We describe a cell-based, microplate colorimetric screen for anti-hepatitis C virus (HCV) drugs that exploits the HCV-JFH1 viral culture system. Antiviral activity was assessed by measuring protection against the HCV-JFH1-induced cytopathic effect (CPE) in Huh7.5.1 cells using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) viability assay. The use of serum-free medium substantially sensitized Huh7.5.1 cells to HCV-induced CPE, causing sufficient cell death to perform colorimetric assays for anti-HCV activity in 96-well plates. As a proof of concept, we carried out a pilot screen of an inhibitor library and identified cyclosporin A and tamoxifen, two compounds with reported anti-HCV activity. Using the assay, we discovered the anti-HCV properties of the plant flavonoids epigallocatechin gallate (EGCG) and 7,8-benzoavone (α-naphthoflavone). Other gallate-type catechins and flavones also displayed anti-HCV activity, but 5,6-benzoavone (β-naphthoflavone), flavanone, and non-gallate catechins were inactive. EGCG apparently acted mainly on HCV entry, although it may also block other steps. In contrast, 7,8-benzoavone was presumed to inhibit later stages of the HCV life cycle. This assay is simple, reliable and cost-effective; does not require any specially engineered cell lines or viruses; and should be useful in the identification of compounds with anti-HCV activity.

Key words hepatitis C virus; 7,8-benzoavone (α-naphthoflavone); epigallocatechin gallate

More than 170 million people worldwide are chronically infected with the hepatitis C virus (HCV) and are at risk for developing liver diseases such as cirrhosis and hepatocellular carcinoma. Vaccines against HCV are not currently available; furthermore, the standard interferon/ribavirin combination therapy is not effective in approximately half of HCV-infected patients, and it has considerable side effects. Thus, there is an obvious and urgent need for new agents that can enhance or replace current HCV therapies.

Screening programs using HCV replicon-based systems have successfully identified compounds that act on HCV RNA replication. However, replicon systems do not reproduce the entire HCV life cycle, and they cannot isolate inhibitors of many important steps such as viral entry, assembly, and egress. HCV cell culture infection models that recapitulate the entire viral life cycle in vitro have greatly enhanced the opportunity for HCV drug discovery. Several reports, including ours, have demonstrated that this model can overcome the limitations of the HCV replicon system and enable the discovery of compounds that target various stages of the HCV life cycle.

Using the HCV cell culture system, we previously developed a tube-capture-reverse transcription-polymerase chain reaction (RT-PCR) assay for screening HCV inhibitors and identified bisindolylmaleimides and indolocarbazoles as inhibitors of HCV replication. Here, we describe another screening method for the detection of anti-HCV activity. This assay measures the inhibition of the HCV-induced cytopathic effect (CPE) in Huh 7.5.1 cells. We show that the use of serum-free medium improves the sensitivity of the assay and permits the identification of drug candidates that may be overlooked in other assays due to their serum-binding capacities. The validated assay was used to evaluate a compound library, in which it successfully identified the anti-HCV activities of cyclosporin A and tamoxifen.

Many biological properties, including anti-oxidative, anti-inflammatory, anti-tumorigenic, anti-bacterial, and anti-viral activities, have been documented for plant flavonoids, particularly the green tea catechin epigallocatechin gallate (EGCG). We tested various flavonoids in the assay and found that EGCG and 7,8-benzoavone (α-naphthoflavone) inhibit HCV infection. Other gallate-type catechins were also active, but non-gallate catechins and 5,6-benzoavone (β-naphthoflavone) did not exhibit prominent anti-HCV activity. EGCG has recently been shown to block HCV entry. Our results using the JFH1 viral culture system also suggested that EGCG mainly targets virus entry but implied that it may also act on other stages of the HCV life cycle. In contrast, 7,8-benzoavone appeared to inhibit post-entry phases.

The assay described here is simple, reliable and cost-effective, does not require any specially engineered viruses or cell lines, and should allow the high-throughput screening of HCV drug candidates.

