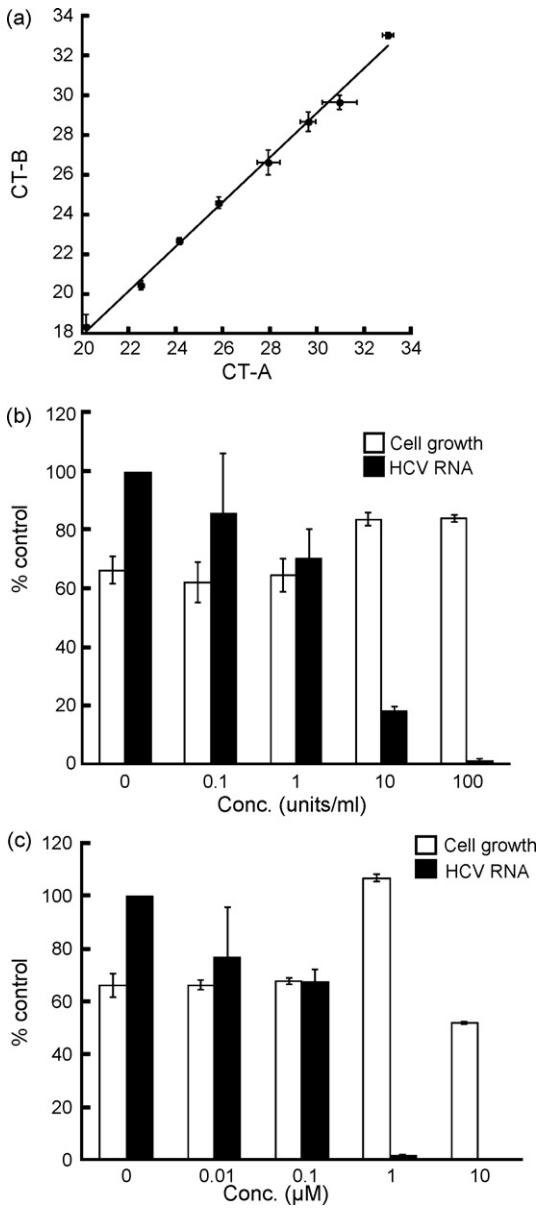

Fig. 1. RT-PCR-based screen for anti-HCV agents using the JFH1 viral culture system. (a) Correlation between CT values from "tube-capture-RT-PCR" (CT-A) and ordinary RNA extraction (CT-B). JFH1 stock solution (3×10^5 ffu/ml) was serially diluted four-fold and quantitative real-time PCR was performed as described under Section 2. CT-A was the average CT of three wells using tube-capture-RT-PCR and CT-B was the average CT of three HCV RNA eluates using a kit (QIAamp Viral RNA mini). (b) and (c) Huh 7.5.1 cells were infected with JFH1 in the presence of the indicated concentrations of IFN-α (b) or cyclosporin A (c). HCV RNA in the medium (closed (black) columns) was assessed by "tube-capture-RT-PCR" as described under Section 2. Open (white) columns represent percentage of cell growth compared with that of control cells without virus and compound. Columns, mean of triplicate wells; bars, SD.

