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Screening for hepatitis C virus antiviral activity with a cell-based secreted alkaline phosphatase reporter replicon system

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Abstract

We describe a phased screening system for discovery of compounds with antiviral activity against hepatitis C virus (HCV). The primary assay utilizes dicistronic subgenomic HCV replicons in which the upstream cistron was modified to express the human immunodeficiency virus (HIV) tat protein. When these replicons are stably transfected into Huh-7-derived cells that express secreted alkaline phosphatase (SEAP) under transcriptional control of the HIV long terminal repeat promoter, there is a strong correlation between intracellular HCV RNA abundance and the activity of SEAP secreted into the culture medium. Thus, active compounds are easily identified by direct enzymatic quantification of SEAP in the medium without post-assay processing. Compounds that reduce SEAP activity without causing cellular toxicity are next evaluated in a second Huh-7-derived cell line constitutively expressing SEAP under control of the tat-HIV promoter axis, independent of HCV RNA replication. This specificity control identifies compounds that cause reductions in SEAP that are unrelated to suppression of HCV RNA replication. Compounds showing HCV-specific activity in primary assays are next evaluated by real-time RT-PCR to directly quantify reductions in HCV RNA. We have found excellent agreement between the SEAP and RT-PCR assays. This phased system provides an efficient and cost-effective screen for compounds with antiviral activity against HCV.

Keywords: Hepatitis C virus; Antiviral activity; Alkaline phosphatase

1. Introduction

It is estimated that more than 170 million people worldwide are infected with hepatitis C virus (HCV). In the majority of cases the initial viral infection fails to resolve and a chronic infection is established. While the chronic HCV infection can remain asymptomatic, it can also result in serious long-term liver damage including progressive fibrosis, cirrhosis and hepatocellular carcinoma (Lauer and Walker, 2001; Hoofnagle, 2002). In fact, HCV is believed to result in more than 100,000 cases of liver cancer annually worldwide and accounts for at least 40% of liver transplants in the United States (Poynard et al., 2003; Davis, 2004). In many developed countries the incidence of new HCV infections has declined in the last decade as a result of blood screening and other public health efforts. However, the clinical burden of disease is likely to increase in the future as a result of the extended period that typically exists between acquisition of the infection and the development of clinically significant liver disease. Thus, in the United States, the clinical burden of those infected prior to these measures being implemented is expected to increase in the years ahead. In addition new HCV infections remain relatively common in some high risk groups, including intravenous drug users (Garfein et al., 1998; Harder et al., 2004), and, shared risk factors have resulted in a substantial population with HCV and human immunodeficiency virus (HIV) coinfection, a situation known to result in

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both a more rapid progression to liver disease, and in an increased incidence of vertical HCV transmission from mothers to offspring (Sherman, 2004; Verucchi et al., 2004).

Currently, treatment options for those infected with HCV are limited and even with the most successful treatment regimen, a combination of pegylated interferon-α with ribavirin, a significant proportion of patients fail to achieve a sustained virologic response (Fried and Hadziyannis, 2004). Thus, there is a very real need for new antiviral agents with activity against HCV. Drug discovery efforts have been hampered, however, by a number of factors including the lack of a fully permissive cell culture system allowing in vitro virus propagation, and the absence of a well-established animal model of chronic HCV infection. The development of replication-competent subgenomic replicon RNAs, in which most of the structural protein coding sequence is replaced by sequence encoding neomycin phosphotransferase (Lohmann et al., 1999), has been a huge impetus for antiviral development. Recently, we (Yi et al., 2002) described the construction of modified subgenomic genotype 1b HCV replicons derived from the HCV-N (Ntat2aneoRG) and Con-1 (Btat2NaneoSI) strains of HCV. These dicistronic replicons encode the HIV tat protein in the upstream cistron between the HCV 5′NTR and a picornoviral 2A proteinase sequence fused to the neomycin phosphotransferase selectable marker, while the second cistron contains the HCV NS3-5B segment under the translational control of the encephalomyocarditis (EMCV) IRES. When these replicons are transfected into En5-3 cells (a clonal cell line derived from Huh7 cells) that express secreted alkaline phosphatase (SEAP) under the transcriptional control of the HIV LTR promoter, tat protein produced as a result of replication of the replicon interacts directly with the LTR RNA transactivating SEAP production and secretion into the culture supernatant. The SEAP activity present in the extracellular fluids correlates closely with the abundance of intracellular HCV RNA (Yi et al., 2002). These replicon containing cell lines are valuable reagents for antiviral screening since they allow reductions in viral RNA replication to be measured by direct enzymatic quantification of SEAP in the supernatant medium, without the need for post assay processing. Here, we describe an efficient and cost-effective phased antiviral screening system that utilizes these SEAP replicons for primary antiviral screening.

