A novel small-molecule inhibitor of hepatitis C virus replication acts by suppressing signal transducer and activator of transcription 3

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Objectives: Hepatitis C virus (HCV) infects hepatocytes and causes liver damage. The aim of this study was to identify new classes of host-targeting anti-HCV compounds that may provide novel approaches for antiviral treatment regimens.

Methods: Cell culture-derived HCV (HCVcc), replicons and pseudoparticles were used in combination with high-throughput screening, reporter gene assays and cytotoxicity and signalling pathway analyses.

Results: A small-molecule inhibitor of HCV, N-(cyclopropyl(phenyl)methyl)thieno[2,3-d]pyrimidin-4-amine, designated IB-32, was identified by screening a compound library with a Jc1-luc HCVcc assay. By using various virus models, HCV replication was identified as the predominant step of IB-32’s action. IB-32 inhibited HCVcc (genotype 2a) and HCV replicons (genotype 1b) at low nanomolar ranges (with IC50s of 40±8 and 100±15 nM, respectively). IB-32 was found to be non-toxic when tested against a panel of human cell lines in vitro at the effective antiviral dose. Mechanistically, IB-32 strongly inhibited STAT3 (Tyr705) phosphorylation, a necessary cellular factor for HCV replication and a pivotal therapeutic target for multiple cancers. Furthermore, the inhibition of HCV replication by IB-32 was augmented in cells with STAT3 knockdown. In contrast, the inhibitory effect of IB-32 was attenuated in cells overexpressing a constitutively active form of STAT3.

Conclusion: The results presented here identify a promising STAT3-targeting anti-HCV therapeutic candidate. This novel small molecule could be further optimized and developed for use as both an antiviral and an anti-cancer drug.

Keywords: Hepatitis C virus, inhibitor, N-(cyclopropyl(phenyl)methyl)thieno[2,3-d]pyrimidin-4-amine, IB-32, STAT3

Introduction

An estimated 150 million individuals worldwide are infected with hepatitis C virus (HCV).1 Chronic HCV infection results in serious liver diseases, including cirrhosis and hepatocellular carcinoma.2 A preventative vaccine is not yet available;3 however, potent direct antiviral agents (DAAs) offer a promising approach to the clinical cure of this stubborn pathogen, which has afflicted people for many decades. Currently, inhibitors of HCV NS3/4A protease (boceprevir, telaprevir and simeprevir) and NS5B polymerase (sofosbuvir) have been approved by the US FDA for IFN-containing or IFN-free regimens.4,5 However, issues with DAAs exist with regard to the development of drug-resistant variants, side effects, response differences between individuals, risk of drug–drug interactions and high cost.6,7 Undoubtedly, anti-HCV agents with novel mechanisms of action are still needed for the further improvement of HCV treatment.

As with all viruses, HCV depends on host proteins for viral entry, uncoating, replication, virion assembly and egress.8 Thus, host-targeting antivirals provide a promising approach for anti-HCV therapeutic development. Indeed, targeting these host cofactors may impose a higher barrier to viral resistance and also offer pan-genotypic coverage of all HCV genotypes and serotypes.9,10 More importantly, as certain host molecules are necessary for both virus replication and virus-induced host pathogenesis,11 host-targeting antivirals may yield anti-HCV therapies that both halt HCV replication and abrogate the other pathogenic effects of HCV.

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To this end, we conducted high-throughput screening for new classes of host-targeting anti-HCV compounds and identified a novel thienopyrimidine small-molecule HCV inhibitor, designated IB-32. We also investigated the potential anti-HCV mechanisms of IB-32.

Materials and methods

Cells and reagents

The human hepatoma cell line Huh7.5.1 was provided by Dr Francis V. Chisari (Scripps Research Institute, La Jolla, CA, USA). The 2–3+ cell line harbouring the HCV genotype 1b replicon genome was provided by Dr Stanley Lemon (University of Texas Medical Branch, Galveston, TX, USA). G418 (500 µg/ml) was routinely added to the 2–3+ cells to maintain viral RNA replication. The above cells were cultured in DMEM supplemented with penicillin and streptomycin, 1% non-essential amino acid and 10% FBS (Gibco, Carlsbad, CA, USA) at 37°C in a 5% CO₂ atmosphere. Primary fetal liver cells (PFLCs) were prepared as described previously. IL–6 was obtained from Sino Biological Inc. (Beijing, China). STAT3 inhibitors STA-21 and S31-201 were from Santa Cruz Biotechnology and Selleck (Shanghai, China), respectively. Antibodies were obtained from Cell Signaling Technology (STAT3, phospho-STAT3-Tyr705), Thermo Scientific (HCV Core) and Sigma (Flag, α-tubulin and ß-actin). Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). The antibodies used in the Supplementary data are described in the SI Materials section, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

