Discovery of Potent Hepatitis C Virus NS5A Inhibitors with Dimeric Structures

Julie A. Lemm, a John E. Leet, b Donald R. O’Boyle II, a Jeffrey L. Romine, c Xiaohua Stella Huang, d Daniel R. Schroeder, d Jeffrey Alberts, e, l Joseph L. Cantone, d Jin-Hua Sun, a Peter T. Nower, a Scott W. Martin, c Michael H. Serrano-Wu, c,2 Nicholas A. Meanwell, c Lawrence B. Snyder, c and Min Gao*a

1 Department of Virology, a Synthesis & Analysis Technology Team, b Medicinal Chemistry, c Discovery Analytical Sciences, d and Discovery Biotransformation e

Bristol-Myers Squibb Research, 5 Research Parkway, Wallingford, Connecticut, 06492

1 Present address: Eli Lilly, Indianapolis, IN

2 Present address: Novartis Institute for BioMedical Research, Cambridge, MA

*Corresponding author. Mailing address: 5 Research Parkway, Wallingford, CT 06492.

Phone: 203-677-6692. FAX 203-677-6088. email: min.gao@bms.com

Running title: NS5A inhibitors derived from an intermolecular reaction
Abstract
The exceptional \textit{in vitro} potency of the HCV NS5A inhibitor BMS-790052 has translated into an \textit{in vivo} effect in proof-of-concept clinical trials. Although the EC$_{50}$ of the initial lead, the thiazolidinone BMS-824, was $\sim$10 nM in the replicon assay, it underwent transformation to other inhibitory species after incubation in cell culture media. The biological profile of BMS-824, including EC$_{50}$, CC$_{50}$ and resistance profile, however, remained unchanged, triggering an investigation to identify the biologically active species. HPLC biogram fractionation of a sample of BMS-824 incubated in media revealed that the most active fractions could readily be separated from the parental compound and retained the biological profile of BMS-824. From mass spectral and NMR data, the active species was determined to be a dimer of BMS-824, derived from an intermolecular radical-mediated reaction of the parent compound. Based upon an analysis of the structural elements of the dimer deemed necessary for anti-HCV activity, the stilbene derivative BMS-346 was synthesized. This compound exhibited excellent anti-HCV activity and showed a similar resistance profile to BMS-824, with changes in compound sensitivity mapped to the N-terminus of NS5A. The N-terminus of NS5A has been crystallized as a dimer, complementing the symmetry of BMS-346 and allowing a potential mode of inhibition of NS5A to be discussed. Identification of the stable, active pharmacophore associated with these NS5A inhibitors provided the foundation for the design of more potent inhibitors with broad genotype inhibition. This culminated in the identification of BMS-790052, a compound that preserves the symmetry discovered with BMS-346.
Introduction

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B hepatitis worldwide, which affects more than 3% of the world’s population. Of those infected with HCV, ~70% proceed to a chronic state which can lead to severe liver diseases including fibrosis, cirrhosis, or hepatocellular carcinoma (1, 7). There is currently no vaccine against HCV and no generally effective therapy for all HCV genotypes. The current optimal therapy is pegylated interferon-α in combination with ribavirin, a regimen associated with significant side effects and limited efficacy in the most prevalent patient population consisting of genotype 1 (3). Therefore, there is an urgent need for the development of more effective, HCV-specific antiviral therapies with fewer side effects.

In the search for more efficacious, safer HCV therapies, the most actively pursued antiviral targets have been the NS3 protease and NS5B RNA-dependent RNA polymerase, both essential enzymes for the replication of HCV (2, 11, 12). Exciting progress has been demonstrated in clinical trials with multiple HCV NS3 serine protease inhibitors, as well as with both nucleoside and non-nucleoside polymerase inhibitors. However, due to the error-prone nature of the HCV polymerase, HCV is a highly heterogeneous virus and resistance variants exist as part of the viral quasispecies. It is widely recognized that combinations of drugs with different resistance profiles are likely to be required to effectively suppress the emergence of resistant virus and achieve a sustained viral response. Thus, agents that inhibit HCV replication via novel targets are of considerable interest.