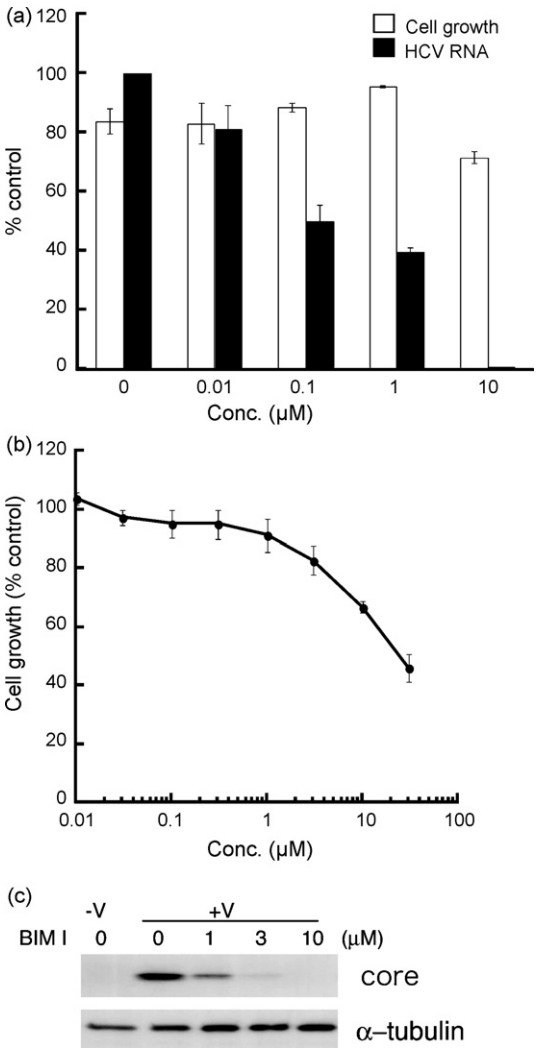

Fig. 2. BIM I inhibits HCV. (a) Effects of BIM I on HCV JFH1 RNA. Huh 7.5.1 cells were infected with JFH1 in the presence of the indicated concentrations of BIM I and assayed for HCV RNA and cell growth as in Fig. 1. Columns, mean of triplicate wells; bars, SD. (b) Effects of BIM I on growth of Huh 7.5.1 cells. (c) Effects of BIM I on HCV core protein in cells. Cells were infected with JFH1 at an MOI of 0.2 in the presence of the indicated concentrations of BIM I and cultured for 2 days. Cells were lysed and subjected to western blotting as described under Section 2. "V" indicates infection with HCV.

infected cells, almost to the level of uninfected cells (Fig. 2a, white columns). Recovery of cell growth was also observed with INF-α or cyclosporin A treatment (Fig. 1b and c). BIM I reduced cell growth of uninfected cells only at concentrations of 1 μM or higher (Fig. 2b). BIM I also inhibited the production of the HCV core protein with marginal effects on host α-tubulin levels (Fig. 2c).

3.3. BIM I inhibits HCV replication

To our knowledge, the anti-HCV effects of BIM I or other PKC inhibitors have not been reported. Because the majority of current HCV drug screening relies on replicon-based models, we investigated the possibility that BIM I targets a step in the HCV life cycle that is not included in the replicon systems, such as attachment, entry or release. We treated two subgenomic replicon cells with BIM I and examined the amount of NS5A protein.

As shown in Fig. 3a and b, BIM I dose-dependently reduced NS5A in both 1b and 2a subgenomic replicon cells, but not the host GAPDH. The results indicate that BIM I inhibits a process involved in the replication of HCV subgenomic replicons. However, although the NS5A level appeared to be more vulnerable, cell growth was substantially suppressed by BIM I (Fig. 3c). Whereas a significant difference between the IC_{50} for HCV RNA and cell growth was observed in the HCV cell culture system, the reduction of NS5A in replicon cells overlapped with the effects on cell growth.

To further elucidate the stage of the HCV life cycle affected by BIM I, Huh 7.5.1 cells were inoculated with higher titers of JFH1 (MOI 2) and then treated with 3 μM BIM I, starting at different time points after infection. JFH1 appeared to complete the life cycle in about 48 h, judging from the expression profiles of viral RNA and proteins in cells (Fig. 4a). When BIM I was added at the time of infection,

