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In vitro antiviral activity of SCH446211 (SCH6), a novel inhibitor of the hepatitis C virus NS3 serine protease

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***Background*: Current hepatitis C virus (HCV) therapies may cure ~60% of infections. They are often contraindicated or poorly tolerated, underscoring the need for safer and more effective drugs. A novel, α-ketoamide-derived, substrate-based inhibitor of the HCV serine protease (SCH446211) was developed. Compared with earlier reported inhibitors of similar chemical class, it has a P_1'–P_2' extension which provides extended interaction with the protease active site. The aim of this study was to evaluate the *in vitro* antiviral activity of SCH446211.**

***Methods*: Binding constant of SCH446211 to HCV NS3 protease was measured with the chromogenic substrate *in vitro* cleavage assay. Cell-based activity of SCH446211 was evaluated in replicon cells, which are Huh-7 hepatoma cells stably transfected with a subgenomic HCV RNA as reported previously. After 72 h of incubation with SCH446211, viral transcription and protein expression were measured by real-time RT–PCR (TaqMan), quantitative *in situ* hybridization, immunoblot and indirect immunofluorescence.**

***Results*: The binding constant of SCH446211 to HCV NS3 protease was 3.8±0.4 nM. HCV replication and protein expression were inhibited by SCH446211 in replicon cells as consistently shown by four techniques. In particular, based on quantitative real-time RT–PCR measurements, the IC_{50} and IC_{90} of SCH446211 were estimated to be 40±20 and 100±20 nM (n = 17), respectively. Long-term culture of replicon cells with SCH446211 reduced replicon RNA to <0.1 copy per cell. SCH446211 did not show cellular toxicity at concentrations up to 50 μM.**

***Conclusions*: SCH446211 is a potent inhibitor of HCV protease *in vitro*. Its extended interaction with the HCV NS3 protease active site is associated with potent *in vitro* antiviral activity. This observation is potentially a useful guide for development of future potent inhibitors against HCV NS3 protease.**

Keywords: chronic hepatitis, antiviral therapy, HCV replicon, *in situ* hybridization

Introduction

Chronic infection with hepatitis C virus (HCV) affects 170 million people worldwide. HCV shows a remarkable tendency to establish persistent infections and chronic liver disease, ultimately leading to cirrhosis and hepatocellular carcinoma.[1] Current standard therapy with peg-interferon and ribavirin has a sustained virological response (SVR) rate of 70–80% in genotypes 2 or 3 in 24 weeks of therapy.[2] Its results are less satisfactory with genotype 1, SVR is ~50% and requires longer treatment (48 weeks), although a 24 week schedule has been shown to be effective in a subgroup of patients with low viral load who achieved very rapid virological response.[3] Both interferon-α (IFN-α) and ribavirin cause significant side effects, often leading to dose reduction or premature discontinuation of therapy. Lack of a complete virological response, relapse and toxicity concerns still

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represent major barriers to treatment in a substantial proportion of patients. Thus, more effective and better tolerated drugs are needed to treat chronic hepatitis C.

HCV is a member of the *Flaviviridae* family with a positive-stranded RNA genome of ~9.6 kb.[4] Its genome encodes a 3000 amino acid polyprotein, which is processed co- or post-translationally by host and viral proteases.[5] The NS3 serine protease, comprising the 189 N-terminal amino acids of protein NS3, is essential to HCV replication. It forms a heterodimer with NS4A, which is a cofactor for protease activity. Following the *cis* cleavage of NS3-NS4A site, NS3 protease cleaves the NS4A-NS4B, NS4B-NS5A and NS5A-NS5B sites to release the non-structural proteins.[6–10] The NS3 serine protease is constituted of two six-stranded β-barrel trypsin-like folds, defining a crevice in which substrate interactions with the catalytic triad take place.[11,12] The shallowness and solvent accessibility of this pocket have made the development of effective inhibitors a challenging task.