Production of cell culture-derived HCV (HCVcc)

JFH-1 genome RNA was in vitro transcribed with RiboMAX Large Scale RNA Production Systems (Promega, Madison, WI, USA), purified with a MEGAclear Kit (Ambion) and transfected into naive Huh7.5.1 cells with TransMessenger Transfection Reagent (Qigene). The transfected cells were cultured and routinely expanded for 10 days and the collected virus-containing supernatants were pooled and further used to inoculate naive Huh7.5.1 cells. This supernatant collection/naive cell inoculation process was repeated for at least five cycles (7 days for each cycle) to produce a large volume of HCVcc stock with relatively high viral titre. To produce firefly luciferase HCV reporter viruses (Jc1-Luc HCVcc), linearized pFK-Luc-Jc1 plasmid with a T7 promoter was transfected into naive Huh7.5.1 cells with Lipofectamine LTX (Invitrogen) and the cells were then infected with a T7 RNA polymerase-encoding vaccinia virus at 8 h post-transfection. Culture supernatants were collected and filtered through 0.1 µm filters (Millipore, Billerica, MA, USA) twice to remove the vaccinia virus and obtain Jc1-Luc HCVcc.

High-throughput antiviral and cytotoxicity assays

A library of 40,000 compounds was screened against infectious Jc1-Luc HCV (genotype 2a) (J6/JFH-1). This library is a combination of synthetic (synthesized by Enamine, Ukraine) and natural (plants, actinomycetes and fungi) products from the Institute of Medicinal Biotechnology (Beijing, China). These compounds were kept as stock solutions in 96-well plates at 10 mg/ml in 100% DMSO. In brief, Huh7.5.1 cells were seeded into 96-well plates at 20,000 cells/well and incubated overnight. Subsequently, the cells were infected with Jc1-Luc HCVcc (moi=0.5) for 8 h, and the medium was then replaced with fresh medium containing a final compound concentration of 20 µg/ml for 48 h. For primary screening, compounds that caused a 50% reduction in luciferase levels were progressed to secondary screening. For secondary screening, the compound was added to wells using a five-point, 3-fold serial dilution series with four replicate treatments per dose. Antiviral effects were evaluated by measuring luciferase activity (Promega, Madison, WI, USA), and toxicity/cell viability was assayed using the MTT assay.

HCV infection and treatment

The Huh7.5.1 cells were seeded in six-well plates at a density of 3 × 10^5 cells/cm². After 6 h of incubation, the cells were infected with HCV viral stock (moi=0.5) for 8 h and subsequently treated with IB-32 or solvent as a control. The culture medium was removed at 72 h after inoculation, and the intracellular RNA was extracted. Total intracellular proteins were also extracted. Intracellular HCV RNA was quantified with a one-step quantitative reverse-transcription–PCR (qRT–PCR) kit (Invitrogen). The HCV core protein was detected by western blotting (see below).

HCV pseudotyped particles and the viral entry assay

Packaging of HCV pseudotyped particles (HCVpp) and vesicular stomatitis virus G protein (VSV–G)–pseudotyped lentivirus (VSVpp) was performed as previously described. Briefly, HEK293T cells were cotransfected with the envelope-deficient HCV genome PNL4-3-Luc.R–E– and a plasmid expressing glycoproteins of HCV or VSV, respectively. Viral supernatants were collected at 48, 60, and 72 h post-transfection and filtered. To conduct the HCVpp infection assay, Huh7.5.1 cells were seeded in a 48-well plate at a density of 5 × 10^4 cells/well the day before infection. The next day, 200 µl of supernatant containing HCVpp or VSVpp was added to each well in the presence of 8 µg/ml Polybrene and 2 µl of 2M HEPES (pH 7.55) and then spin-infected for 1.5 h in a tabletop centrifuge (Beckman; 2500 g, 30°C), followed by another 1.5 h of incubation in a CO₂ incubator. The cells were lysed at 48 h post-infection and assayed with the luciferase assay system.