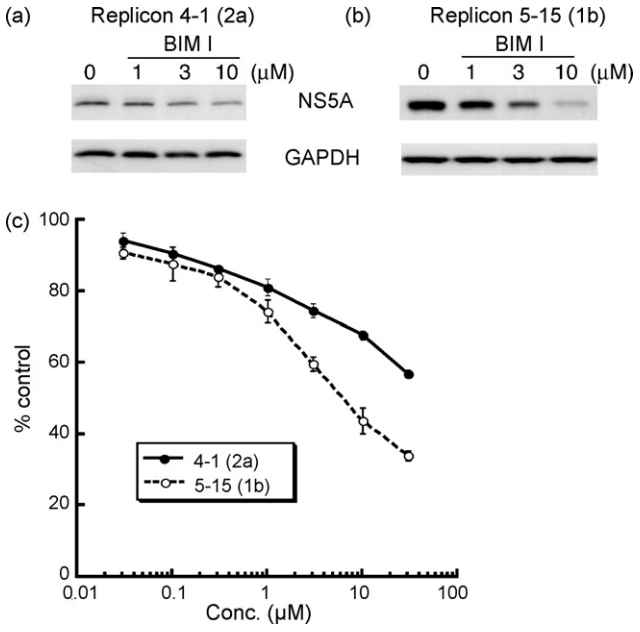

Fig. 3. Effect of BIM I on subgenomic replicon cells. (a) Subgenomic replicon cells harboring genotype 2a (4-1) (a) and genotype 1b (5-15) (b) were treated with BIM I for 2 or 4 days, respectively. Cells were lysed and analyzed by western blotting with anti-NS5A antibody or anti-GAPDH antibody. (c) Effect of BIM I on cell growth of the two replicon cells. Cells were incubated for 2 (4-1) or 4 days (5-15) with BIM I, and cell growth was measured by MTT assay.

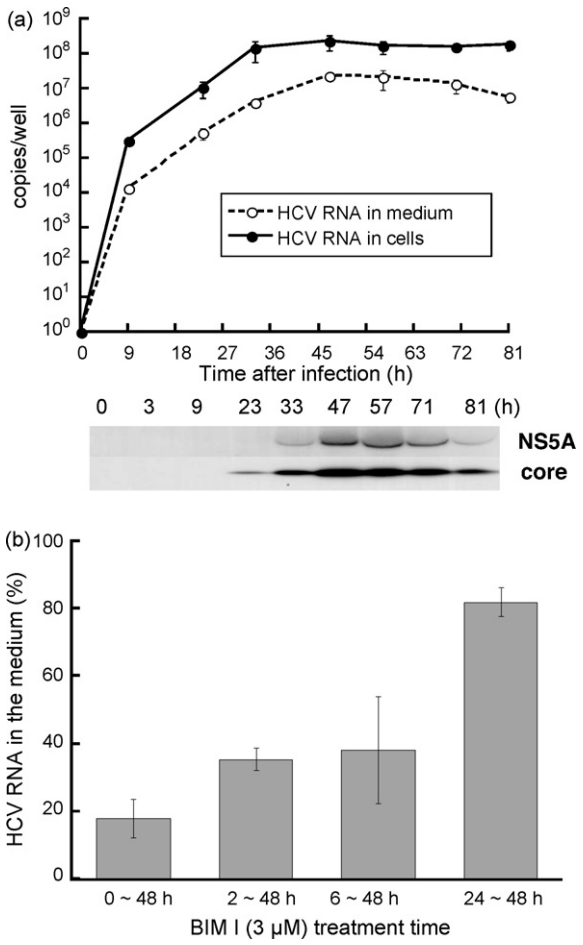

Fig. 4. (a) Expression profile of the HCV RNA and proteins. Huh 7.5.1 cells were infected with HCV as described above, but at an MOI of 2. The cells and medium were harvested at the indicated time and analyzed for HCV RNA and proteins. The core and NS5A protein were detected in the cell lysate. (b) Effect of time of addition. BIM I (3 μM) was added to Huh 7.5.1 cells just before and 2, 6, or 24 h after HCV addition at an MOI of 0.2. After 48 h of incubation following virus infection, the HCV RNA in the medium was extracted and measured with quantitative real-time RT-PCR. The indicated values represent the averages for two independent experiments.

the amount of viral RNA in the medium after 48 h decreased to less than 20% of control cells without inhibitor treatment. Addition of BIM I at 6 h post-infection still resulted in a reduction of viral RNA to 30% of control, but after 24 h the antiviral activity of BIM I was significantly diminished and only a modest decline to about 80% was observed (Fig. 4b). These results suggest that interference with RNA synthesis or translation of viral proteins accounts, at least in part, for the anti-HCV activity of BIM I.

