

Discovery and Early Clinical Evaluation of BMS-605339, a Potent and Orally Efficacious Tripeptidic Acylsulfonamide NS3 Protease Inhibitor for the Treatment of Hepatitis C Virus Infection.

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Purification of the HCV NS3/4A Protease Complex

Full length genomic HCV constructs (GT1a (H77 strain), GT1b (Con1 strain), and BMS-HCV-FL) were used to amplify the NS3/4A protease coding region by PCR. The amplicons were cloned into the Xba1 and Xho1 regions of the pET21b vector (Novagen, Madison, WI) and transformed into *E. coli* BL21(DE3) pLysS cells (Novagen) for high yield expression. For protein purification purposes cells were grown in a chemically defined media composed of per liter of water: 20 mM glucose, 20 mM (NH₄)₂SO₄, 100 mM K₂HPO₄ pH 7.0, 5 μM biotin, 7 μM thiamine, 0.5% Casamino acids, 0.5 mM MgSO₄, 0.5 mM CaCl₂, 13 μM FeSO₄, 1 μM ZnCl₂ and 100 μg/mL ampicillin. Briefly, NS3/4A expression was induced at 0.8 OD with 0.5 mM IPTG at 20 °C for 22 h. The cells were resuspended in lysis buffer (10 mL/g cell paste) consisting of 25 mM HEPES, pH 7.5, 20% glycerol, 500 mM NaCl, 0.5% Triton-X100, 1 μg/mL lysozyme, 5 mM MgCl₂, 1 μg/mL DNaseI, 5 mM β-Me. The cell suspension was sonicated and clarified by ultracentrifugation at 23,500 x *g* for 1 h at 4 °C. Imidazole was added to the supernatant to a final concentration of 15 mM and the pH adjusted to 8.0. The crude protein extract was loaded on to an Ni-NTA (Quiagen) column, pre-equilibrated with buffer B (25 mM HEPES, pH 8.0, 20% glycerol, 500 mM NaCl, 0.5% Triton-X100, 15 mM imidazole, 5 mM β-Me). The column was washed with 15 column volumes of buffer C (same as buffer B except with 0.2% Triton-X100). The protein was eluted with 5 column volumes of 200 mM imidazole in buffer C. Fractions containing the NS3/4A complex were pooled and loaded on to a Superdex-S200 column, pre-equilibrated with buffer D (25 mM HEPES, pH 7.5, 20% glycerol, 300 mM NaCl, 0.2% Triton-X100, 10 mM β-Me). Fractions containing the NS3/4A protease complex were pooled and concentrated to approximately 0.5 mg/mL. Purification was monitored by SDS-PAGE and enzyme activity measured by the *in vitro* HCV NS3 protease FRET assay (Section 2.3). Fractions were flash frozen and stored at -80°C. The purity of the NS3/4A protease complex was judged to be greater than 90% by SDS-PAGE and mass spectrometry analyses.

Cell lines

HuH-7 cells were obtained from Dr. Ralf Bartenschlager, University of Heidelberg, Heidelberg, Germany and were propagated using DMEM supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine.

The HCV 1b strain Con 1 replicons used in these studies were generated at BMS. The coding sequence of the published HCV replicon was synthesized by Operon Technologies, Inc. (Alameda, CA) using the sequence set forth in EMBL Accession No. AJ242652, nucleotides 1801 to 7758. The functional replicon was then assembled in plasmid pGem9zf(+) (Promega, Madison, WI) using standard molecular biology techniques. The replicon used to create the 1b con 1 replicon cell line consists of a bacteriophage T7 RNA polymerase promoter, followed by the 5'-untranslated region of the HCV genome, fused to the first 12 amino acids of the HCV Core protein. This sequence is followed by, in order, i.) the neomycin phosphotransferase gene, which confers resistance to G418, ii.) the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) sequence, iii.) HCV NS3 to NS5B genes, and iv.) the HCV 3' untranslated region. The replicon used to create a cell line with a luciferase gene is identical to that described above, except that a cassette of the gene encoding humanized Renilla Luciferase protein fused in-frame to 7 amino acids loosely based on the WT NS4A-NS4B cleavage site and an Ascl restriction site (nucleotide sequence ATGGAGGAGGCCGCCAGCCACCTGGGGCGCGCCGGGCGCGCC), has been introduced upstream of the neomycin phosphotransferase gene. A mutation was also introduced into the NS5A gene (S2204I) to improve replication in cell culture.