MATERIALS AND METHODS

Materials Epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, cyclosporin A and tamoxifen were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Catechin gallate and gallocholate gallate were purchased from Nagara Science Co., Ltd. (Nagase, Japan). Flavone, flavanone, 5,6-benzoavone, and 7,8-benzoavone were obtained from Wako Pure Chemicals (Osaka, Japan). The SCADS Inhibitor Kit I was provided by the Screening Committee of...
Anticancer Drugs, supported by a Grant-in-Aid for Scientific Research on Innovative Areas in the Scientific Support Programs for Cancer Research, from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The insulin-transferrin-selenium-X (ITS-X) supplement was purchased from Invitrogen (Grand Island, NY, U.S.A.). The anti-NS5 monoclonal antibody was obtained from Austral Biologicals (San Ramon, CA, U.S.A.), and antibodies against extracellular-regulated kinase 1 (ERK1) and ERK2 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Anti-core monoclonal antibody (2H9) has been described previously. Huh 7.5.1 cells were kindly provided by Francis V. Chisari. The pJFH1 plasmid, containing a full-length cDNA corresponding to the JFH1 isolate, was used to produce HCV in culture. 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 d post-infection and stored at −80°C until use. The cells harboring the genotype 1a full-genomic replicon (RCYM1) and the genotype 2a subgenomic replicon (clone 4-1) have been described previously.

HCV CPE Inhibition Assay Huh 7.5.1 cells were suspended in 1:1 Dulbecco's modified Eagle's medium: Nutrient Mix F-12 (DMEM/F12) supplemented with 0.5% ITS-X and seeded in wells of collagen-coated 96-well plates at a density of 5000 cells per well. Cells were infected with HCV-JFH1 virus stock at a multiplicity of infection (MOI) of 1. A duplicate plate without the virus was prepared in parallel to assess the cytotoxicity of the compounds. After 4 d, cell growth was monitored using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. MTT solution (15 µL at 5 mg/mL in phosphate buffered saline) was added and incubated for 4 h. The resulting formazan was extracted by adding 100 µL of 20% sodium dodecyl sulfate (SDS) in 10 mM HCl, and the absorbance was monitored after 24 h at 570 nm with a reference wavelength of 690 nm.

Immunoblotting Analysis Huh 7.5.1 cells were seeded in 24-well collagen-coated plates at a density of 1.5×10^5 cells per well and infected with HCV-JFH1 at an MOI of 0.5. Drug treatment was initiated at various timepoints before and after infection. At 48 h after infection, cells were fixed with 10% trichloroacetic acid and lysed with 9× urea, 2% Triton X-100 and 2% lithium dodecyl sulfate. The lysates were neutralized with 2× Tris and analyzed by immunoblotting. Replicon cells were seeded in 24-well collagen-coated plates at a density of 5.0×10^4 cells per well, treated with the compounds for 72 h, and processed for immunoblotting as described above.

RESULTS

Assay Development As reported by Sekine-Osajima, infection with HCV-JFH1 induces CPE in Huh 7.5.1 cells. We postulated that compounds with anti-HCV activity would protect cells from CPE and improve cell viability. Thus, by measuring the recovery of cell growth from HCV-induced CPE in microtiter well plates, it should be possible to screen for inhibitors of HCV.

To increase the sensitivity of the primary screen and identify as many compounds with anti-HCV activity as possible, we reduced the concentration of serum in the assay medium; many compounds are known to bind to serum proteins and thus may not show activity in media containing high concentrations of serum. We found that Huh 7.5.1 cells can proliferate on a collagen-coated surface in DMEM/F12 supplemented only with insulin and transferrin at a rate fully comparable to the growth in serum-containing medium.

The infection of Huh 7.5.1 cells cultured in serum-free medium with JFH1 caused marked CPE. HCV-mediated CPE was also observed in serum-supplemented medium, but cells were less susceptible and required several-fold higher viral titers to achieve equivalent growth inhibition (Fig. 2). We chose an MOI of 1 in serum-free medium as the condition for screening. Although there is some serum carry over from the virus stock, at this MOI, the final serum concentration is generally reduced more than 100-fold compared to serum-supplemented medium. Cell growth usually decreased to less than 10% of the control, as measured by MTT assay.

To test the ability of the assay to identify HCV inhibitors, we performed a test screen using an inhibitor kit obtained from the Screening Committee of Anticancer Drugs (SCADS inhibitor kit 1). This kit consists of 92 compounds with various biological activities. Cyclosporin A and tamoxifen, two compounds with known anti-HCV activity, were identified as HCV inhibitors in the assay, providing a proof-of-concept for anti-HCV screening. As shown in Fig. 3, infection with JFH1 at an MOI of 1 reduced the growth of Huh 7.5.1 cells to less than 7% of the control in this experiment. In the presence of 2.5 µM cyclosporin A and tamoxifen, cell growth recovered to 73% and 71% of the control, respectively.