The lack of a robust tissue culture system and a small animal model had hindered the pre-clinical evaluation of HCV inhibitors. The recent development of *in vitro* HCV infection systems[13–15] will provide an opportunity to evaluate HCV inhibitors in the entire HCV life cycle. Earlier development of HCV replicon has proved to be an invaluable tool to evaluate new inhibitors of HCV replication.[16–18] Recently, proof-of-concept clinical trials were reported with HCV NS3 protease inhibitors BILN-2061, VX-950 and SCH503034.[19–21] All these three compounds have submicromolar IC_{90} in the replicon assay and markedly reduced serum viral load in patients chronically infected with HCV. Resistance mutations against each of these inhibitors were developed. A156T/V conferred strong resistance to all three compounds, while D168V was resistant to BILN2061 and remained sensitive to VX-950 and SCH503034. The overlapping and distinct resistance profiles emphasizes the importance in versatility of inhibitors to optimize potency and reduce the emergence of resistance. Here, we report the *in vitro* antiviral activity of SCH446211 (SCH6), a new ketoamide peptidomimetic inhibitor of NS3. Our results demonstrate that SCH446211 is a potent inhibitor of HCV protease *in vitro*. Its extended interaction with HCV NS3 protease is associated with potent *in vitro* antiviral activity and its resistance profile is also discussed.

Materials and methods

HCV NS3/NS4A protease chromogenic assay

The continuous chromogenic assay for HCV protease was reported previously.[22] Briefly, protease $NS4A_{21-32}$-GSGS-$NS3_{3-181}$ was added to assay buffer containing peptide substrate linked to chromophore Ac-DTEDVVP(Nva)-O-PAP. The peptide sequence is derived from the NS5A-NS5B junction where the C-terminal group is coupled to chromophoric phenylazophenol (PAP). Serial diluted inhibitor was mixed with protease. The assay was performed at 30°C in 96-well microtitre plates. The reactions were monitored at 30 s intervals for 1 h by reading the absorbance at 370 nm in a Spectromax Plus microtitre plate reader (Molecular Devices, Sunnyvale, CA, USA). The data were fitted to the two-step slow-binding inhibition model of Morrison and Walsh $P = v_s t + (v_0 - v_s)(1 - e^{-kt})/k$ using SAS version 8.0 (SAS Institute Inc.) The overall K_i^* was calculated from the estimated steady-state velocities $\{v_s = V_{max} S/[K_m(1 + I/K_i^*)]\}$.

Replicon cells and treatment with SCH446211

The replicon cell clone 16 contains identical HCV replicon RNA sequences as reported,[16] except for the incorporation of adaptive mutation(s) S1179I.[17] The replicon clones were generated by transfection of replicon RNA followed by 0.5 mg/mL of G418 selection. The replicon cells were routinely maintained with DMEM medium supplemented with 4 mM L-glutamine, 1.8 mM sodium bicarbonate, 1× non-essential amino acids and 1 mM sodium pyruvate (Mediatech, VA, USA) on collagen-coated plates (BD Biosciences Pharmingen, CA, USA).

For real-time RT–PCR (TaqMan), 4000 cells were seeded in a 96-well plate in DMEM medium containing 0.5 mg/mL of G418. SCH446211 was added to the medium in concentrations from 5 μM to 10 nM in the presence of 5% FCS, 0.5 μg/mL of G418 and 0.5% DMSO. SCH446211 and medium were refreshed every day for 72 h.

For *in situ* hybridization, immunoblot and immunofluorescence, replicon cells were plated at 3×10^5 cells per 100 mm tissue culture dish. After 24 h, SCH446211 was added to the cells at final concentrations of 50, 100 and 500 nM with 10% fetal bovine serum, 0.5% DMSO and 1 mg/mL of G418 (all from Invitrogen, Basel, Switzerland). SCH446211 was refreshed every 24 h. After 72 h, the cells were trypsinized and processed.

Real-time RT–PCR

The 96-well plates were aspirated and washed. Cell-cDNA buffer (Ambion, TX, USA) (30 μL) was added to each well and heated at 75°C for 5 min. Lysate (1 μL) was added to real-time RT–PCR (TaqMan) reactions containing 1× RT–PCR master mix (Applied Biosystems, CA, USA), RNase inhibitor, 50 μM 5B forward (5′ATGGACAGGCGCCCTGA) and reverse (5′TTGATGGGCAGC-TTGGTTTC) primers, 5B probe (5′CACGCCATGCGCTGCGG-FAM) and 1× GAPDH primer and probe mixture (Applied Biosystems). The PCR reactions were run on an ABI PRISM 7900HT Sequence Detection System using the following program: 48°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s followed by 60°C for 1 min. Amplification of HCV RNA was linear over five logs and the detection sensitivity was estimated to be 500 RNA copies per reaction with cell lysate and 10 copies per reaction with purified RNA.