The replicon cell line representing the genotype 1a H77 strain was generated by using recombinant PCR to replace the sequences encoding from nucleotide 2049 of the NS3 gene through NS5B in the con 1 replicon with sequences representing the genotype 1a H77 strain. This was achieved by using the

restriction enzyme sites BsrG1 and Xba1. The neomycin phosphotransferase gene has also been replaced by a cassette of the gene encoding humanized Renilla Luciferase protein fused in-frame to 7 amino acids loosely based on the WT NS4A-NS4B cleavage site and an Ascl restriction site (nucleotide sequence ATGGAGGAGTGCGCCAGCCACCTGGGGCGCGCCGGGCGCGCC) followed by the gene encoding for Blasticidin resistance. Mutations have been introduced into the NS3 helicase domain (P1496L) and the NS5A gene (S2204I) to improve replication in cell culture.

HCV NS3/4A protease enzyme assay:

The *in vitro* HCV NS3/4A protease assay was performed using purified recombinant NS3/4A protease and measures the fluorescence change associated with the cleavage of a fluorescence resonance energy transfer (FRET) labeled substrate over time. Assays are performed in 96-well microtiter plates (Costar 3631) and read in a Molecular Devices fluorescent plate reader (excitation at 360 nm and emission at 490 nm). The assay buffer is composed of 50 mM HEPES pH 7.5, 0.15 M NaCl, 15% glycerol, 0.1% Triton X-100, 10 mM β -Me, 1 nM NS3/4A protease and compound with a final concentration of 1% DMSO. Inhibitors were serially diluted in reaction buffer in 10% DMSO. The reaction was initiated by addition of an NS3 FRET-labeled peptide substrate (Ac-Asp-Glu-Asp(EDANS)-Glu-Glu-Abu[C(O)-O]-Ala-Ser-Lys(DABCYL)-NH₂) to a final concentration of 2.5 μ M. This substrate¹ is loosely based on the HCV NS4A/B cleavage site with an ester bond incorporated between the P1 and P1' residues instead of an amide bond. Due to ready trans-esterification of the scissile bond to the acyl-enzyme intermediate, the substrate shows very high k_{cat}/K_m values. The concentration of compound required to yield a 50% inhibitory response compared to compound-free samples (IC₅₀) was calculated by using the exponential form of the median effect equation where $IC_{50} = 100 - [(\delta F_{inh}/\delta F_{con}) \times 100]$.

Cell-based HCV replicon assay:

To determine antiviral activity, HCV replicon cells were incubated in 96-well plates in the presence of compound for 4 days. The 50% effective concentration (EC_{50}) was calculated by using the exponential form of the median effect equation where $EC_{50} = 100 - [(\delta F_{inh}/\delta F_{con}) \times 100]$. For replicons containing a humanized Renilla Luciferase reporter, luciferase activity was assayed using the Promega Enduren kit. Enduren Luciferase Reagent, dissolved in DMSO, was diluted 1:2000 in pre-warmed dilution medium containing DMEM and 2 mM L-glutamine. Enduren was added to each plate well at a final concentration of 15 μ M. Plates were then incubated for 1 h at 37°C in 5% CO₂ before transfer to room temperature for 5 min prior to reading on a Perkin-Elmer TopCount set for Luminescence. For replicons lacking a reporter gene, NS3 protease activity was used as an indirect measure of the amount of HCV replicon RNA present within cells. Following a 4 day incubation with compound, NS3 protease activity was measured using a FRET-labeled peptide substrate (10 μ M final concentration, described in detail above), diluted in Promega 1x Cell Lysis Buffer (catalogue number E153A) to which an additional 150 mM NaCl was added prior to use. Cells were washed 3 times with 150 μ l per well D-PBS, all remaining D-PBS was removed, and 25 μ l of the above buffer containing the FRET-labeled peptide was added per well. Protease activity was measured using a Cytofluor 4000 or Molecular Devices Gemini instrument set to wavelength 340 nm (excitation) and 490 nm (emission). Linear rates obtained during 15 cycles in kinetic mode were used in EC_{50} calculations.

pKa Determinations:

A Sirius GLpKa instrument was used to determine the pKa of BMS-605339 in the pH range between 2 and 11. Both the spectrophotometric² and the potentiometric titration protocols³ were used. The compound was solubilized in water with varying concentrations of methanol, and the apparent pKa was determined as a function of the cosolvent content. The pKa in water was then determined by extrapolation of these values to purely aqueous conditions.