2. Materials and methods

2.1. Cell lines

EN5-3 cells and the HCV SEAP-replicon cell lines Ntat2aNeo(RG) and Btat2aNeo(SI) have been described previously (Yi et al., 2002). Low passage cell stocks of all lines were stored frozen (−160 °C). Cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 400 μg/ml G418, 100 IU/ml penicillin, 100 μg/ml streptomycin (all Mediatech, Inc., Herndon, VA, USA) and 2 μg/ml Blastocidin S (ICN Biomedicals, Inc., Aurora, OH, USA). All cell lines were confirmed to be mycoplasma free.

2.2. Compounds

7-Chloro-5-(2-pyrryl)-3*H*-1,4-benzodiazepin-2(*H*)-one (NSC 66020) was supplied by the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID. Interferon α-2b was purchased from UTMB pharmacy. The ketoamide peptidomimetic NS3/4a protease inhibitor SCH6 (Foy et al., 2003) was kindly provided by the Schering Plough Research Institute (Kenilworth, NJ, USA). The macrocyclic protease inhibitor BILN-2061 was synthesized from the published structure (Lamarre et al., 2003). All compounds were dissolved in DMSO prior to use.

2.3. Primary in vitro SEAP activity screening

Five serial five-fold dilutions of test compounds were made from initial DMSO stocks and each concentration plated in triplicate in 96-well plates. Low passage HCV replicon-bearing cells or Et2AN cells were then trypsinized, counted and seeded into the wells at a final concentration of 1×10^4 cells/well. The final concentration of DMSO in all wells was maintained at 1%. The plates were incubated at 37 °C in a CO_2 incubator for 4 days. The culture supernatant was then removed from each well and heat inactivated for 30 min at 65 °C. SEAP activity was measured using the Phospha-Light Chemiluminescent Reporter Assay (Tropix, Bedford, MA, USA) and a TR717 microplate luminometer with WinGlow software (Tropix/PE Applied Biosystems, Bedford, MA, USA). EC50 values were calculated by comparing SEAP present in drug-treated and untreated wells using the GraphPad Prism 4.0 software package.

2.4. Primary cytotoxicity screening

The cytotoxicity produced by compounds during primary screening was evaluated by MTT assay at the end of the incubation period. Briefly, following removal of the culture supernatant, 100 μl of a 1 mg/ml solution of MTT in RPMI-1640 (Mediatech Inc.) was added to each well. The plates were incubated at 37 °C for 3 h. The MTT solution was completely aspirated and 120 μl/well isopropanol added. The plates were rocked for 10 min at ambient temperature and the OD_{560} measured using a VERSAmax turntable microplate reader with SOFTmaxPRO 4.0 software. CC50 values were calculated by comparing drug-treated and -untreated wells using GraphPad Prism 4.0 software.

2.5. RNA isolation and cDNA synthesis

Total RNA was isolated from 96-well plate cultures of the HCV replicon cell lines and the ET2AN control cells using the Aurum96 kit (Bio-Rad). Approximately 5×10^6 cells

were present in each well based upon the GAPDH levels observed in the RT-PCRs (assumption of ~10,000 copies/cell). Growth medium from each well was aspirated prior to the addition of 150 μl of lysis solution provided with the kit followed by vigorous pipetting. Each lysate was processed subsequently following the recommendations of the manufacturer including a DNase I treatment step. Total RNA was eluted into 80 μl of organic carbon-free water and then converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad). For each well, 5 μl of RNA (representing 6.25% of the sample or $\sim 3 \times 10^5$ cells) was transcribed to cDNA (final volume of 10 μl) using random hexamer and oligo dT priming in an iCycler (Bio-Rad) programmed for 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C, with an infinite hold at 4 °C. The remaining RNA samples were stored at −80 °C for future analyses. cDNA samples were stored at −20 °C.