The difference in cycle numbers (CT) needed to amplify the NS5B and GAPDH to the threshold level (ΔCT), was plotted against the log of compound concentrations and fitted to the sigmoid dose–response model using SAS version 8.0 (SAS Institute) or PRISM (Graphpad Software Inc.). IC_{50} and IC_{90} indicate the drug concentrations needed to achieve 2-fold (50%) and 10-fold (90%) inhibition, respectively, compared with no treatment.

HCV RNA by in situ hybridization

Cells were collected in 150 μL of PBS after drug treatment; 20 μL (~50 000 cells) was layered onto poly-L-lysine-coated slides (Kindler GmbH & Co. Freiburg, Germany) and dried. The slides were fixed, washed and denatured. NS3 coding region was cloned into pGM vector under the T7 promoter, linearized with *Spe*I (Promega, Catalys AG, Wallisellen, Switzerland) and used for *in vitro* synthesis of 1028 base long, [^{35}S]CTP-labelled RNA of antigenomic polarity, according to standard protocols (specific activity: 0.1–0.2×10^8 cpm/μg of RNA). The *in situ* hybridization procedure followed standard protocols. Cells were stained with H&E and visually inspected for autoradiographic silver grain density assessment. At least 20 cells were counted by two independent observers, and results expressed as mean (±SD) autoradiographic silver grain

number per cell, after subtracting the average grain number per untransfected cell, hybridized and processed in parallel. Differences between experiments were assessed by the Student's *t*-test.

Antibodies

A rabbit polyclonal antibody was raised against NS3 protein. A monoclonal antibody 5B-3B1 directed against HCV NS5B protein and the antibody against β-actin were kindly provided by Dr D. Moradpour (Lausanne, Switzerland) and Dr C. Chaponnier (Geneva), respectively.

Immunoblot

Cells were lysed in 250 mM Tris–HCl, pH 6.8, 500 mM DTT, 10% SDS, 0.5% Bromophenol Blue and 50% glycerol. Samples were boiled, loaded onto a 12% polyacrylamide gel and separated by electrophoresis. Proteins were transferred onto nitrocellulose membrane (Millipore, Milian, Geneva, Switzerland). Membranes were probed with primary antibody at 1:12, 1:1000 and 1:10 000 dilutions for anti-NS5B, anti-β-actin and anti-NS3 antibodies, respectively, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Reinach, Switzerland) diluted 1:3000 in washing buffer. Proteins were revealed by chemiluminescence using a commercially available kit (ECL, Amersham Pharmacia).

Indirect immunofluorescence

Replicon cells grown on coverslips in 6-well plates were fixed, washed and incubated with the primary antibody diluted 1:1000 in PBS, 2% bovine serum albumin (BSA), 1.2% Triton X-100 for 2 h at room temperature. After rinsing in PBS, cells were incubated for 2 h at RT with a rhodamine-conjugated anti-rabbit antibody (Jackson ImmunoResearch) diluted 1:100 in PBS/0.5% BSA. After rinsing, the coverslips were mounted onto a microscope slide with 90% glycerol, 200 mM Tris–HCl, pH 8, 0.02% sodium azide, 2% DABCO (Calbiochem, Juro AG, Luzern, Switzerland).

Cell toxicity

Cells (4000) were treated with SCH446211 at concentrations ranging from 50 µM to 10 nM in 96-well plates. SCH446211 was refreshed every day for the first 3 days and once for the last 3 days during the 6 day incubation period. The MTS assay (Promega) was performed at various time points up to 6 days.