Synthesis of Intermediates:

1-Chloro-5-fluoroisoquinoline. ¹H-NMR (400 MHz, CDCl₃) δ 8.33 (d, *J* = 5.9 Hz, 1 H), 8.14 (d, *J* = 8.6 Hz, 1 H), 7.84 (d, *J* = 5.6 Hz, 1 H), 7.62 (td, *J* = 8.2, 5.4 Hz, 1 H), 7.43 (ddd, *J* = 9.7, 7.8, 0.9 Hz, 1 H); LCMS (Method A): *t*_R = 1.38 min; LCMS (ESI) *m/z* calcd. for C₉H₅ClFN: 181.01; found: 182.09 (M+H)⁺.

4-(1-Chloroisoquinolin-5-yl)morpholine. ¹H-NMR (400 MHz, CDCl₃) δ 8.26 (d, *J* = 5.87 Hz, 1 H), 8.06 (d, *J* = 8.56 Hz, 1 H), 7.91 (d, *J* = 5.87 Hz, 1 H), 7.60 (m, 1 H), 7.32 (d, *J* = 7.58 Hz, 1 H), 3.97 (m, 4 H), 3.09 (m, 4 H.); LCMS (Method D): *t*_R = 2.75 min; LCMS (ESI) *m/z* calcd. for C₁₃H₁₃ClN₂O: 248.07; found: 249.13 (M+H)⁺.

1-Chloro-8-methylisoquinoline. ¹H-NMR (400 MHz, CDCl₃) δ 8.19 (d, *J* = 5.6 Hz, 1 H), 7.68-7.66 (m, 1 H), 7.58-7.52 (m, 2 H), 7.62 (d, *J* = 7.2 Hz, 1 H), 3.06(s, 3 H); LCMS (Method A): *t*_R = 1.41 min; LCMS (ESI) *m/z* calcd. for C₁₀H₈ClN: 177.03; found: 178.13 (M+H)⁺.

1-Chloro-6-(methoxymethoxy)isoquinoline. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.25 (d, $J = 7.6$ Hz, 1H), 8.18 (d, $J = 5.6$ Hz, 1H), 7.48 (d, $J = 5.6$ Hz, 1H), 7.37-7.34 (m, 2H), 5.32 (s, 2 H), 3.51 (s, 3 H); LCMS (Method A): $t_R = 1.33$ min; LCMS (ESI) m/z calcd. for $\text{C}_{11}\text{H}_{10}\text{ClNO}_2$: 223.04; found: 223.99 (M+H) $^+$.

1-Chloro-6-(1H-pyrazol-1-yl)isoquinoline. $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 8.49-8.44 (m, 2H), 8.32-8.23 (m, 4H), 7.83-7.82 (m, 2H); LCMS (Method F): $t_R = 1.35$ min; LCMS (ESI) m/z calcd. for $\text{C}_{12}\text{H}_8\text{ClN}_3$: 229.67; found: 229.98 (M+H) $^+$.

1-Chloro-7-methylisoquinoline. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.20 (d, $J = 5.6$ Hz, 1H), 8.09 (s, 1H), 7.73 (d, $J = 8.3$ Hz, 1H), 7.57 (d, $J = 8.6$ Hz, 1H), 7.54 (d, $J = 5.6$ Hz, 1H), 2.58 (s, 3H); LC-MS (Method C): $t_R = 1.99$ min; LCMS (ESI) m/z calcd. for $\text{C}_{10}\text{H}_8\text{ClN}$: 177.03; found: 177.95 (M+H) $^+$.

1-Chloro-6-fluoroisoquinoline. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.44-8.39 (m, 1H), 8.30 (dd, $J = 5.6, 0.5$ Hz, 1H), 7.59 (dd, $J = 5.6, 0.5$ Hz, 1H), 7.5 -7.44 (m, 2H); HRMS (+TOF MS) calcd. for $\text{C}_9\text{H}_5\text{ClFN}$ [(M+H) $^+$] 182.0165, found: 182.0167.