With the development of HCV replicon and virus systems, it is now possible to identify inhibitors targeting non-enzymatic proteins via cell-based screens. The use of a
cell-based replication assay includes essential functions that previously could not be evaluated with *in vitro* enzyme assays. Inhibitors that target HCV NS5A, a protein for which there is no known enzymatic function, provide an interesting example of this approach. NS5A is a multi-functional protein required for several stages of the viral life cycle. It is a membrane-associated phosphoprotein (9, 18) thought to be involved in interferon resistance that also has been shown to interact with a number of host proteins, although its precise role in HCV replication is unknown (14). NS5A has recently been validated as a clinically-relevant target (6) and inhibitors targeting this protein are actively being pursued in clinical trials.

We recently reported the identification of compounds that inhibit HCV replication in cell-based assays and target NS5A (10). One such compound, BMS-824, is a potent and specific inhibitor of HCV RNA replication with an EC$_{50}$ of ~10 nM. Studies to further characterize this compound revealed that BMS-824 was not stable in media yet anti-HCV activity was maintained. In this report we describe the use of an HCV bioactivity chromatogram assay (referred to herein as “biogram” (4)) to isolate and identify two trace constituents from incubations of BMS-824 in assay media that demonstrate exceptionally potent HCV inhibition in replicons.
Materials and Methods

Cell culture and compound. Both bovine viral diarrhea virus (BVDV) and HCV replicon cell lines were isolated as previously described (10, 15) and maintained in Dulbecco’s modified Eagle medium (DMEM) with 100 U/ml penicillin/streptomycin, 10% fetal bovine serum (FBS) and 0.3 – 0.5 mg/ml Geneticin (G418). Huh-7 cells cured of a Con1 replicon were generated as previously described (10) and propagated in DMEM with penicillin/streptomycin and 10% FBS. Compounds used in this study were synthesized at Bristol-Myers Squibb.

Cell culture cytotoxicity and HCV inhibition assays. To assess HCV inhibitory activity, HCV replicon cells were plated at a density of $10^4$ per well in 96-well plates in DMEM media containing 10% FBS (assay media). Following incubation overnight, compound or HPLC fractions (detailed below) were added to cell plates and incubated at 37°C for 3 days prior to assaying for cytotoxicity and HCV inhibition. Cell viability was measured using an alamar blue assay and $CC_{50}$ values, the concentration of compound which caused a 50% reduction in cell viability, were calculated using the median effect equation.

HCV inhibition was measured using a fluorescence resonance energy transfer assay (FRET) which was performed as previously described (15). Briefly, after staining with alamar blue, replicon cell plates were washed with phosphate-buffered saline and then used for FRET assay by the addition of 30 µl of the FRET peptide assay reagent per well. The assay reagent consisted of 1x-luciferase cell culture lysis buffer, 150 mM NaCl and 20 µM FRET peptide. The plate containing assay reagent was then read in a kinetic mode in a Cytoflour 4000 instrument which had been set to 340 excite/490 emission,
automatic mode for 20 cycles. EC_{50} values were calculated as the concentration of compound which caused a 50% reduction in HCV FRET activity.

**Isolation of resistant replicons.** Selection of resistant replicon cells was performed by growing genotype 1b replicon cells in media containing a concentration of 5 µM BMS-346. Media containing compound was added to monolayers of HCV 1b-377-neo replicon cells at ~25% confluence in the presence of 0.5 mg/ml G418. Replicon cells maintained in the presence of DMSO were used as a control. After 5-6 weeks, control DMSO-selected replicon cells and compound-selected cells were tested for compound sensitivity using the HCV replicon FRET assay.

cDNA cloning. Total RNA was isolated from both DMSO- and compound-selected cell lines using Trizol (Gibco-BRL) according to the manufacturer’s protocol. To generate HCV cDNAs, the NS5A gene was amplified using the SuperScript One-Step RT-PCR kit (Gibco-BRL,) and primers targeting the NS4B and NS5B genes. Reaction products were cloned directly into pCR2.1-TOPO using a TOPO TA cloning kit (Invitrogen) and the DNA sequence of the NS5A coding region was determined for multiple clones.

**Transient replication assays.** RNA transcripts of HCV replicons containing a luciferase reporter gene were synthesized in vitro using Scal-digested DNAs and the T7 MegaScript transcription kit (Ambion) according to the manufacturer’s directions. For transient replication assays, subconfluent cured Huh-7 cells in a 35-mm dish were transfected with 2.5 µg of RNA transcript using DMRIE-C (Invitrogen) according to the manufacturer’s directions. Four hours later, transfectant was removed and replaced with DMEM + 10% FBS with or without compound then incubated at 37°C. At various time points, cells
were harvested and luciferase activity determined using the Renilla Luciferase Assay kit (Promega).