Results

Structural analysis

SCH446211 has unique features that can lead to enhanced activity in both enzyme and replicon assays. Different from BILN 2061, which is a macrocyclic inhibitor occupying the P_3 to P_1' area of the enzyme surface, SCH446211 contains a α-ketoamide electrophilic trap (Figure 1a). Compared with VX-950 and SCH503034, which are peptidomimetric inhibitors spanning from P_4 or P_3 to P_1', respectively, SCH446211 extends from P_3 towards the P_2' side of the active site (Figure 1b). The peptidic core of SCH446211 binds to the protease through a series of hydrogen bonding interactions. Crystallographic analysis shows that in

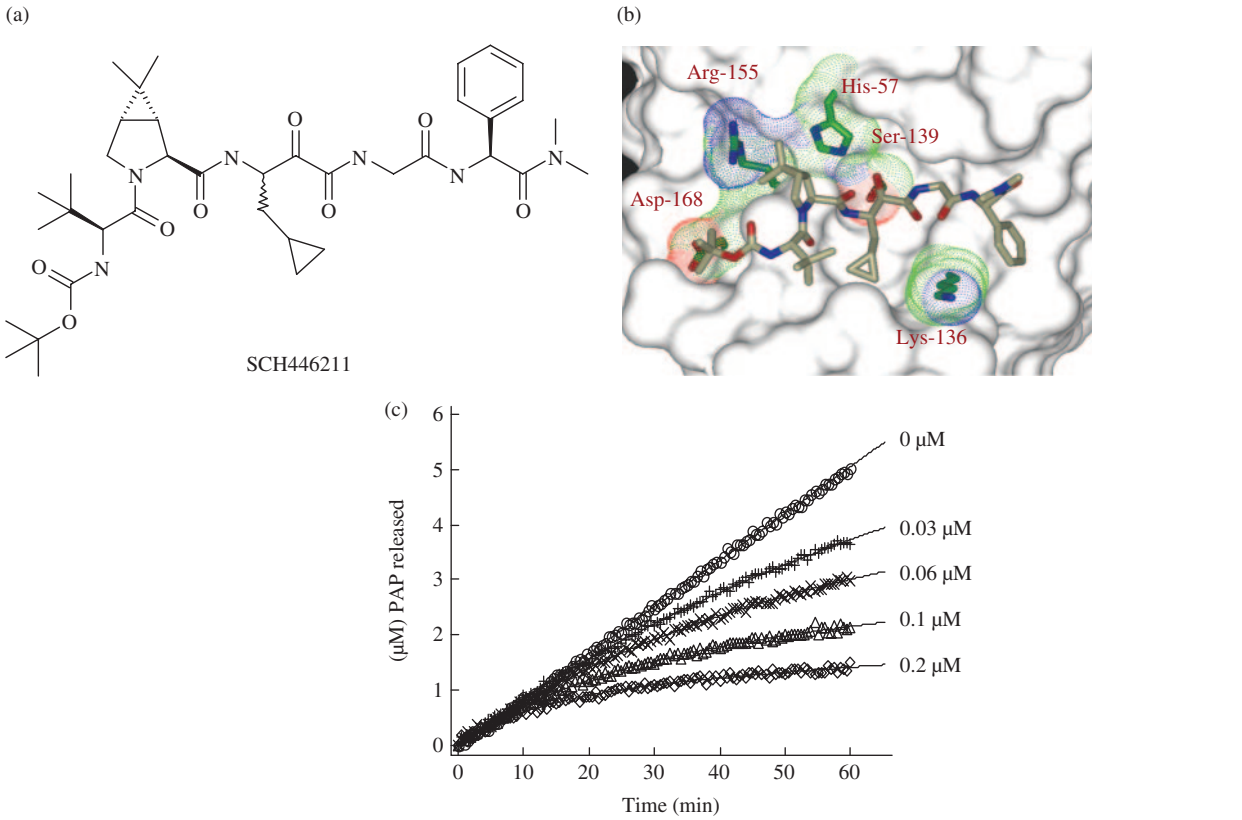

Figure 1. SCH446211 chemical structure (a) and X-ray structure of SCH446211 bound to protease (b). Time course of peptide hydrolysis by the single chain HCV NS3 protease in the presence of SCH446211 (c). The reaction was initiated by adding SCH446211 and enzyme mixture to substrate (see the Materials and methods section).