Identification of Plant Flavonoids as Inhibitors of HCV We then used the assay to evaluate the potential anti-HCV activities of various compounds. As shown in Figs. 3 and 4, various plant flavonoids, including the green tea gallate catechin EGCG and 7,8-benzo-flavone, reversed the CPE of HCV. The growth of Huh 7.5.1 cells recovered from less than 7% to 90% and 84% in the presence of 10 µM EGCG or 2.5 µM 7,8-benzo-flavone, respectively. The morphology of cells infected in the
presence of EGCG or 7,8-benzo flavone was indistinguishable from that of control cells without virus (not shown). Other gallate-type catechins (catechin gallate, gallocatechin gallate, and epicatechin gallate) were approximately as active as EGCG (Fig. 3). Flavone also displayed a protective effect (Fig. 4), although only at concentrations approximately 10-fold higher than that of 7,8-benzo flavone, but 5,6-benzo flavone, flavanone (not shown), and the non-gallate-type catechins epicatechin and epigallocatechin did not show significant anti-HCV activity (Fig. 3).

Effects of Serum on Anti-HCV Activities of Various Compounds We next performed the assay in a medium containing 5% FBS to assess the effects of serum on the anti-HCV activities of the identified compounds. To obtain CPE comparable with that observed in serum-supplemented medium (Fig. 3), cells were infected at an MOI of 4 in serum-supplemented medium.

In the absence of HCV infection, cyclosporin A was
somewhat more toxic to Huh 7.5.1 cells in serum-free medium than in serum-supplemented medium. However, growth recovery from HCV-induced CPE did not appear to be noticeably influenced by the increase in MOI (not shown) or by the addition of serum (Fig. 5). Other compounds did show anti-HCV activity and cytotoxicity in the presence of serum but required higher concentrations to achieve equivalent activities.

In serum-free medium, tamoxifen showed the highest HCV-inhibitory activity at 2 µM (Fig. 2). The increase in MOI from 1 to 4 slightly reduced the anti-HCV activity of tamoxifen (not shown). In 5% serum, the most effective dose of tamoxifen shifted to highest tested concentration of 8 µM, although the maximum observed growth improvement (6.6% to 91%) was better than that in serum-free medium. The cytotoxic concentrations also changed. In serum-free medium, tamoxifen was highly toxic to Huh 7.5.1 cells at concentrations
greater than 4 µM (Fig. 2), but in the presence of serum, the cells still showed 78% growth at 8 µM (Fig. 5).

At 2.5 µM, 7,8-benzoflavone clearly protected cells from HCV-induced CPE in serum-free medium, but was completely inactive in the presence of 5% serum. Even at 20 µM, cell growth only recovered from 6.6% to 41% in serum-supplemented medium. The cytotoxic concentrations also changed; the 50% growth inhibitory concentrations in the absence of HCV infection without and with serum were 4.2 µM and 26 µM, respectively.

Among the compounds tested, EGCG was most influenced by serum addition and increased MOI. For example, 5 µM EGCG stimulated recovery from 7% to 80% at an MOI of 1 (Fig. 3), but no significant improvement could be observed at an MOI of 4 (not shown). The highest anti-HCV activity of EGCG at an MOI of 4 was observed at 20 µM (recovery from 3.1% to 78% of control), and its activities were further attenuated by the addition of serum. In 5% serum-supplemented medium, growth only recovered from 6.1% to 15% upon the addition of 20 µM EGCG, and the optimum concentration was 80 µM or higher (Fig. 5).

The results indicate that serum proteins may mask the anti-viral activities of some compounds and that the use of serum-free medium can improve the sensitivity of a cell-based screen.

**EGCG and 7,8-Benzoflavone Target Different Steps of the HCV Life Cycle**

To determine which step of the HCV life cycle is blocked by flavones, we examined the effect of the time of addition. Compounds were added either immediately before HCV infection or at 2 h after infection, when the entry process had presumably been completed. The activity of entry inhibitors would be expected to decrease when added after the completion of entry, whereas compounds that interfere with post-entry steps should still be effective.

The protective effects of tamoxifen and EGCG decreased when added at 2 h after infection, suggesting that these compounds mainly target the early phases of infection (Fig. 6). By contrast, the anti-HCV activities of cyclosporin A and 7,8-benzoflavone were not affected by time of addition.