**Extraction and HPLC bioactivity chromatogram (biogram).** To begin isolation of active components derived from BMS-824, BMS-824 (5 µM final) was incubated in assay media at 37°C for 48 h in an initial 6 ml pilot experiment. After 48 h, 6 ml acetonitrile was added to the incubation and the resulting suspension was centrifuged. A 100 µl aliquot of the supernatant was subjected to high performance liquid chromatography (HPLC) fractionation. HPLC (C18) conditions: Analytical: Agilent HP-1100, Waters X-Terra 5 µm (C18) column, 4.6 x 150 mm; mobile phase: 0.01% trifluoroacetic acid-acetonitrile gradient (8), flow rate 1.2 mL/min. Ultraviolet (UV) 254 nm detection. For the HPLC biogram (replicon assay) analyses, fractions were collected into Beckman 96-deepwell plates using a Gilson 215 liquid handler and dried under vacuum using a Savant speed-vac. The dried material was resuspended in media and a portion of it tested for inhibition in the replicon assay. The procedure was repeated using an enriched acetonitrile extract, which was prepared by freezing of the aqueous media – acetonitrile supernatant at -20°C, followed by recovery of the upper, acetonitrile phase, evaporation to dryness, and reconstitution in 200 µl methanol. In this manner, bioassay of all fractions revealed activity that correlated with a distinct yet minor late eluting UV detectable peak. The incubation procedure was scaled up (2 L, 5 µM BMS-824 in assay media at 37°C, 5% CO₂, 95% humidity for 48 hours with the bottle cap closed). The incubation was extracted with 2L acetonitrile, followed by centrifugation (Beckman GS-6R, 5000 rpm, 20 min). The centrifuged aqueous media – acetonitrile solutions were frozen at -20°C and the resulting upper acetonitrile extract recovered. The crude
acetonitrile extract was dissolved in methanol-water 65:35 (20 ml) and extracted twice with equal volumes of chloroform that had been presaturated with methanol-water 65:35. The biogram fractionation on the enriched chloroform extract was conducted as follows:

Agilent HP-1100, YMC Pro-C18 5 µm column, 4.6 x 150 mm; mobile phase: 0.01% trifluoroacetic acid-acetonitrile linear gradient 60:40 to 10:90 v/v over 20 min, held at 0.01% trifluoroacetic acid-acetonitrile 10:90 for 5 min., 1.2 mL/min flow rate, UV detection at 254 nm. In this manner, bioassay of all fractions revealed two active UV peaks (17.8 min “peak 4” and 19.0 min “peak 6”). The chloroform extract was subjected to preparative HPLC: Beckman System Gold workstation, YMC Pro-C18 5 µm column, 20 x 150 mm, mobile phase: 0.01% trifluoroacetic acid-acetonitrile linear gradient 60:40 to 10:90 v/v over 20 min, held at 0.01% trifluoroacetic acid-acetonitrile 10:90 for 5 min., 20 mL/min flow rate, UV detection at 254 nm. The replicon active peaks 4 and 6 were manually collected and submitted for biological evaluation.

A second scale up incubation was conducted at higher concentrations of BMS-824 (100 µM) in 1 L assay media. A solution of BMS-824 (60 mg/60 mL DMSO) was added to the media and the media was divided into 4 x 500 ml Erlenmeyer shake flasks, sealed with a semi-permeable membrane (bio-wrap) and placed in an incubator/orbital shaker at 37°C, 100 rpm, for 67 h. Work up and isolation as described above yielded sufficient amounts of replicon active components for structure elucidation (Peak 4: 1.1 mg; Peak 6: 1.1 mg).