To conduct the HCVcc entry assay, IB-32 was added at a final concentration of 1 µM under the following three conditions. (i) Preincubation: IB-32 was added to cells for 4 h at 37°C followed by washing four times with growth medium before virus infection. (ii) Coinoculation: the mixture of IB-32 and virus was added to cells for 4 h at 37°C followed by washing four times with growth medium. (iii) Post-inoculation: cells were first infected for 4 h at 37°C followed by washing four times with growth medium. IB-32 was added and incubated with the cells for the duration of the experiment. At 72 h post-infection, intracellular HCV RNA was quantified by qRT–PCR as described above.

RNA interference

All of the small interfering RNAs (siRNA), including the scrambled negative control siRNA (siCtrl), were purchased from RiboBio (Guangzhou, China). The sequences of CD81 siRNA (siCD81) and STAT3 siRNAs (siSTAT3) were as follows: siCD81, 5′-GGGACCAAGUGCGAGAUU-3′; siSTAT3-1, 5′-CAUCUGCCUAGAUAGCCUA-UdTdT-3′; siSTAT3-2, 5′-UGACAGGUUCUGUCA-3′. siRNA transfection was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions.

Immunofluorescence staining and confocal microscopy

Cells cultured on glass slides were fixed with 4% paraformaldehyde in PBS at room temperature for 30 min. After two washes with PBS, the fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and incubated with the primary antibody overnight at 4°C. After three washes with PBS, the cells were further incubated with Dylight fluorescent dyes (Thermo Fisher Scientific, Inc., USA) for 1 h at room temperature. The nuclei were stained with DAPI. After two washes with 0.1% Triton X-100 in PBS and three washes with PBS, the cells were analysed using a Zeiss LSM 700 laser confocal microscopy system (Carl Zeiss, Inc., Thornwood, NY, USA).
Calculation of anti-HCV potency (IC_{50}, IC_{90}), cytotoxicity (CC_{50}) and selectivity index (SI)

To determine anti-HCV potency, cytotoxicity and the SI, Huh7.5.1 cells were seeded in 96-well plates (~30% confluence). The potency of the compounds [IC_{50} and 90% inhibitory concentration (IC_{90})] was calculated by determining relative infection efficiency levels in the presence of serial compound dilutions at 72 h post-infection by the luciferase assay described above. Parallel uninfected cell cultures treated with the same compound dilutions were used to determine CC_{50} values by the MTT assay. The IC_{50}, IC_{90} and CC_{50} values were interpolated graphically from the dose–response curves. The SI was calculated by dividing the CC_{50} value by the IC_{50} value.

Immunoblotting

Western blotting was performed according to the standard protocols of our laboratory, as reported elsewhere.

Statistical analysis

Bar graphs were plotted to show the mean ± standard deviation (SD) of at least two independent experiments. The statistical analyses were performed using Prism 5. A P value of <0.05 by Student’s t-test was considered statistically significant.

Results

Identification of IB-32 by screening a small-molecule library using the HCVcc system

To identify new host-targeting inhibitors of HCV, Huh7.5.1 cells and the Jc1-luc HCVcc (J6/JFH1) system were used to screen >40,000 novel-structure compounds. This HCVcc model was used because it provided the maximum possibility of identifying inhibitors for every stage of the HCV life cycle. The primary screens identified 60 candidate compounds that inhibited >50% of luciferase activity at 20 μg/mL. After cytotoxicity analysis by the MTT assay, 10 compounds were identified that showed no toxicity at relatively high concentrations. Of the 10 compounds, 4 suppressed Jc1-luc HCVcc, with IC_{50} values ranging from 0.06 to 4.81 μM and SIs ranging from 5.6 to >300. Of the remaining anti-HCV compounds, one of the most active was a thieno[2,3-d]pyrimidine with the structural formula N-(cyclopropyl(phenyl)methyl)thieno[2,3-d]pyrimidine-4-amine (C_{16}H_{15}N_{3}S, molecular weight 281.38), here designated IB-32 (Figure 1a). IB-32 was then synthesized for further studies, as depicted in Figure 1(b). Briefly, cyclopropyl (phenyl) methanol was prepared by the reduction of phenyl ketone with NaBH₄, and the intermediate was then treated with SOCl₂ to produce cyclopropyl(phenyl)chloromethane. Finally, N-(cyclopropyl(phenyl)methyl)thieno[2,3-d]pyrimidine-4-amine was synthesized by mixing thieno[2,3-d]pyrimidine-4-amine with cyclopropyl(phenyl)chloromethane in the presence of Et₃N in the appropriate solvents.