4-Chloro-7-methoxy-2-phenylquinoline. $^1\text{H-NMR}$ (400 MHz, DMSO-d_6) δ 8.26-8.30 (m, 2H), 8.19 (s, 1H), 8.08 (d, $J = 9.2$ Hz, 1H), 7.49-7.59 (m, 4H), 7.36 (dd, $J = 9.2, 2.6$ Hz, 1H), 3.97 (s, 3H); LCMS (Method D): $t_R = 2.97$ min; LCMS (ESI) m/z calcd. for $\text{C}_{16}\text{H}_{12}\text{ClNO}$: 269.06; found: 269.90 (M+H) $^+$.

1-Chloro-4-methylisoquinoline. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.35 (d, $J = 8.31$ Hz, 1 H), 8.11 (s, 1 H), 7.94 (d, $J = 8.31$ Hz, 1 H), 7.78 (t, $J = 7.70$ Hz, 1 H), 7.68 (t, $J = 7.70$ Hz, 1 H), 2.59 (s, 3 H); LCMS (Method A): $t_R = 1.41$ min; LCMS (ESI) m/z calcd. for $\text{C}_{10}\text{H}_8\text{ClN}$: 177.03; found: 178.15. $(\text{M}+\text{H})^+$

1-Chloro-4-methoxyisoquinoline . $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.6 (d, $J = 8.4$ Hz, 1 H), 8.2 (d, $J = 8.4$ Hz, 1 H), 7.80 (s, 1 H), 7.73-7.70 (m, 2 H), 4.05 (s, 3 H); LCMS (Method A): $t_R = 1.45$ min; LCMS (ESI) m/z calcd. for $\text{C}_{10}\text{H}_8\text{ClNO}$: 193.03; found: 194.18 $(\text{M}+\text{H})^+$.

1-Chloro-4,6-dimethoxyisoquinoline. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.16 (d, $J = 9.29$ Hz, 1 H), 7.75 (s, 1 H), 7.45 (d, $J = 2.69$ Hz, 1 H), 7.31 (dd, $J = 9.17, 2.57$ Hz, 1 H), 4.05 (s, 3 H) 3.97 (s, 3 H); LCMS (ESI) m/z calcd. for $\text{C}_{11}\text{H}_{10}\text{ClNO}_2$: 223.04; found: 224.37 $(\text{M}+\text{H})^+$.

1,4-Dichloroisoquinoline. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.36 (d, $J = 8.6$ Hz, 1H), 8.34 (s, 1H), 8.23 (d, $J = 8.3$ Hz, 1H), 7.88 (ddd, $J = 8.3, 7.1, 1.2$ Hz, 1H), 7.77 (ddd, $J = 8.3, 7.1, 1.2$ Hz, 1H); LC-MS (Method C): $t_R = 2.11$ min; LCMS (ESI) m/z calcd. for $\text{C}_9\text{H}_5\text{Cl}_2\text{N}$: 196.98; found: 197.90 $(\text{M}+\text{H})^+$.

1,5-Dichloroisoquinoline. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.38 (d, $J = 5.9$ Hz, 1H), 8.29 (dt, $J = 8.6, 1.0$ Hz, 1H), 8.00 (d, $J = 5.9$ Hz, 1H), 7.86-7.79 (m, 1H), 7.60 (dd, $J = 8.6, 7.6$ Hz, 1H); LC-MS (Method C): $t_R = 2.06$ min; LCMS (ESI) m/z calcd. for $\text{C}_9\text{H}_5\text{Cl}_2\text{N}$: 196.98; found: 197.90 $(\text{M}+\text{H})^+$.

1-Chloro-5-methylisoquinoline. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.28 (d, $J = 5.9$ Hz, 1H), 8.22 - 8.15 (m, 1H), 7.70 (dd, $J = 5.9, 1.0$ Hz, 1H), 7.57-7.53 (m, 2H), 2.67 (s, 3H); LC-MS (Method C): $t_R = 1.99$ min; LCMS (ESI) m/z calcd. for $\text{C}_{10}\text{H}_8\text{ClN}$: 177.03; found: 177.95 (M+H) $^+$.