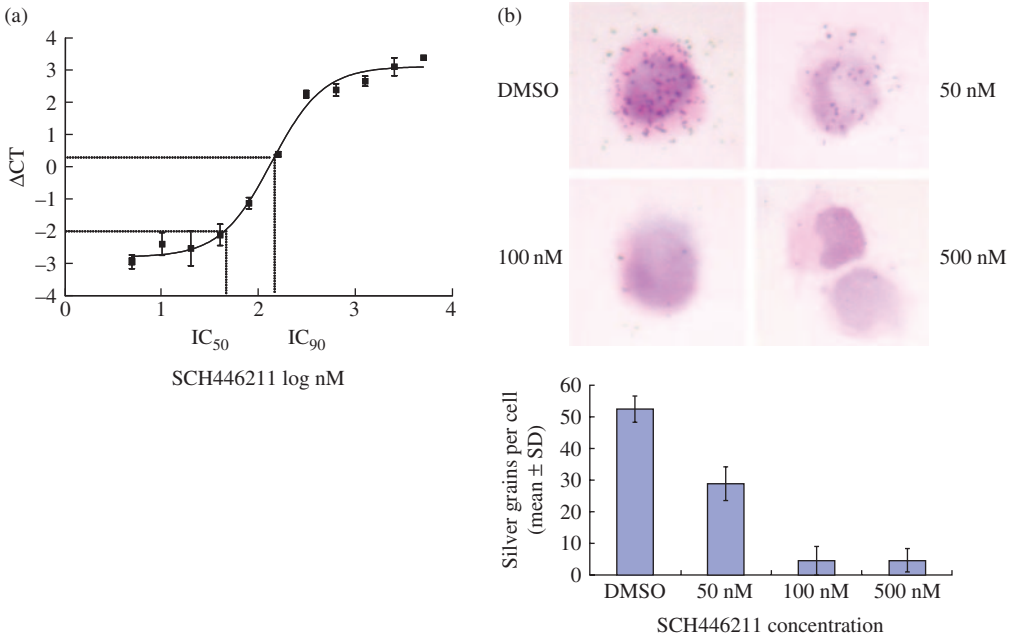

Figure 2. SCH446211 inhibits HCV replication in subgenomic replicon cells. (a) Real-time RT–PCR analysis of replicon RNA. The cells containing HCV replicon were treated with SCH446211 at concentrations from 5 μM to 10 nM for 72 h. Cells were lysed at the end of treatment and the HCV RNA level was measured relative to GAPDH RNA. The difference in threshold cycle number between HCV RNA and GAPDH RNA (ΔCT) was plotted against the concentration of SCH446211. IC_{50} and IC_{90} are determined, respectively, as the concentrations corresponding to 1 and 3.2 ΔCT increase from the baseline as determined with no drug control. (b) Detection of HCV RNA by radioactive *in situ* hybridization. Top: cells were fixed and stained with haematoxylin and eosin after 72 h of incubation in the presence of DMSO or of increasing concentrations of SCH446211, as specified. Original magnification, ×100. Bottom: Quantitative detection of HCV RNA (expressed as average number of silver grains per cell ± SD) by radioactive *in situ* hybridization.

addition to the covalent bond formed after the attack of Ser-139 to the ketoamide moiety, Thr-42, Lys-136 and Ala-157 also form hydrogen bonds with SCH446211. The P′ residue wraps around the side chain of lysine 136. Most notably, the P_1'–P_2' moiety forms a C-clamp locking Lys-136 in place,[23,24] resulting in extensive hydrophobic interaction that can be translated into potent binding activity.

In vitro inhibition of NS3 protease

SCH446211 inhibited NS3 cleavage of the chromophore PAP-linked peptide substrate in a time- and dose-dependent manner (Figure 1c). The inhibitor binding constant K_i^* was estimated to be 3.8 ± 0.4 nM (n = 18, 95% CI 3–6 nM). Human neutrophil elastase (HNE) is also a serine protease which prefers a hydrophobic residue at the P_1 position. The binding constant of SCH446211 to HNE is estimated to be 1.5 ± 0.2 μM (n = 3, 95% CI 1.0–2.2 μM), ∼1000-fold weaker compared with that to HCV protease.

Ex vivo potency of SCH446211

SCH446211 binds to HCV NS3 protease and blocks polyprotein processing which results in inhibition of HCV RNA replication. The clone 16 dicistronic replicon cells were dosed with SCH446211 at concentrations from 5 μM to 10 nM every 24 h for 3 days. Dose–response curves were generated and the drug concentrations necessary to suppress replicon RNA level by 50% (IC_{50}) and 90% (IC_{90}) were estimated to be 40 ± 30 nM (95% CI) and 100 ± 40 nM (95% CI) (n = 17). A representative experiment is shown in Figure 2(a). SCH446211 was also evaluated in three independent dicistronic replicon clones, monocistronic replicon cells (containing only HCV IRES, kindly provided by R. Bartenschlager) as well as full-length replicon cells[25] and the results were comparable (data not shown).