IB-32 inhibits HCV RNA replication

Next, the anti-HCV efficacy and cytotoxicity of IB-32 were evaluated. IB-32 strongly suppressed Jc1-luc HCV infection at 72 h in a concentration-dependent manner (Figure 2a) without inducing host cell toxicity, as measured by the MTT assay (Figure 2b). The IC_{50} and IC_{90} values of the compound measured by the luciferase activity assay were determined to be 40 ± 8 and 500 ± 20 nM, respectively, and the CC_{50} of IB-32 was estimated to be 10000 ± 40 nM (Figure 2c). Based on calculation using the concentration dependency of IB-32, the compound exhibited an SI value of >250. In addition, the JFH-1 HCV RNA replication level was significantly suppressed after incubation with the compound for 72 h, as determined by qRT–PCR analysis (Figure 2d). The HCV core protein was also gradually decreased with increasing concentration of IB-32 (Figure 2e). The results in Figure 2(f) and (g) show that IB-32 suppressed JFH-1 HCV RNA replication in a time-dependent manner; a similar inhibitory effect on JFH-1 HCVcc RNA replication was observed using immunofluorescence staining with an antibody against the HCV core protein in Huh7.5.1 cells treated with IB-32 (Figure 2h). These results suggest that IB-32 is a potent HCV inhibitor in vitro.

HCV replication is the step primarily inhibited by IB-32

To further delineate the individual steps in the HCV life cycle that are affected by IB-32, the effects of IB-32 on viral entry, replication and progeny virus production were evaluated. HCVpp and HCVcc entry was measured to determine the effect of IB-32 on viral entry. As shown in Figure 3(a), IB-32 treatment had no effect on HCVpp entry into hepatocytes. For HCVcc entry, IB-32 was added at different timepoints during HCVcc inoculation of Huh7.5.1 cells and the results are shown in Figure S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org), which clearly demonstrates that IB-32 had no effects on HCVcc entry. However, after treatment of the 2–3 × 10⁷ cell line harbouring the HCV genotype 1b replicon
genome with serial concentrations of IB-32 for 3 days, the abundances of HCV RNA and viral protein expression decreased in a concentration-dependent manner (Figure 3b and c). Furthermore, the results in Figure 3(d) and (f) show that IB-32 also suppressed HCV replication in a time-dependent manner. Finally, the treatment of HCVcc-infected Huh7.5.1 cells with
transfected cells were used to inoculate naive Huh7.5.1 cells. After 2 days of incubation, total cellular RNA was isolated using TRIzol reagent for qRT–PCR. Also exhibited CC 50 values of 17327 (Figure 4c) and 18680 (Figure 4d). HepG2, with a CC 50 value of 16100 cell line, IB-32 was non-toxic in another human liver cell line, different tissues. Similar to the observations using the Huh-7.5.1 cell line, IB-32 was non-toxic in another human liver cell line, HepG2, with a CC50 value of 16 100 ± 530 nM (Figure 4a). IB-32 also exhibited CC50 values of 17 327 ± 490 nM in a human cell line derived from lung carcinoma (A549) (Figure 4b), 14 284 ± 280 nM in a human rhabdomyosarcoma cell line (RD) (Figure 4c) and 18 680 ± 190 nM in a cervical cancer cell line (HeLa) (Figure 4d). The results generated from these cell-based assays likely represent the bona fide inhibition of viral replication and are not false-positive observations resulting from cell toxicity.