1-Chloro-5-methoxyisoquinoline. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.25 (d, $J = 5.9$ Hz, 1H), 7.97 (d, $J = 5.9$ Hz, 1H), 7.88 (d, $J = 8.6$ Hz, 1H), 7.57 (t, $J = 8.1$ Hz, 1H), 7.04 (d, $J = 7.8$ Hz, 1H), 4.01 (s, 3H); LC-MS (Method C): $t_R = 1.91$ min; LCMS (ESI) m/z calcd. for $\text{C}_{10}\text{H}_8\text{ClNO}$: 193.03; found: 193.90 (M+H) $^+$.

1-Chloro-6-methylisoquinoline. $^1\text{H-NMR}$ (400 MHz, CD_3Cl) δ 8.26 - 8.08 (m, 2H), 7.56 (s, 1H), 7.48 (m, 2H), 2.53 (s, 3H); LCMS (Method A): $t_R = 1.92$ min; LCMS (ESI) m/z calcd. for $\text{C}_{10}\text{H}_8\text{ClN}$: 177.03; found: 177.95 (M+H) $^+$.

1-Chloro-6-ethylisoquinoline. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.21 (d, $J = 8.6$ Hz, 1H), 8.14 (d, $J = 5.6$ Hz, 1H), 7.74 (br s, 1H), 7.68 (br. s., 1H), 7.62 (br. s., 1H), 2.86 (q, $J = 7.6$ Hz, 2H), 1.39-1.18 (m, 3H); (Method A): $t_R = 1.64$ min; LCMS (ESI) m/z calcd. for $\text{C}_{12}\text{H}_{10}\text{ClN}$: 191.05; found: 192.24 (M+H) $^+$.

1-Chloro-6-methoxy-3-phenylisoquinoline. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ 8.21 (d, $J = 9.2$ Hz, 1H), 8.09 (d, $J = 7.3$ Hz, 2H), 7.89 (s, 1H), 7.54-7.36 (m, 3H), 7.30-7.19 (m, 1H), 7.12 (d, $J = 2.4$ Hz, 1H), 3.97 (s, 3H); LCMS (Method A): $t_R = 1.99$ min; LCMS (ESI) m/z calcd. for $\text{C}_{16}\text{H}_{12}\text{ClNO}$: 269.06; found: 269.86 (M+H) $^+$.

6-(tert-Butyl)-1-chloroisoquinoline. ¹H-NMR (400 MHz, CD₃OD) δ 8.26 (d, *J* = 8.8 Hz, 1H), 8.18 (d, *J* = 5.6 Hz, 1H), 8.00-7.84 (m, 2H), 7.80-7.70 (m, 1H), 1.54-1.29 (m, 9H); LCMS (Method A): t_R = 2.01 min; LCMS (ESI) *m/z* calcd. for C₁₃H₁₄ClN: 219.08; found: 220.00 (M+H)⁺.

tert-Butyl ((2S)-1-((2S,4R)-4-((7-methoxy-2-phenylquinolin-4-yl)oxy)-2-((1-(methylsulfonyl)carbamoyl)-2-vinylcyclopropyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamate (3). ¹H NMR (500MHz, CD₃OD) δ 8.36 (d, *J* = 9.2 Hz, 1H), 8.15 - 8.06 (m, *J* = 7.6 Hz, 2H), 7.84 - 7.72 (m, 3H), 7.68 (br. s., 1H), 7.57 (br. s., 1H), 7.43 (d, *J* = 7.0 Hz, 1H), 5.88 (br. s., 1H), 5.84 - 5.72 (m, 1H), 5.33 (d, *J*=17.1 Hz, 1H), 5.17 (d, *J* = 10.1 Hz, 1H), 4.73 - 4.56 (m, 2H), 4.27 - 4.14 (m, 2H), 4.13 - 3.99 (m, 3H), 3.23 (br. s., 3H), 2.85 - 2.75 (m, 1H), 2.53 - 2.39 (m, 1H), 2.34 - 2.19 (m, 1H), 1.93 (dd, *J* = 7.9, 5.5 Hz, 1H), 1.53 - 1.42 (m, 1H), 1.22 (s, 9H), 1.06 (br. s., 9H); LCMS (Method D): t_R = 2.71 min; LCMS (ESI) *m/z* calcd. for C₃₉H₄₉N₅O₉S: 763.33; found: 764.25 (M+H)⁺.

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