By *in situ* hybridization, HCV replicon RNA was inhibited by 50% ($P < 0.001$) and 90% ($P < 0.001$) when treated with 50 and 100 nM SCH446211, respectively (Figure 2b), as compared with untreated cells. Similar results were obtained with different replicon clones (data not shown).

With the inhibition of HCV RNA replication, the non-structural proteins expressed from HCV replicon genome were also reduced. Immunoblot analysis with antibodies against HCV NS3 and NS5B indicated that increasing concentrations of SCH446211 were associated with a progressive decrease in both proteins (Figure 3a, left and middle panels), and the levels of β-actin remained constant (Figure 3a, right panel). The decrease in both viral protein expression levels started from 50 nM SCH446211, and continued in a dose-dependent manner. However, trace amounts of NS5B were still recognized by specific antibodies at 500 nM of SCH446211, most likely due to incomplete degradation of existing proteins.

Immunofluorescence assays were carried out using the anti-NS3 polyclonal antibody. In the absence of the drug, the antibody revealed a granular staining pattern that surrounded the nucleus and extended through the cytosol (Figure 3b). No nuclear or plasma membrane staining was observed. The fluorescence signal showed a strong decrease upon incubation with increasing concentrations of SCH446211. The most dramatic decrease was

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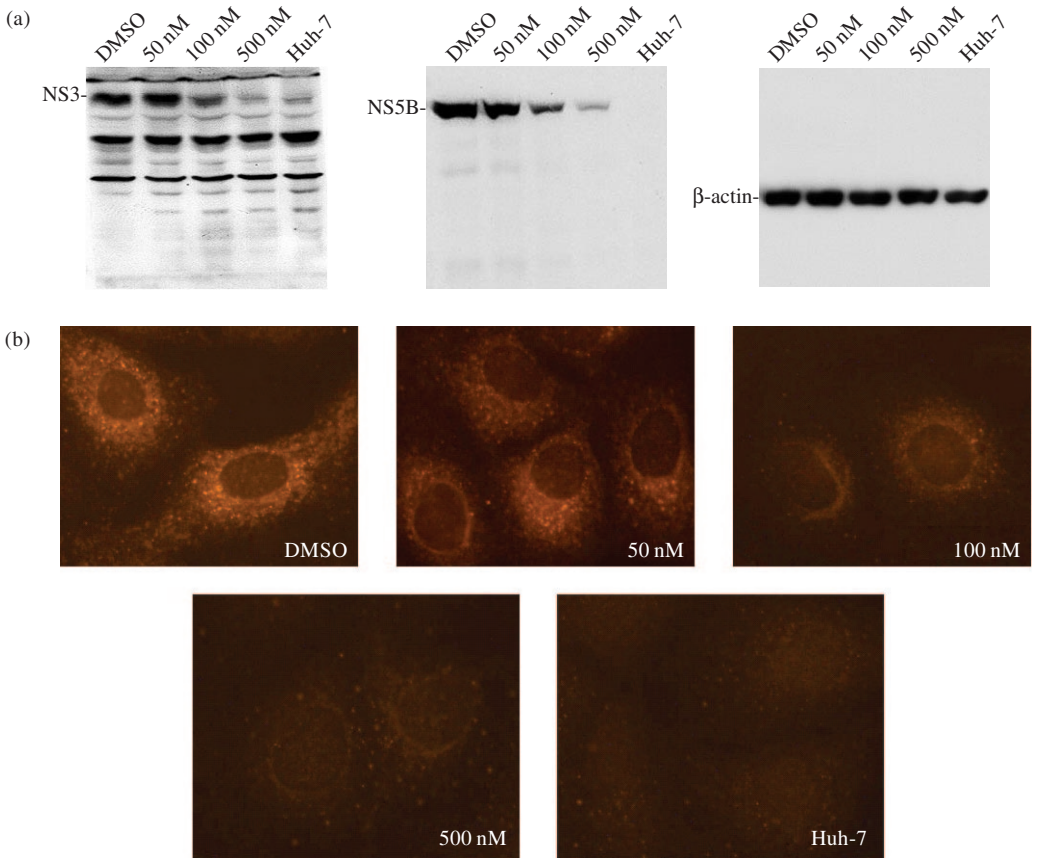