**Cytotoxicity of IB-32 in different cell types**

To rule out the possibility that IB-32 is toxic to cells, cytotoxicity was evaluated against a panel of cell lines from an array of different tissues. Importantly, it is becoming clear that STAT3 plays a crucial role during the HCV life cycle. Thus, our findings encouraged us to further delineate the possible mechanism of the inhibition of STAT3 by IB-32.

We first sought to determine the endogenous phospho-STAT3 (Tyr705) level in Huh7.5.1 cells and 2–3+ replicon cells in response to IB-32 treatment. After 24 h of treatment at the indicated concentrations, ranging from 0 to 1 μM, Huh7.5.1 cell and 2–3+ replicon cell lysates were prepared for western blot analyses. IB-32 significantly inhibited the phosphorylation of STAT3 (Tyr705) in a concentration-dependent manner when compared with the untreated control, but the levels of total STAT3 remained unchanged (Figure 5a and b); this inhibition was also rapid and time dependent. A 20-min treatment was sufficient to block activation (Figure 5c), suggesting the possible direct inhibition of STAT3 phosphorylation by IB-32.

Next, we investigated the influence of IB-32 on the IL-6-induced phosphorylation of STAT3 (Tyr705). Huh7.5.1 cells were incubated with the indicated concentrations of IB-32 for 2 h before stimulation with IL-6 (30 ng/mL) for 15 min, and whole-cell lysates were processed for western blot analyses. As shown in Figure 5(d), the IL-6-induced phosphorylation of STAT3 (Tyr705) was suppressed by IB-32 treatment in a concentration-dependent manner.

IB-32 suppressed progeny virus infectivity in culture supernatants (Figure 3f).

**IB-32 suppresses STAT3 phosphorylation**

To examine the possible intracellular pathways influenced by IB-32, we tested the activation status of a series of signalling molecules, including MAP kinases, PI3 K/Akt, Jak/Stat, Wnt and TGF-β (Figure S2). Ultimately, STAT3 (Tyr705) phosphorylation was found to be significantly suppressed upon IB-32 treatment.

**A novel HCV replication inhibitor**

HCV replication is the step primarily inhibited by IB-32. Huh7.5.1 cells were infected with VSVpp (VSV-pseudotyped lentivirus) and HCVpp in the presence of serially diluted IB-32 for an entry assay. The results are calculated relative to DMSO-treated cells. (b) 2–3+ cells were incubated with IB-32 for 3 days. Total cellular RNA was isolated using TRIzol reagent for qRT–PCR analyses of the HCV genome. (c) Cell lysates from the samples described above (b) were analysed by western blotting. (f) Huh7.5.1 cells were infected with JFH-1 HCVcc (moi = 0.5) for 8 h, followed by various concentrations of IB-32 for 72 h. Supernatants from the transfected cells were used to inoculate naive Huh7.5.1 cells. After 2 days of incubation, total cellular RNA was isolated using TRIzol reagent for qRT–PCR analyses of the HCV genome. The data represent means ± SD (n = 3, NS, not statistically significant). P values were determined using Student’s t-test.
Because Huh-7 cells are highly transformed tumour cells, to better understand the regulation of IB-32 on STAT3 phosphorylation, the effects of IB-32 in cultured PFLCs were also investigated. In accordance with the results observed in hepatoma cells, phosphorylation of STAT3 (Tyr705) was suppressed by IB-32 treatment in a concentration-dependent manner in cultured PFLCs (Figure 5e).

Previous studies suggested that STAT3 could be activated by HCV.\textsuperscript{18,20,22} We further determined whether IB-32 was able to decrease STAT3 activation induced by HCV infection. As shown in Figure 5(f), STAT3 phosphorylation induced by HCV infection could be efficiently inhibited by IB-32.

In conclusion, constitutive and IL-6- or HCV-induced phosphorylation of STAT3 (Tyr705) was inhibited by IB-32.