Immunoblot analysis of the HCV core protein supported the results of the MTT assay. Compounds were added at various time points before and after infection, and the levels of the HCV core protein were analyzed at 48 h after infection. As shown in Fig. 7, tamoxifen and EGCG completely blocked core expression if added at or before the time of infection, but addition later than 1 h post-infection resulted in markedly decreased efficacy. On the contrary, 7,8-benzoflavone and cyclosporin A were still effective even when added 4 h after infection. Our data suggested that EGCG and tamoxifen mainly inhibited the early steps of the HCV life cycle, such as attachment and entry, whereas 7,8-benzoflavone and cyclosporin A blocked later stages.

**Effects of Inhibitors on Replicon Cells**

We next compared the effects of cyclosporin A, tamoxifen, EGCG and 7,8-benzoflavone on cells that harbor a genotype 2a subgenomic or a genotype 1b full-genomic replicon. Cells were

![Fig. 6. The Effects of Time of Addition on the Anti-HCV Activity of Cyclosporin A (CsA), Tamoxifen (TMX), 7,8-Benzoflavone (7,8-BF) and EGCG](image)

Compounds were added immediately before (shaded bars) or 2 h after (closed bars) HCV infection, and cell growth was monitored after 4 d. Open bars represent cell growth in the absence of HCV-infection. Cell growth is presented as a percentage of mock-infected cells without inhibitors. The values presented are the means±S.D. of quadruplicate wells. The concentrations used were: cyclosporin A, 2 µM; tamoxifen, 5 µM; 7,8-benzoflavone, 2.5 µM; EGCG, 5 µM.

![Fig. 7. The Effect of Time of Compound Addition on the Expression of Core Protein](image)

Huh 7.5.1 cells were seeded in 24-well plates. Compounds were added at the indicated times before and after infection. Cells were fixed 48 h after infection and analyzed by immunoblotting with an anti-core antibody; anti-ERK antibody was used to confirm equal loading. –V indicates control without infection.
treated with the inhibitory compounds for 72 h, and the levels of NS5 or core proteins were analyzed by immunoblotting. Replicon replication is completed within cells, so compounds that target steps other than RNA replication, such as attachment or entry, should not show activity. We hypothesized that cyclosporin A and 7,8-benzo-β-flavone would be effective against replicon cells and that tamoxifen and EGCG would not be effective.

As expected, treatment with cyclosporin A and 7,8-benzo-β-flavone noticeably reduced the level of NS5 in 2a subgenomic replicon cells (Fig. 8a). EGCG showed little effect, but tamoxifen appeared to show a moderate level of activity. In the genotype 1b full-genomic replicon cells, cyclosporin A and 7,8-benzo-β-flavone also reduced the amount of core protein, although 7,8-benzo-β-flavone appeared to be somewhat less effective (Fig. 8b). However, tamoxifen was completely inactive and even appeared to enhance core expression at some concentrations. Unexpectedly, EGCG displayed considerable activity, implying that it may inhibit stages of the HCV life cycle other than entry. The results indicate that the efficacy and even the mechanism of action of inhibitors may differ with viral genotype.

DISCUSSION

We established a microtiter plate method for anti-HCV drug discovery that measures the increased viability of infected cells. This method enables the screening of inhibitors that target all stages of the HCV life cycle, including steps that cannot be recognized by replicon systems, such as viral attachment, entry, and egress. An assay based on a similar principle using an engineered cell line has recently been described. In that study, an HCV NS3-4A protease-cleavable derivative of Bid, which renders cells highly susceptible to HCV infection, was expressed in Huh 7 cells to increase the sensitivity of the assay. Here, we showed that the use of serum-free medium substantially enhanced HCV-induced CPE, and obviated the need for specially engineered cells. The mechanism of increased CPE in serum-free medium is not clear, but it is likely that serum proteins and/or lipids confer resistance to HCV-infection.

Many compounds are known to bind to serum proteins and may not show activity in a cell-based assay in the presence of high concentrations of serum. For example, EGCG has been reported to form water-soluble complexes with bovine serum albumin. We showed that the anti-HCV activity of EGCG is diminished in the presence of serum. Thus, serum-free medium offers various benefits to screening programs. It increases the sensitivity of the assay by allowing the use of lower MOIs and by enabling the detection of compounds with high serum protein-binding capacity. Compounds that bind to serum proteins with high affinity may have undesirable
pharmacodynamic properties, but the object of primary screening is to identify as many potentially active compounds as possible, and pharmacodynamic properties can be improved by chemical modifications. Although serum-free medium does have certain negative aspects such as enhanced toxicity of some compounds, we anticipate that its use will expand the range of identified compounds and increase the possibility of discovering anti-HCV drug candidates.