3.4. HCV inhibition by bisindolylmaleimide and indolocarbazole compounds does not involve PKC

Since the discovery of staurosporine as a broad-spectrum protein kinase inhibitor, a variety of bisindolylmaleimide and indolocarbazole inhibitors with different potencies and selectivity have been developed. BIM I is one such compound that is highly specific for PKC and is broadly used to analyze PKC-mediated events. To gain insight into the relevance of the PKC inhibitory spectrum and antiviral activity, panels of different bisindolylmaleimide and indolocarbazole compounds were tested in the assays. Contrary to our expectation, no correlation was found between the ability to inhibit PKC and HCV.

Another bisindolylmaleimide PKC inhibitor without the N-dimethylaminopropyl chain, BIM IV, displayed similar significant anti-HCV activity (Fig. 5). Other structurally related pan- and isozyme-specific PKC inhibitors such as BIM II, Ro31-8220 (BIM IX), LY333,531 and 3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione (PKCβ Inhibitor, Calbiochem) or an indolocarbazole compound K252c also inhibited HCV replication (not shown). However, as shown in Fig. 5, the non-PKC-inhibitory analog BIM V (Toullec et al., 1991) and arcyriaflavin A, an indolocarbazole compound with no reported effects on PKC (Zhu et al., 2003), were also effective in reducing HCV RNA. BIM V was actually more potent than BIM I. Whereas the effect of BIM I on HCV overlapped with cytotoxicity in this particular experiment, BIM V was virtually nontoxic at a dose (1 μM) that reduced HCV RNA to less than 20% of control. The results indicate that a broad range of bisindolylmaleimide and indolocarbazole compounds inhibit HCV replication, albeit in a PKC-independent manner.

4. Discussion

HCV replicon systems have made significant contributions in HCV research and drug development. Nevertheless, as drug screening tools, replicon systems have limitations because they are not capable of identifying inhibitors of several important events in the viral life cycle. The use of HCV cell culture systems should overcome the drawbacks of the replicon systems and facilitate the identification of inhibitors with novel mechanisms of action. Actually, it has recently been shown that use of an infectious HCV system identified inhibitors that a replicon-based screen did not recognize (Zhang et al., 2008).

We developed a simple screening method for HCV inhibitors that measures the viral RNA released from JFH1-infected cells. The assay does not require specially engineered viruses. The "tube-capture-RT-PCR" method obviates the need for labor-intensive RNA isolation and significantly increases the efficiency of screening. The validity of the assay was confirmed by successful identification of known