**IB-32 inhibition of HCV replication is STAT3 dependent**

We hypothesized that, if IB-32 truly inhibits HCV by suppressing STAT3 (Tyr705) phosphorylation, IB-32 should be more effective in cells in which STAT3 is knocked down by siRNA. To address this, the effect of the siRNA knockdown of STAT3 on HCV infection was first confirmed in our system. As shown by the luciferase assay in Figure 6(a), STAT3 silencing led to significant inhibition of HCV infectivity. The results in Figure 6(b) show that total STAT3 decreased by \( \sim 40\% - 60\% \) and that the HCV core protein was concomitantly reduced. Moreover, the inhibition by IB-32 in STAT3 siRNA-knockdown cells was also assayed. Huh7.5.1 cells were transfected with siRNAs (siCtrl and siSTAT3-1) for 2 days, infected with HCV (moi=0.5) and then treated with IB-32 at the indicated concentration for 48 h. The results in Figure 6(c) show that the inhibitory effect of IB-32 on HCV in STAT3-knockdown cells was augmented compared with the control-treated cells. The results were confirmed at the protein level (Figure 6d).

It has been reported previously that constitutively activated STAT3 increases HCV replication.\textsuperscript{18} We therefore assessed whether the overexpression of a constitutively active form of STAT3 could reverse the IB-32-mediated inhibition of HCV replication. The pMIR-STAT3C-cFlag vector expressing a constitutively active form of the STAT3 molecule, STAT3-C, was transiently transfected into Huh7.5.1 cells. STAT3-C has two substituted cysteine residues at positions A661 and N663 of the WT STAT3, which allows the formation of disulphide bonds between STAT3 monomers and results in a constitutively active form of STAT3.\textsuperscript{23} Using the JC1-luc reporter system, we showed that HCV replication was inhibited by \( 60\% - 70\% \) in IB-32-treated cells. However, HCV replication was significantly restored (60\% versus 20\% at 0.125 \( \mu \text{M} \) of IB-32, 70\% versus 30\% at 0.0625 \( \mu \text{M} \) of IB-32, of inhibition) in cells exogenously expressing constitutively activated STAT3 (Figure 6e). The results in Figure 6(f) also show that the overexpression of constitutively activated STAT3 rescued HCV replication at the protein level (Figure 6f).

Next, the effect of IB-32 together with STAT3-specific inhibitors was investigated. Two commercial STAT3 inhibitors, STA-21 and S3I-201, were used. STA-21 binds to the SH2 domain of STAT3.

\begin{figure}
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\caption{Cytotoxicity of IB-32 in different cell types. (a) HepG2, (b) A549, (c) RD and (d) HeLa cells were treated with the indicated concentrations of IB-32 for 72 h and cell viability was determined by MTT assays. Data on cell viability are expressed as percentages relative to vehicle-treated cells. The data are means \( \pm \) SD (\( n = 3 \)).}
\end{figure}
and specifically prevents dimerization of STAT3 and DNA binding. S3I-201 targets the SH2 domain and blocks STAT3-dependent transcription activity. Huh7.5.1 cells were infected with HCV for 8 h and then treated with IB-32, STA-21 or S3I-201, either alone or in combination at the indicated concentration for 48 h. Remarkably, the anti-HCV effect of combination of IB-32 with STA-21 or S3I-201, compared with the IB-32, STA-21 or S3I-201 treatment alone, was enhanced (Figure 6g and i). The results were confirmed at the protein level (Figure 6h and j). Collectively, these observations further confirm that the IB-32 inhibition of HCV replication is STAT3 dependent.

Discussion

The HCV interaction with host factors represents an attractive target for drug discovery. The aim of this study was to identify new classes of anti-HCV compounds, particularly ones targeting virus–host interactions. Such compounds may be critical for patients who fail to respond to current DAA treatment or harbour drug-resistant HCV isolates. Using a high-throughput screening approach, we were able to identify compounds that significantly inhibited HCV replication. A small-molecule inhibitor, IB-32, which belongs to the group of thienopyrimidine chemical derivatives, was selected for further study based on its efficient anti-HCV activity, low cytotoxicity, favourable physicochemical properties and novel chemical structure.