2.6. Quantitative real time RT-PCR for HCV and human GAPDH

Real-time PCR reactions were performed in an iCycler system equipped with real time optics (Bio-Rad). In 96-well format, 25 μl reactions were prepared using 1 μl of the template cDNA (representing 3×10^4 cells), 12.5 μl of 2× iQ Supermix (Bio-Rad), 5 pM of forward and reverse primers for the HCV NTR and human GAPDH and 3.75 pM of an HCV-specific TaqMan probe and 7.5 pM of a probe for human GAPDH. The HCV probe was labeled with FAM on the 5′-end and Black Hole quencher #1 on the 3′-end while the GAPDH probe was labeled with TET on the 5′-end and Black Hole quencher #1 on the 3′-end (Qiagen). The HCV-F (nts 130–146) and HCV-R (nts 272–290) primers were designed to recognize a conserved region of the 5′-non-translated region common to all HCV genotypes (nucleotide positions are relative to the sequence of GenBank accession number AF139594). The primers for human GAPDH (forward nts 153 to 174 and reverse nts 258 to 274; GenBank accession number M17851) were designed to allow co-amplification of the two targets in multiplex fashion.

Following an initial 3 min incubation at 95 °C, PCR was carried out for 50 replicate cycles consisting of 20 s at 95 °C and 1 min at 62 °C followed by an infinite hold at 4 °C. Random samples of the products of PCR reactions were separated on a 2% agarose gel to confirm the nature of the product.

Cloned amplimers (pGEM-TEZ, Promega) were utilized as synthetic standards for quantification by parallel amplification of 10-fold serial dilutions of both plasmids. Copy numbers were extrapolated from the plot of threshold crossing and starting quantity (copy number). Reactions with perfect efficiency should establish a linear relationship that has a slope of −3.3. Based on this prediction, the PCR assay efficiency and correlation coefficient were determined for each run. Acceptable ranges were considered to be efficiencies of 90–110% and correlation coefficients of no less than 0.95.

3. Results

3.1. Construction of a SEAP-specificity control cell line

Non-cytotoxic compounds that reduce SEAP production in the screening assay could do so without activity against HCV. This would occur if the compound inhibited the encephalomyocarditis (EMCV) internal ribosome entry site (IRES) that drives translation of the second cistron within the replicon, the foot-and-mouth disease (FMDV) 2a proteinase that cleaves tat2A from the tat2ANeo precursor expressed from the upstream cistron of the replicon, the tat-LTR axis driving SEAP transcription, or the reporter enzyme itself. To identify such false positive compounds, a specificity control cell line, Et2AN, was created from En5-3 cells by stably transforming these cells under G418 selection with the plasmid pEt2AN shown in Fig. 1A. In Et2AN cells, the tat2ANeo mini-polyprotein, present in the SEAP-replicon cells, is expressed constitutively under the control of the CMV immediate early (IE) promoter, with translation of the mini-polyprotein directed by the ECMV IRES. Any suppression of SEAP production occurring in SEAP-replicon cells that is due to one of the non-specific mechanisms described above rather than to a specific effect on replication of the HCV replicon should thus be reproduced in Et2AN cells.

To demonstrate the utility of this specificity control, we tested the known HIV tat inhibitor NSC 66020 in the screening system. Fig. 1B shows that the NSC 66020 reduced SEAP production in HCV replicon Ntat2aNeo(RG) cells in a non-cytotoxic manner, but also produced a similar reduction in SEAP production in the Et2AN cells. EC50 values for the reduction in SEAP production are shown in Table 1. The low ratio of the two values (3.37) indicates that reductions in SEAP were not due to a specific suppression of HCV replication. These results were confirmed by RT-PCR analysis, as shown in Fig. 1B and Table 2.