Figure 3. Detection of HCV NS3 and of NS5B proteins by immunoblot (a) and immunofluorescence (b). (a) The replicon cells were treated with indicated concentrations of SCH446211 for 72 h and then cells were analysed by immunoblot analysis with antibodies against NS3 (left) and NS5B (middle). The level of β-actin (right) in each sample was used as a control. (b) Detection of HCV NS3 in replicon cells after 72 h of incubation with DMSO or increasing concentrations of SCH446211, as depicted. Untreated Huh7 cells are shown for comparison. Original magnification, ×64.

observed between 50 and 100 nM, whereas no signal was detected at 500 nM.

SCH446211 acts rapidly and eliminates HCV RNA from replicon cells

Time course studies which followed the HCV replicon RNA levels showed that replicon RNA started to decrease after 24 h of SCH446211 treatment. The increase in potency for a given dose over time reflects the decay of existing RNA. Based on the time course of 50× IC_{90} dose, the replicon RNA half-life is estimated to be 12 h (Figure 4).

To assess the effect of prolonged exposure of replicon cells to SCH446211, clone 16 cells were dosed with 0.8×, 5× and 50× IC_{90} SCH446211 in the absence of G418 selection and total RNA was isolated at days 6, 11 and 14. Replicon RNA was below detection limit in the samples treated with 5× and 50× IC_{90} on day 14, estimated to be <0.1 copy per cell. When 0.5 mg/mL of G418 was added to cells treated with 5× and 50× IC_{90}, no cells survived the selection, indicating cure of replicon RNA from these cells.

SCH446211 did not show toxic effects on the cells. No changes in morphology and growth rate was noted when clone 16 cells were treated with up to 10 μM of SCH446211 during the 14 day study. In a second study, cells were treated with up to 50 μM of SCH446211 for 6 days and no cytotoxicity was observed by the MTS assay (data not shown).

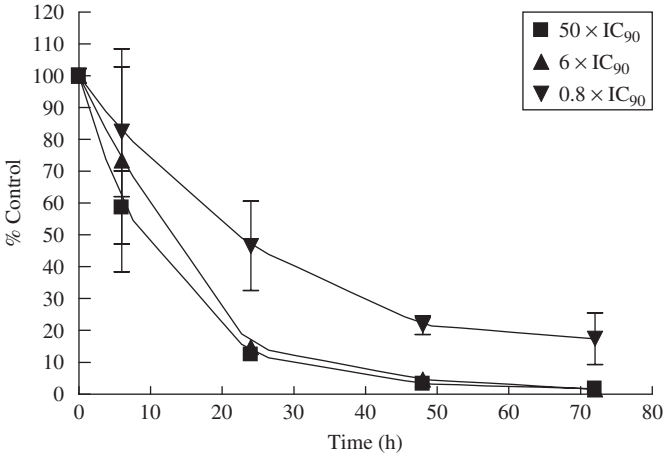

Figure 4. SCH446211 acts quickly on replicon cells. Replicon cells were treated with SCH446211 and were collected at 6, 24, 48 and 72 h for real-time RT–PCR analysis.

When G418 was added to the SCH446211 treated cell culture to select for resistant replicon cells, cells were isolated only from the replicon cells dosed with 0.8× IC_{90}, not at 5× or higher doses, indicating that higher doses may have advantages in reducing

resistance and/or highly resistant replicon may be less fit in the presence of cured cells. Others have reported resistance at higher doses.[26] It is possible that the lack of G418 in the first 2 weeks of treatment, which applied no selection pressure on replicon cells, favoured the growth of the cured replicon cells in culture over cells bearing replicon RNA. The IC_{90} of the resistant cells was measured to be 700 nM, ~7-fold increase compared with the parental replicon cells. Sequence analysis of the RT–PCR products from the NS3 protease domain indicated mixed populations at several amino acid positions including the previously reported A156,[27,28] confirming that SCH446211 inhibited NS3 protease.