Thienopyrimidines occupy a special position among fused pyrimidines because they are structural analogues of biogenic purines and can be considered as potential nucleic acid antimetabolites. Their derivatives possess remarkable activity, such as antifungal, antibacterial, insecticidal, hypnotic, anti-allergic and antiviral activity. Although thienopyrimidine derivatives have already been reported to have antiviral activity against several other viruses, such as HIV-1 and herpes simplex virus, anti-HCV thienopyrimidine compounds have not yet been reported. Here, we demonstrate for the first known time that a thienopyrimidine-bearing compound, N-(cyclopropyl(phenyl)-methyl)thieno[2,3-d]pyrimidin-4-amine, has a potent antiviral effect on HCV replication. Cell-based evaluations showed that IB-21 was capable of inhibiting the replication step of HCV but not viral entry. This notion is supported by evidence that IB-32 inhibits viral genomic RNA replication in 2–3+ replicon cells but does not interfere with the transduction of pseudotyped virus into host cells. More efficient inhibitory activity was found for genotype 2a JFH1 HCVcc than genotype 1b Con1 replicon. Overall, the IC50 values ranged from 40 to 250 nM, with an ideal
Figure 6. IB-32 inhibition of HCV replication is STAT3 dependent. (a) Huh7.5.1 cells were transfected with the indicated siRNA, followed by infection with Jc1-luc (moi = 0.5) for 8 h. At 3 days post-infection, HCV infectivity was determined by measuring luciferase activity. (b) Total cell lysates harvested from cells treated as described in (a) were immunoblotted with the indicated antibodies. (c) Huh7.5.1 cells were transfected with the indicated siRNA followed by infection with Jc1-luc (moi = 0.5) for 8 h; the cells were then treated with the indicated concentration of IB-32 for 48 h. HCV infectivity relative to siCtrl-transfected cells treated with control (DMSO) is expressed as the fold change. (d) Total cell lysates harvested from cells treated as described in (c) were immunoblotted with the indicated antibodies. (e) Huh7.5.1 cells were transiently transfected with the vector pMIR-STAT3C-cFlag expressing a constitutively active form, STAT3-C, and control vector, followed by infection with Jc1-luc (moi = 0.5) for 8 h. The cells were then treated with the indicated concentration of IB-32 for 48 h. HCV infectivity relative to control-treated cells is expressed as fold change. (f) Total cell lysates harvested from cells treated as described in (e) were immunoblotted with the indicated antibodies. (g and i) Huh7.5.1 cells were infected with Jc1-luc (moi = 0.5) for 8 h and then treated with IB-32 in combination with STA-21 (g) or S3I-201 (i) at the indicated concentration for 48 h. HCV infectivity relative to DMSO-treated cells (control) is expressed as the fold change. (h and j) The corresponding cell lysates as described in (g) and (i) were prepared and immunoblotted with the indicated antibodies. Data are means ± SD (n = 3).
selectivity index. These data suggest that IB-32 offers an excellent opportunity for further optimization and may be a potential therapeutic agent for HCV.

STAT3 plays an important role in all facets of the HCV life cycle. It has been documented that HCV NS5A and core protein activate STAT3 via oxidative stress. Constitutive STAT3 activation results in cellular transformation. Importantly, STAT3 is a proviral host factor for HCV via the positive regulation of microtubule (MT) dynamics. The mechanistic basis of IB-32 antiviral activity is largely unknown and needs to be elucidated. Our exploration of IB-32-regulated intracellular signalling pathways indicated that IB-32 suppresses both constitutively active and IL-6-induced STAT3 phosphorylation at Tyr705. Furthermore, the inhibitory effect of IB-32 on HCV in STAT3 siRNA-knockdown cells was augmented compared with the control-treated cells, whereas the overexpression of constitutively active STAT3 substantially rescued HCV replication in the presence of IB-32. However, the overexpression of constitutively active STAT3 only partially rescued HCV replication in the presence of IB-32. Thus, we cannot exclude the possibility that additional, as-yet-unrecognized mechanisms, such as other host factors or pathways, contribute to the efficacy of this compound. Studies to evaluate this in greater detail are ongoing in our laboratories. Collectively, these results demonstrate that IB-32 inhibits HCV by suppressing STAT3 (Tyr705) phosphorylation. Based on these results, we propose a model illustrating the mechanism of IB-32 antiviral activity, as shown in Figure 7. In conclusion, we present the novel STAT3 inhibitor IB-32, which efficiently suppresses HCV infection with little cytotoxicity, via an action mode that is distinctly different from that of the marketed anti-HCV drugs, with the potential advantage of decreased drug-resistant mutations.