3.2. Utility of the SEAP-replicon screening assay for identifying compounds active against HCV

To demonstrate that the SEAP-replicon screening system was able to identify compounds active against HCV replication, we tested three compounds with known antiviral activity against HCV. Fig. 2A shows that treatment with interferon α-2b reduced SEAP production in Ntat2aNeo(RG) cells in a dose-dependent manner in a time course study with optimal activity being seen after 96 h incubation. Fig. 2B shows that interferon α-2b had similar activity against the other genotype 1b replicon cell line Btat2aNeo(SI), but that SEAP production was not reduced in the Et2AN control cells. This suggests that the reduction in SEAP expression occurred as a result of specific activity against HCV replication. After 96 h of incubation, the total SEAP accumulated in the supernatant of untreated Ntat2aNeo(RG) (5000 ± 300 relative light units; mean ± S.D.) or Btat2aNeo(SI) (2500 ± 200 relative light units) replicon cells was at least 100-fold greater

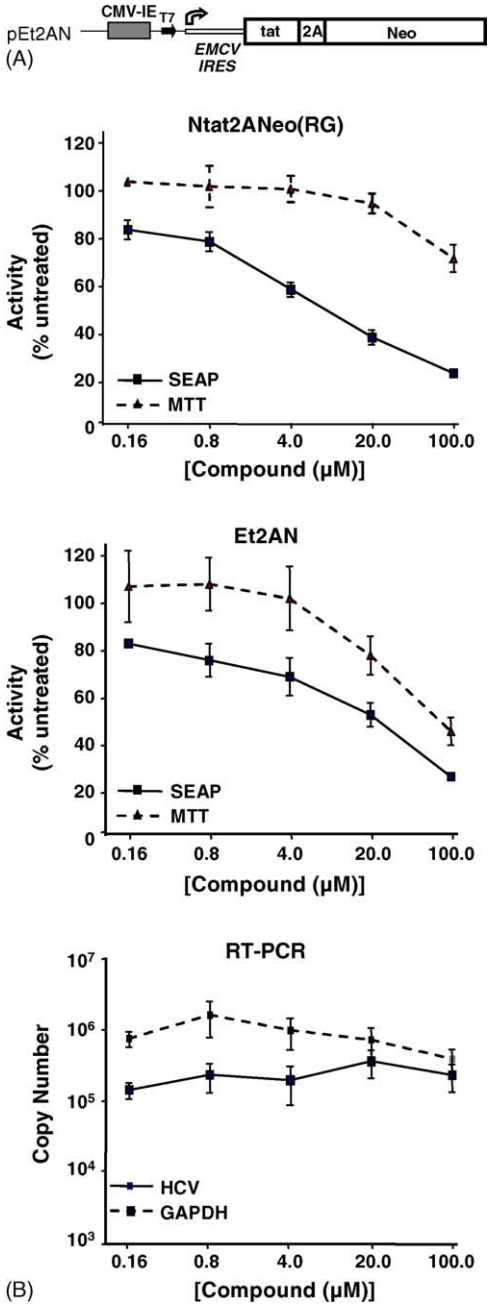

Fig. 1. The Et2AN specificity control cell line. (A) Organization of plasmid pEt2AN showing the tat2ANeo minipolyprotein under control of the CMV IE promoter and EMCV IRES, respectively. (B) NSC-66020 (an HIV tat inhibitor) reduces SEAP production in both the HCV SEAP-replicon Ntat2aNeo(RG) cells and in the specificity Et2AN cells indicating non-specific activity. This was confirmed by the lack of reduction in HCV RNA by quantitative RT-PCR.

than that seen in the supernatant of non-replicon containing cells (25 ± 2 relative light units). Further, in preliminary studies (data not shown) we determined that there was no reduction in SEAP activity in supernatant incubated for 96 h at 37 °C indicating that any antiviral effect seen during primary screening was not being confounded as a result of SEAP degradation during incubation. Results of primary screening with interferon α-2b and two specific small molecules, peptidomimetic inhibitors of the NS3/4A protease of HCV, SCH6 and BILN-2061, and both genotype 1b HCV replicons are summarized in Table 1. As shown, both of these compounds specifically reduced SEAP production in both of the HCV genotype 1b replicon cell lines.