**High-resolution MS, NMR.** Electrospray ionization-high-resolution mass spectrometry (ESI-HR-MS) data were conducted with a Micromass QTOF-2 mass spectrometer, Ion mode: positive; resolution 9,500 FWHM; Lock mass: LEU-enkephalin m/z 556 (M + H)^+
MS/MS: collision energy 22 eV; Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker DRX 500 MHz spectrometer equipped with a 5-mm TXI cryoprobe. Proton and carbon chemical shifts are reported in ppm relative to TMS ((1) acetonitrile-d₃ (4) acetone-d₆ solvent) (Figure 4). HPLC Co-injection analyses were conducted using the following methods: Waters Sunfire C-18 column, 5 µm, 4.6x150 mm. Mobile phase – 10 mM ammonium acetate – acetonitrile 95:5 (A solvent) – 10 mM ammonium acetate – acetonitrile 5:95 (B solvent) 85:15 (A:B) to 0:100 (A:B) over 25 minutes, held at 100% B solvent for 5 min.; flow rate 1.2 mL/min, UV (254 nm); Waters Acquity UPLC BEH C18 or Shield RP18, 1.7 µm, 2.1x50 mm column. Mobile phase – 10 mM ammonium acetate – acetonitrile or methanol 95:5 (A solvent) – 10 mM ammonium acetate – acetonitrile or methanol 5:95 (B solvent) 100:0 (A:B) to 0:100 (A:B) over 4 minutes, held at 100% B solvent for 1 min.; flow rate 0.5 mL/min (methanol method) or 0.83 ml/min (acetonitrile method), UV (220 nm).
Results

Characterization of HCV NS5A inhibitors. As part of the characterization of the NS5A inhibitor BMS-824 (Fig. 1) (10), it became apparent that under certain conditions this compound exhibited some chemical reactivity, leading to experiments designed to ascertain its chemical behavior in media. Toward this end, BMS-824 was incubated in cell-free assay media for 72 hours under conditions used for the replicon assay. After incubation, media containing compound was extracted with acetonitrile and examined by HPLC fractionation. A control sample of BMS-824 which was not incubated in media gave a single UV detectable peak in the HPLC trace. In contrast, the parent compound could not be detected by HPLC in the sample of BMS-824 incubated in media, providing evidence of parent compound reactivity under assay conditions (data not shown). The major products formed from BMS-824 (1) were identified as an oxidation product (2) and a thiourea derivative (3) derived from 2 by hydrolysis (Fig. 1). When tested in the HCV replicon assay, these products exhibited poor activity (EC$_{50}$ >10 µM, data not shown), thus raising the possibility that undetected amounts of other highly potent component(s) may be present. To assess this possibility, BMS-824 was incubated with media for various time intervals (0 – 120 h) and the media subjected to the 3 day HCV replicon assay to determine if the HCV inhibitory activity remained. The EC$_{50}$ remained constant for all time points, indicating that, after 5 days of incubation, the potency was maintained even though parent compound was no longer detectable (data not shown). This suggested that an active, stable component(s) was generated from the parent compound during incubation in media and that the resulting new species was a potent HCV inhibitor that could potentially be isolated and characterized.
**Identification of fractions with HCV activity.** To identify the source of the potent inhibition, we first used a replicon HPLC biogram assay to determine which fractions contained anti-HCV activity. The fraction collection utilized a time-based protocol, resulting in a direct relationship between an anti-HCV active well’s position in the plate and a corresponding area on the HPLC chromatogram. Following incubation of BMS-824 and extraction, the sample was subjected to HPLC fractionation (Fig. 2) and 80 fractions were collected and evaluated in the HCV replicon assay. To ensure that any activity we observed was specific for HCV, the fractions were tested against the Y93H BMS-824-resistant replicon cell line and evaluated for cytotoxicity. BMS-824 and an HCV NS3 protease inhibitor were used as controls for the assay and yielded the expected inhibitory profiles. Incubation of cells with 50 nM BMS-824 gave 72 and 0% inhibition on the wild-type and Y93H replicon cells, respectively, while the protease inhibitor gave similar levels of inhibition on both cell lines. Upon testing all 80 fractions, HCV inhibition (>35% inhibition) was reproducibly detected in fractions 56, 61 and 62 (Fig. 2). These fractions showed no significant activity toward the Y93H replicon, suggesting cross-resistance to BMS-824, with no overt cytotoxicity. Under the fractionation conditions used, a distinct UV peak is not detectable in the region of interest (Frs. 61-62); however it is clear from the biogram results that the active component(s) eluted later than parent compound, BMS-824 (1) (Fr. 56).