Drug development is a long and arduous journey. Our compound, IB-32, shows good anti-HCV activity in the low nanomolar range. Furthermore, we reveal that IB-32 possesses the potential for selective/preferential targeting. It inhibited HCV mainly by mainly suppressing host factor STAT3 (Tyr705) phosphorylation via a mode of action different from that of the marketed anti-HCV drugs, with the potential advantage of decreased drug-resistant mutations. We do not exclude additional, as-yet-unrecognized target(s). Safety, rather than mere activity and selectivity, is another major limiting factor in drug development. STAT3 is constitutively activated in the liver of HCV-positive patients.

Figure 7. Model illustrating the mechanism of IB-32 antiviral activity. Illustration of our model outlining the STAT3 signalling cascade and identifying the specific target (or targets) acted upon by IB-32. STAT3 becomes activated in hepatocytes via oxidative stress, cytokines and growth factors, thus allowing tubulin polymerization and enhanced MT activity. HCV is then able to establish a productive infection. IB-32 suppresses STAT3 (Tyr705) phosphorylation, disrupts tubulin polymerization and inhibits HCV replication. The question mark indicates that we still need to ascertain other target(s) of IB-32.
Overactive proteins offer the prospect of pharmacological inhibition. Importantly, previous studies reported that antisense oligonucleotides against STAT3 killed tumour cells but had little effect on the animal.28,29 With this in mind, STAT3 has become an ideal drug target. Our experiments also demonstrated that suppressing STAT3 (Tyr705) phosphorylation by IB-32 is safe in vitro. In conclusion, IB-32 shows great feasibility for clinical use in the treatment of HCV patients.

In addition, aberrant activation of STAT3 occurs in many human cancers and promotes tumour progression. The activation of STAT3-mediated tumorigenesis involves the dysregulation of gene expression, leading to cell proliferation and resistance to apoptosis, as well as tumour angiogenesis, invasion and migration.10 Given its importance in cancer, STAT3 is the focus of anti-cancer drug discovery efforts and has gained much attention.28 Some small-molecule STAT3 inhibitors have been reported. Sorafenib, a tyrosine protein kinase inhibitor approved for use in advanced renal cell carcinoma and hepatocellular carcinoma patients, has been shown to inhibit STAT3 activation.31 Therefore, it is feasible that treatment with IB-32 will have an important role in carcinoma patients.

In spite of the rapid development of antiviral agents, antiviral resistance remains a challenge for the treatment of HCV infection due to the inherent characteristics of HCV RNA replication, and preventing and treating antiviral resistance are important for the development of novel antiviral strategies.32 HCV propagation is dependent on host cellular cofactors; moreover, HCV interferes with host cell signalling pathways, causing pathogenic effects such as steatosis, hypercholesterolaemia and insulin resistance/diabetes.33 Hence, targeting host cellular factors that are critical for both HCV replication and viral pathogenesis may yield antiviral and antipathogenic effects. The cyclophilins (CYPAs) are one such example of a host target antiviral for chronic hepatitis C. Targeting host factors in general shows promise as they have a high barrier to drug resistance and in most cases are pan-genotypic. The CYP inhibitor alisporivir is under Phase III clinical investigation and shows potent anti-HCV effectiveness in patients coinfected with HCV and HIV.34 In fact, HCV modulates and depends on lipid metabolism, enhancing lipogenesis for the establishment of efficient viral infection, and cholesterol-lowering statins are not only effective in reducing steatosis and retarding fibrosis in viral and non-alcoholic fatty liver disease 1 (NAFLD) patients, nicely exemplifying the notion stated above.11 These facts justify the assertion that therapeutic intervention to activate STAT3 by IB-32 treatment may have a place in the treatment of chronic HCV infection and HCV-related hepatocellular carcinoma in the future.

Taken together, our data suggest that the small molecule IB-32 inhibits HCV by suppressing STAT3 (Tyr705) phosphorylation. The involvement of STAT3 as a mediator in proviral and pathogenic pathways positions IB-32 as a potentially critical and druggable candidate, not only as an antiviral but also as an antipathogenic agent.

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Transparency declarations
None to declare.

Supplementary data
Supplementary Methods, Figures S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References

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