In our trial screening, we identified active compounds with several different modes of action, including the plant flavonoids EGCG and 7,8-benzoﬂavone. The green tea catechin EGCG is most likely the best-studied plant flavonoid, and it is known to exert multiple biological effects. It has been reported to inhibit various viruses through a number of mechanisms. The documented anti-viral activities of EGCG include the inhibition of the influenza virus, human immunodeﬁciency virus type 1 (HIV-1), Epstein–Barr virus, herpes simplex virus, and hepatitis B virus. In vitro, EGCG inhibited various viral enzymes, such as adenosine triphosphate adenyl kinase, influenza A RNA polymerase, and HIV-1 integrase. EGCG bound to CD4 on T cells and prevented its interaction with HIV gp120. EGCG has been shown to possess activity against the HCV NS3 serine protease and NS5B, although the actual impact of these effects on HCV replication have not been confirmed.

EGCG has recently been reported to inhibit the entry of HCV. EGCG inhibited the entry of all genotypes tested (1a, 1b and 2a), but it was much less active against vesicular stomatitis virus (VSV) entry. The present study also suggested that the attenuation of the replication of genotype 2a HCV by EGCG is mainly due to entry inhibition. We tested the effect of EGCG on the entry of other genotypes using pseudoparticles and, as reported, observed that it inhibited the entry of all HCV genotypes (not shown). In our hands, however, the entry of VSV was blocked at comparable concentrations, demonstrating that EGCG was not speciﬁc to HCV. It has also been reported that EGCG, EC, and ECG had no obvious inhibitory activity on HCV. Although EGCG and EC were not effective in our assay, ECG and the other gallate-type catechins GCG and CG showed anti-HCV activities similar to that of EGCG. Time-of-addition experiments suggested that these gallate-type catechins all mainly target viral entry, at least for genotype 2a HCV (not shown). It is likely that the gallate moiety is essential for the anti-HCV activity of catechins. The influence of the third hydroxyl group in the B-ring appeared to be marginal, at least in the present study.

EGCG had little effect on the expression of HCV proteins in genotype 2a replicon cells, a context that does not involve viral entry, further supporting the theory that EGCG is an entry inhibitor. However, EGCG substantially reduced the level of HCV proteins in genotype 1b replicon cells, implying that it may also target post-entry phases. Given that green tea catechins display diverse biological effects, it is conceivable that the mechanism of anti-HCV action is multimodal and that the main target may differ with genotypes.

In contrast to EGCG, 7,8-benzoﬂavone inhibited HCV replication even when added after the completion of the entry phase, and it effectively reduced the amount of NS5 in genotype 2a replicon cells, suggesting that it acts on post-entry phases. Furthermore, 7,8-benzoﬂavone displayed genotype selectivity and was not as active against the genotype lb replicon. 7,8-Benzoﬂavone has been reported to inhibit aromatase and breast cancer resistant protein (BCRP) and modulate aryl hydrocarbon receptor (AhR) signaling. Whether any of these biological activities are associated with the anti-HCV activity of 7,8-benzoﬂavone remains to be elucidated.

Other plant flavonoids with reported anti-HCV activity include naringenin and quercetin. Although the mechanisms of action of these compounds are not completely deﬁned, naringenin acted as a PPAR α agonist to block virus assembly. The inhibition of HCV particle production by quercetin was attributed at least partially to the reduction of HSP40 and HSP70 that is potentially involved in IRES translation. More recently, quercetin was found to inhibit NS3 activity.

In our assay, 7,8-benzoﬂavone was more active than naringenin or quercetin in inhibiting HCV (not shown). We are currently testing various plant ﬂavonoids to gain insight into the structure/activity relationship.

In conclusion, using the JFH1-HCV viral culture system, we developed a simple and cost-effective microplate colorimetric assay that will allow the high-throughput screening for HCV inhibitors that target various phases of the viral life cycle. The use of serum-free medium sensitized Huh 7.5.1 cells to HCV-induced CPE and eliminated the need for specially engineered cells or viruses. We anticipate that this assay will facilitate the discovery of compounds with anti-HCV activity and assist drug development. We have extended the screening to large chemical libraries and revealed anti-HCV activity in a wide variety of compounds.

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REFERENCES