**Isolation of active components.** Following incubation of BMS-824 in media, the material was extracted using specific modifications to facilitate separation (Materials and Methods) and the enriched sample subjected to HPLC fractionation. A critical
enrichment step prior to HPLC involved extraction of the complex media matrix with acetonitrile, followed by centrifugation and freezing at -20°C to facilitate phase separation. The unfrozen acetonitrile extract was recovered and further refined using a chloroform-methanol-water partitioning step (i.e. modified Folch method (5)). The HPLC-UV peaks in the enriched material consisted of starting material (P3), plus four major peaks and one minor peak between P4 and P6 (Fig. 3). Each fraction was tested for anti-HCV activity on both the wild-type and Y93H resistant cells which show reduced susceptibility to BMS-824 (Table 1). Peak fractions were compared to control BMS-824 which yielded an EC$_{50}$ ~ 10-fold higher in this experiment than normally observed (10). This was interpreted as a reduced ability to convert to an active species in the three day replicon assay. As a control, a media only extraction/separation was performed as above and tested in parallel. The control extraction did not yield peaks of similar intensity nor did the gradient contain any significant inhibitory activity (data not shown). As shown in Table 1, peaks 3, 4, and 6 from the sample containing incubated compound exhibited significant anti-HCV activity, with no detectable cytotoxicity. EC$_{50}$ values for these peaks ranged from 43 nM to 600 pM. Importantly, these three peaks showed 30 - ~3,800 -fold resistance on the Y93H cell line, suggesting that they contained components related to the parent compound BMS-824. Additional fractions representing the entire gradient were tested and did not contain any significant antiviral activity (data not shown). Parent compound BMS-824 (1) was identified in UV peak 3, the same peak in which control non-incubated BMS-824 also eluted. An oxidized form (2) of BMS-824 was identified in UV peak 2 and a thiourea component (3) was identified in UV peak 1. These peaks were of less interest due to their reduced HCV inhibitory activity compared to the other peaks.
and were not pursued further. Peaks 4 and 6 did not correspond to BMS-824 parent yet contained very potent anti-HCV activity; in fact peak 6 had more potent HCV activity than parent compound, indicative of conversion to a more active species.

Structure elucidation. A second scale-up was performed in order to generate a sufficient quantity of the BMS-824-derived active species to allow structure determination. From ESI-HR-MS the molecular formulae for 4 and 6 were determined to be C_{62}H_{52}N_{8}O_{8}F_{2}S_{2} (observed masses: 4 and 6: m/z 1139.3379 (M+H)^+, calculated 1139.3396). The NMR spectrum (500 MHz, acetone-d$_6$) of 4 revealed that all of the substituents; phenacetyl, alanine, fluorobenzene, furan, and the thiazolidinone ring, that were in the starting material BMS-824 (1) were present. Key differences, however, included the loss of the benzylic methine proton (δ5.33, BMS-824, (1)), and a $^{13}$C resonance shift from δ51.4 (1) to δ70.2 (4) (Fig 4). From these data it was surmised that 4 is a symmetrical (homo) dimer of BMS-824, with dimerization occurring at the benzylic carbon, and this was confirmed by mass spectral data (Fig. 5). In the course of optimizing NMR experimental conditions (i.e. solvent, temperature), we observed that upon prolonged NMR data acquisition with 6 in acetonitrile-d$_3$ at 55°C, this compound, similarly shown to be a homodimer of BMS-824 (1), converted to 4. This was concluded based on co-injections on HPLC with orthogonal methods, by proton NMR, and mass spectral data. We therefore propose that 4 and 6 are atropisomers rather than diastereomers. The gross structure of 4 is depicted in Fig. 6A and further studies regarding its formation and the stereochemistry at the dimer linkage are currently under investigation.
Biological profile of a dimeric inhibitor. Due to the dimeric structure of 4 it appeared that the symmetry of the molecule was important for achieving potent HCV inhibitory activity. To test this hypothesis, a simplified dimeric stilbene derivative BMS-346 (Fig. 6B) was synthesized based on the precise SAR associated with the amino acid moiety (17). When tested in the HCV replicon assay, BMS-346 had an EC$_{50}$ of 86 pM (Table 2), an approximately 70-fold enhancement in potency compared to BMS-824. In contrast, the EC$_{50}$ of BMS-346 on the BVDV replicon was >10 µM, demonstrating that the dimeric inhibitor has excellent potency as well as selectivity for HCV. Resistance of the Y93H replicon to BMS-346 indicated the mechanistic relatedness of this compound to BMS-824 and suggested that NS5A is also the target of this compound (Table 2).

