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 P1′–P2′ 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 IC50 and IC90 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. 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. 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. 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...
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 tryspin-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 systems13–15 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 IC₅₀ 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 NS4Aₓ₁–ₓ₂-GSGS-NS3ₓ₁–ₓ₃ was added to assay buffer containing substrate linked to chromophore Ac-DTEDVVVF(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 = vₒ + (vₒ – vᵣ)(1 – e⁻kt) using SAS version 8.0 (SAS Institute Inc.) The overall Kᵣ was calculated from the estimated steady-state velocities {vᵣ = V_maxS/K_s(1 + 1/Kᵣ)}.
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 P3 to P1₀ area of the enzyme surface, SCH446211 contains a α-ketoamide electrophilic trap (Figure 1a). Compared with VX-950 and SCH503034, which are peptidomimetic inhibitors spanning from P₄ or P₃ to P₁₀, respectively, SCH446211 extends from P₃ towards the P₂₀ 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).
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 P0 residue wraps around the side chain of lysine 136. Most notably, the P1–P2 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 P1 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% (IC50) and 90% (IC90) 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 cells25 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...
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 × IC₉₀ 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 × IC₉₀, 5 × IC₉₀, and 50 × IC₉₀ 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 × IC₉₀ 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 × IC₉₀, 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 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 Hu7 cells are shown for comparison. Original magnification, ×64.

**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 × IC₉₀, 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. 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$_{50}$ 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, 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, the development of alternative, efficacious antivirals is warranted. Several compounds have been reported to block the NS3 protease activity. They include both peptidomimetic and non-peptidomimetic inhibitors, which may bind either to the S site or to the prime site, generally not used by natural substrates. In addition, short RNA molecules have also been reported to inhibit the NS3 protease activity. Recently, small molecular protease inhibitors were shown to inhibit HCV RNA replication in replicon cells and proof-of-concept clinical trials have been reported. SCH446211 is a new $\alpha$-ketoamide-derived, substrate-based inhibitor of the HCV serine protease. Its potency was also confirmed with the newly developed HCV infection system and its IC$_{50}$ in the infection system was reported to be 190 nM. 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$_{50}$ is 100 ± 40 nM (95% CI). In comparison, VX-950 and SCH503034, which are also peptidomimetic inhibitors, respectively, spanning from P$_4$ to P$_3$ to P$_1$$^\alpha$, have binding constants of 7 nM and 14 ± 1 nM, respectively. In the same 72 h replicon assay used to test SCH446211, the IC$_{50}$ for SCH503034 was reported to be 400 nM (95% confidence interval, 200–700 nM; n = 23). The IC$_{50}$ for VX-950 was determined as 830 ± 190 nM in a 48 h replicon assay. This information is potentially a useful guide for future development of potent inhibitors against HCV NS3 protease. This versatility of information is potentially a useful guide for future development of potent inhibitors.

A156T/V and R109K were identified as major resistance mutations to SCH446211 in the genotype 1b replicon cells. R109K was a novel resistance mutation against SCH446211 and it conferred moderate level (3- to 5-fold) resistance. 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. 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). IRF-3 induces the expression of a variety of cellular genes including type I IFNs, which further amplify the antiviral response through the induction of interferon-stimulated genes (ISGs). 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-$\alpha$-based therapy. 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. This study was supported by the Schering-Plough Research Institute.

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Transparency declarations

None to declare.

References