In the phased screening system we utilize, a compound that specifically reduces SEAP production in replicon cell lines in the primary screen undergoes secondary evaluation by RT-PCR to confirm antiviral activity by direct quantification of the impact of treatment on viral RNA levels. The results of these studies with interferon α-2b in both genotype 1b replicon cell lines are shown in Fig. 2B and the EC50 values are summarized in Table 2. The results obtained correlate well with those seen in the primary screen. The antiviral activities of SCH6 and BILN2061 were also confirmed by RT-PCR analysis.

4. Discussion

We previously described the construction of dicistronic subgenomic genotype 1b HCV replicons that expressed the reporter enzyme SEAP in a manner that correlated closely with the intracellular abundance of HCV RNA (Yi et al., 2002). We suggested that these replicons may have potential utility in screening for compounds with antiviral activity against HCV RNA replication. In the studies reported here, we have confirmed the usefulness of these modified replicons and developed an efficient and cost-effective two phase, cell-based, antiviral screening system utilizing a 96-well plate format. During these studies we have shown that the SEAP-replicon-bearing cells thawed from frozen stocks can undergo at least 40 passages in culture without developing mutations that reduce SEAP production.

In the primary phase of this system, stably transfected SEAP-replicon-bearing cells are exposed to putative antiviral agents at various concentrations and the effect on virus replication is measured after 96 h of incubation by quantification of SEAP in the culture medium. The incubation time was selected because our studies showed that antiviral mediated reductions in SEAP increased between 48 and 96 h. However, by 96 h, cell monolayers had reached confluence and, at this point the abundance of replicon RNA relative to cellular GAPDH mRNA is no longer increasing (Yi et al., 2002). Therefore, further incubation does not lead to increased assay sensitivity. This simple enzymatic assay does not require the more expensive and labor-intensive processing required to measure effects on viral RNA by RT-PCR, making it ideal as a primary screen. Others have also described replicon-reporter enzyme systems that have potential for use in antiviral screening. Among the reporter enzymes used in these systems are firefly luciferase (Krieger et al., 2001) and β-lactamase (Zuck et al., 2004). For both of these, enzyme activity is measured in the cells thus one advantage of the system we describe is that, because the reporter enzyme is

Table 1
Activity of compounds in primary SEAP-screening assays

Compound	Replicon	Replicon[a] SEAP EC50	Replicon[b] MTT CC50	SI[c]	Et2AN[d] SEAP EC50	Et2AN/replicon[e] ratio
NSC# 66020	Ntat2ANeoRG	6.5 μM	>100 μM	>15.38	21.9 μM	3.37
Intron alfa 2b	Ntat2ANeoRG	67.6 IU	>500 IU	>7.39	>500 IU	>7.39
	Btat2ANeoSI	40.7 IU	>500 IU	>12.28	>500 IU	>12.28
SCH6	Ntat2ANeoRG	794.3 nM	>10000 nM	>12.59	>10000 nM	>12.59
	Btat2ANeoSI	3311.3 nM	>10000 nM	>3.02	>10000 nM	>3.02
BILN-2061	Ntat2ANeoRG	15.1 nM	>500 nM	>33.11	>500 nM	>33.11
	Btat2ANeoSI	17.4 nM	>500 nM	>28.74	>500 nM	>28.74

[a] SEAP EC50: the concentration required to reduce SEAP production by 50% compared to that in untreated cells.
[b] MTT CC50: the concentration required to reduce MTT values in treated cells to 50% of that in untreated cells.
[c] SI: specificity index, the ratio of the MTT CC50:SEAP EC50 values.
[d] Et2AN SEAP EC50: the concentration required to reduce SEAP production by 50% compared to that in untreated cells of the specificity control line.
[e] Ratio provides a measure of the specificity of antiviral activity of the compound.