Discussion

Since currently available treatments for HCV result in a permanent cure in only ~60% of patients and are often contraindicated or poorly tolerated,[2] the development of alternative, efficacious antivirals is warranted. Several compounds have been reported to block the NS3 protease activity. They include both peptide (i.e. substrate-based)[30–38] and non-peptide[39–41] inhibitors, which may bind either to the S site or to the prime site,[32,33] generally not used by natural substrates. In addition, short RNA molecules have also been reported to inhibit the NS3 protease activity.[42–44] Recently, small molecular protease inhibitors were shown to inhibit HCV RNA replication in replicon cells[37,38] and proof-of-concept clinical trials have been reported.[18,19] SCH446211 is a new α-ketoamide-derived, substrate-based inhibitor of the HCV serine protease. Its potency was also confirmed with the newly developed HCV infection system and its EC_{50} in the infection system was reported to be 190 nM.[45] SCH446211's extended P_1'–P_2' interaction with NS3 protease is translated into tight binding activity to NS3 protease and potent antiviral activity in replicon cells. Its binding constant is 3.8 ± 0.4 nM and its IC_{90} is 100 ± 40 nM (95% CI). In comparison, VX-950 and SCH503034, which are also peptidomimetric inhibitors, respectively, spanning from P_4 or P_3 to P_1', have binding constants of 7 nM and 14 ± 1 nM, respectively.[46,47] In the same 72 h replicon assay used to test SCH446211, the IC_{90} for SCH503034 was reported to be 400 nM (95% confidence interval, 200–700 nM; $n = 23$).[47] The IC_{90} for VX-950 was determined as 830 ± 190 nM in a 48 h replicon assay.[46] This information is potentially a useful guide for future development of potent inhibitors against HCV NS3 protease. This versatility of NS3 inhibitors may pave the way to therapeutic combinations, thus minimizing the risk of selection for drug-resistant viral variants.

A156T/V and R109K were identified as major resistance mutations to SCH446211 in the genotype 1b replicon cells.[25] R109K was a novel resistance mutation against SCH446211 and it conferred moderate level (~3-fold) resistance. This mutation remains sensitive to VX-950 and SCH503034.[25] The lack of cross-resistance to VX-950 and SCH503034 was expected as the unique feature of SCH446211 was its extension toward the P′ side of the active site and interaction with R109. VX-950 and SCH503034 do not make contacts with this residue and therefore their potency was not affected.[25] A156T/V conferred >100-fold resistance to VX-950 and BILN2061 as well as SCH503034.[28,48] This mutation also reduced replicon fitness to 3–5% versus wild-type. The same mutation only conferred ~20-fold resistance to SCH446211. The difference in resistance to different compounds by the same mutation may be explained by that SCH446211 derived its binding energy from contacts from both P and P′ sides of the enzyme.[25] Owing to its extended contacts with NS3 protease, SCH446211 may encounter few strong resistant variants as they would require loss of contacts at both P and P′ sides of the enzyme.

In addition to the direct antiviral activity, SCH446211 was shown to revert the NS3/4A mediated blockade of phosphorylation and effector activity of the interferon regulatory factor-3 (IRF-3).[49,50] IRF-3 induces the expression of a variety of cellular genes including type I IFNs,[49,51] which further amplify the antiviral response through the induction of interferon-stimulated genes (ISGs).[51] The blockade of IRF-3 activation by the HCV NS3/4A protease may significantly affect both the host response to viral infection and the response to IFN-α-based therapy.[51,52] Thus, SCH446211 and other NS3/4A protease inhibitors may counteract not only the cleavage function necessary to proper processing of mature viral proteins, but also the inhibition of the innate host immune response against HCV. Foy *et al.*[49] have shown the likelihood of such dual therapeutic mechanisms, since SCH446211 both restored the host IRF-3 pathway and inhibited viral polyprotein processing. As an additional effect, NS3/4A inhibitors may also counteract the transforming activity of HCV. Transfection with NS3 of different cell lines has shown that this protein may induce tumour formation upon engrafting of transfected cells into nude mice.[53] Non-specific inhibitors of NS3 protease eliminated the transforming activity.[54,55]

In conclusion, SCH446211 is a potent inhibitor of the NS3 protease and it effectively inhibits the HCV subgenomic RNA replication. Its extended interaction with the protease active site is associated with improved antiviral potency in replicon cells and can be a useful guide for future development of potent inhibitors.

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Transparency declarations

None to declare.

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