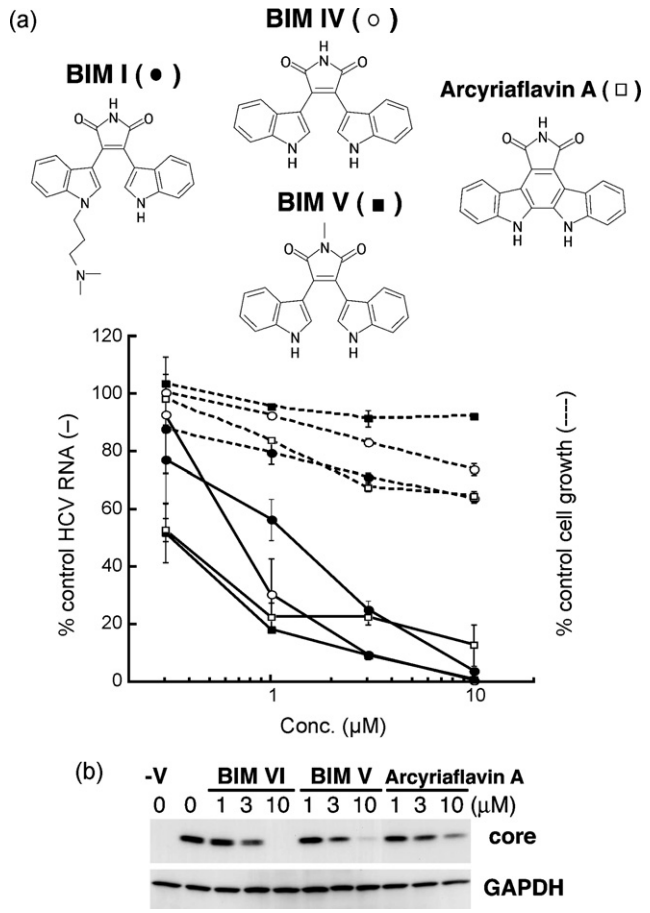

Fig. 5. Effects of bisindolylmaleimide or indolocarbazole compounds on HCV infection. (a) Effects of BIM I, BIM IV, BIM V, and arcyriaflavin A on HCV RNA in the medium. Huh 7.5.1 cells were seeded and infected with HCV at an MOI of 0.01 in the presence of drugs. After 4 days of incubation, the HCV RNA in the medium was extracted and quantified. The relative amounts of HCV RNA with BIM I (closed circle), BIM IV (open circle), BIM V (closed rectangle), and arcyriaflavin A (open rectangle) are represented by solid lines. Cell viability, represented by dotted lines, was determined by MTT assay of a parallel culture without HCV challenge. (b) Effects of BIM IV, BIM V and arcyriaflavin A on core proteins in cells. The cells were infected with JFH1 at an MOI of 0.2 in the presence of the indicated concentrations of compounds and analyzed as described in Fig. 2c. "–V" indicates control without HCV infection.

HCV inhibitors in the pilot screen. In addition, the assay identified the PKC inhibitor BIM I.

BIM I is a widely used compound, and it was somewhat surprising to us that its anti-HCV activity had not been reported. HCV replication in the cell culture system appeared to be considerably more susceptible to BIM I than in the replicon systems, and this is probably why this compound had not been identified as an HCV inhibitor.

Because virus replication is closely linked to host cell growth, HCV inhibition could occur as a result of cell growth inhibition. However, as shown in Fig. 2a, BIM I reduced the cytopathic effect of HCV infection just like interferon-α and cyclosporin A. BIM I, at 1 μM, enhanced the growth of infected cells almost to the level of uninfected cells, presumably because HCV replication, but not cell growth, was inhibited, resulting in a reversal of the cytopathic effect.

Because the PKC inhibitory properties of bisindolylmaleimides and indolocarbazoles have been characterized extensively, we tested a panel of commercially available compounds in the assay to gain insight into the role of PKC in HCV replication. However, no correlation between PKC inhibition and antiviral activity could be found. Anti-HCV activity did not involve PKC inhibition, apparently because a non-PKC-inhibitory analog, BIM V, was also active. Furthermore, PKC inhibitors with different structures, such as calphostin C and H-7, did not show specific inhibition of HCV (data not shown).

Previous studies have indicated that bisindolylmaleimide PKC inhibitors have cellular targets other than PKC. It has been shown that BIM I and Ro31-8220 inhibit p70S6K and p90RSK (Alessi, 1997; Roberts et al., 2005) and that BIM V inhibits p70S6K (Marmy-Conus et al., 2002). Although we did not monitor the activities of these enzymes in our experiments, inhibition of p70S6K is unlikely to be responsible for the anti-HCV effect of PKC inhibitors, because Ishida et al. reported that silencing of p70S6K enhanced HCV RNA abundance (Ishida et al., 2007).

Bisindolylmaleimides and indolocarbazoles have also been reported to inhibit the ATP-binding cassette (ABC) transporters P-glycoprotein and multidrug resistance-associated protein 1 (MRP1), efflux pumps that play important roles in cancer drug resistance (Merritt et al., 1999; Gekeler et al., 1995). More recently, Robey et al. reported that BIMs I, II, III, IV, and V, K252c, and arcyriaflavin A inhibit ABCG2, an ABC half-transporter that confers resistance to various antitumor agents (Robey et al., 2007). Whether ABC transporters play any role in HCV infection awaits further study. We are currently examining the anti-HCV effects of other ABC transporter inhibitors.

In conclusion, we developed a simple infectious HCV system-based assay that can be used for high-throughput screening of HCV inhibitors and identified bisindolylmaleimides and indolocarbazoles. These compounds might represent lead substances for the development of new HCV drugs. Further analysis of the mechanism of HCV-inhibition by these compounds might reveal a new mechanism of regulation of HCV infection.

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