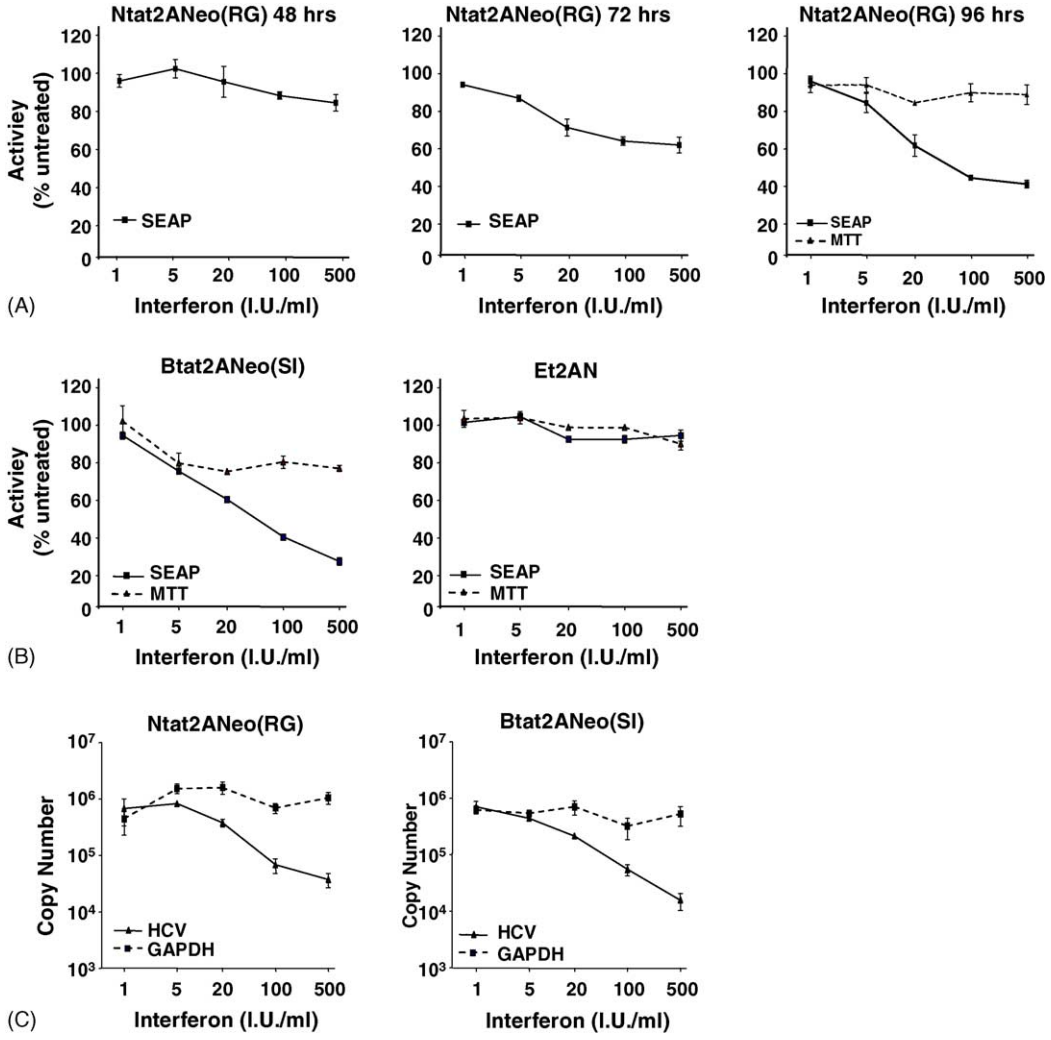

Fig. 2. (A) Effect of interferon α-2b on SEAP production in genotype 1b strain HCV-N (Ntat2aNeo(RG)) after 48, 72 and 96 h exposure. (B) Effect of interferon α-2b on SEAP production in Con1 (Btat2aNeo(SI)) replicon cells and the specificity control Et2AN cells. (C) Confirmation of activity against HCV replicons by quantitative RT-PCR.

Table 2
Activity of compounds in secondary RT-PCR evaluation

Compound	Replicon	Replicon[a] HCV EC50	Replicon[b] GAPDH CC50	SI[c]
NSC# 66020	Ntat2ANeoRG	>100 μM	>100 μM	>1.0
Intron alfa 2b	Ntat2ANeoRG	6.7 IU	>500 IU	>74.6
	Btat2ANeoSI	5.2 IU	>500 IU	>96.1
SCH6	Ntat2ANeoRG	95.5 nM	>10000 nM	>104.7
	Btat2ANeoSI	794 nM	>10000 nM	>12.6
BILN-2061	Ntat2ANeoRG	7.9 nM	>500 nM	>63.3
	Btat2ANeoSI	6.2 nM	>500 nM	>80.6

[a] HCV EC50: the concentration required to produce a 50% reduction in HCV RNA.
[b] GAPDH CC50: the concentration of the compound required to produce a 50% reduction in cellular GAPDH RNA.
[c] SI: specificity index, the ratio of the GAPDH CC50:HCV EC50.

secreted, the cell monolayer can be used to evaluate cytotoxicity, allowing compounds that reduce SEAP production by cellular damage to be more rapidly eliminated from the screening paradigm.

Compounds that reduce SEAP production in a non-cytotoxic manner in the replicon cells are next evaluated in the Et2AN control cells to ensure that activity is HCV-specific. Compounds active against the ECMV IRES, the FMDV 2a proteinase, the tat-LTR axis, or the reporter enzyme will reduce SEAP production in this cell line, indicating that the activity is not HCV-specific. To demonstrate the utility of this specificity control, we tested the known HIV tat inhibitor 7-chloro-5-(2-pyrryl)-3*H*-1,4-benzodiazepin-2(*H*)-one. This compound reduced SEAP production in replicon cells, but did so also in the specificity control Et2AN cells, thus it would have been identified as having non-specific activity in the testing paradigm and would not have progressed to secondary evaluation. For these studies, however, we confirmed that the compound did not posses HCV-specific activity by RT-PCR quantitation of the replicating intracellular HCV RNA. In contrast three compounds with known activity against HCV, interferon α-2b and the NS3/4A proteinase inhibitors, SCH6 and BILN 2061, were all active in replicon-bearing cells but failed to reduce SEAP production in the Et2AN cells at the highest concentration tested. In the screening program, such compounds would progress to secondary evaluation.

Secondary evaluation of antiviral activity utilizes quantitative RT-PCR to directly measure the impact of treatment on intracellular HCV RNA abundance. For RT-PCR, the assay duration is reduced to 72 h. This is because declines in intracellular RNA precede decreases in SEAP in the supernatant media of SEAP-expressing replicon cell lines by approximately 24 h, probably as a result of the kinetic delay in tat signaling of SEAP secretion (Yi et al., 2002). Thus, the 72 h RT-PCR assay closely replicates the results of the 96 h SEAP-screening assay. Changes in cellular GAPDH abundance measured by quantitative RT-PCR during the secondary assay provide a second method of evaluating cellular toxicity. We have shown excellent agreement between the SEAP and RT-PCR assays in detecting antiviral activity. However, the EC50 values generated by RT-PCR are consistently lower than those determined by SEAP reduction. In this regard, it is important to remember that the SEAP assay measures the activity of a reporter enzyme whereas the RT-PCR assay directly measures the impact of antiviral therapy on viral RNA abundance thus we believe that the RT-PCR results provide the most accurate measure of antiviral activity.

In conclusion, we have developed an efficient and cost-effective phased system for the identification of compounds with activity against HCV. The primary screening assays allow the most active and least cytotoxic compounds to be rapidly and inexpensively identified for more complete evaluation using an RT-PCR-based assay. We demonstrate here the utility of this system using replicons derived from two HCV genotype 1b replicons. However, it is well known that there is considerable genetic diversity between viral genotypes (Nolte, 2001) and there is increasing evidence that this diversity is mirrored in differences in responses to antiviral agents (Fried and Hadziyannis, 2004; Thibeault et al., 2004). In this regard, we have recently described the development of a comparable replicon derived from the genotype 1a H77c strain of HCV (Yi and Lemon, 2004). We believe that the addition of such reporter replicons derived from other genotypes will further enhance the value of this screening system for the identification of novel compounds with activity against HCV.

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