To further explore the antiviral activity of BMS-346, the compound was used to select for resistance on genotype 1b HCV replicon cells. Mapping of the BMS-346-resistant cell line revealed an L31V NS5A substitution in 6 out of 6 clones, with two of the clones having L31V linked with a Q54L substitution in NS5A. These are the same substitutions previously identified from selection with BMS-824 (10), suggesting that BMS-346 binds in a similar manner to that of the active component of BMS-824. When tested in a transient replication assay, the single Q54L and L31V mutants conferred 30- to 80-fold resistance to BMS-346, respectively (Fig. 7). However, when the L31V and Q54L mutations are present together, resistance to BMS-346 increased significantly to >400-fold, suggesting that both changes are required to maximally affect compound potency. As expected, none of the NS5A mutants conferred resistance to a control HCV protease inhibitor (data not shown).
The chemical stability of BMS-346 was also examined by incubating the compound in cell media for 72 h at 37ºC followed by HPLC fractionation. Under these conditions, less than 3% degradation of the parent structure was observed, indicating that BMS-346 is stable in media and demonstrating that this novel dimeric species has potent and selective anti-HCV activity.

In summary, the active components in replicon media were identified whose symmetry afforded the necessary insight to prepare inhibitor BMS-346, a compound chemically inert to assay conditions, and the NS5A inhibitory activity of BMS-346 was clearly established. Subsequently, this compound provided the basis for the design of BMS-790052, the first NS5A inhibitor to show clinical efficacy.

Discussion

A multiplexed HCV replicon screen was used to identify specific, non-toxic low molecular weight inhibitors such as BMS-824 that target the NS5A protein. Careful evaluation of potency and specificity in a number of additional assays revealed that BMS-824 was chemically reactive under the assay conditions and transformed into other species in tissue culture media that were responsible for inhibitory activity. It was the surprising finding that anti-HCV activity was maintained in the absence of detectable parent compound that lead us to pursue identification of the exceptionally active components. To do so, a sensitive replicon HPLC biogram assay was used which allowed us to monitor fractions that contained anti-HCV activity. Inclusion of an NS5A-resistant cell line in the experiments to demonstrate cross-resistance was key to
confirming relatedness to BMS-824 and allowing us to identify the peak fractions of interest.

A powerful aspect of the HPLC biogram methodology lies in the ability to use a functional assay to detect biologically active substances in crude matrices that are not readily detectable by physical methods such as, for example, HPLC-UV (4, 8). The present study began by incubating BMS-824 in HCV replicon assay media prior to isolation experiments. Two essential sample enrichment steps enabled the HPLC-UV detection and isolation of two minor peaks that correlated with HCV replicon inhibitory activity, and allowed us to conduct structure determination using conventional spectroscopic techniques. In this manner, the active species was determined to be a larger, dimeric form of the parent molecule derived from a presumed intermolecular reaction rather than a smaller degraded derivative. The dimerization of BMS-824 is currently believed to occur through a radical mechanism and this process is the subject of continuing examination in order to determine the precise reaction pathway and define the stereochemical relationships.

The synthesis of BMS-346, a compound that symmetrizes elements of BMS-824 thought to be critical for HCV inhibitory activity, yielded a compound with excellent activity against HCV (EC$_{50}$ ~86 pM), confirming the hypothesis that potent antiviral activity could be derived from a symmetrical dimeric molecule. This was further demonstrated by extensive SAR studies (17). In addition, resistance generation and mapping yielded similar NS5A resistance substitutions as those identified from selection with BMS-824, implying a common binding site for the 824-derived active species and BMS-346. Based on resistance mapping, these NS5A inhibitors appear to interact, either
directly or indirectly, with the N-terminus of NS5A. The NS5A protein consists of three
putative domains (I, II and III) with the resistance mutations residing in domain I.
Domain I consists of the first 213 amino acids of the protein and contains a membrane
anchoring α-helix in the N-terminal 30 amino acids (16). The solid state structure of
domain I was recently determined and shown to form a dimeric complex via contacts
near the N-terminal ends of the molecules, which can adopt different conformations (13,
19). The dimeric structure of BMS-346 complements the symmetry observed in domain
I, suggesting a functional role that allows association with the NS5A protein across the
dimer interface. The mechanism of action of NS5A inhibitors, like the role of the NS5A
protein during the HCV viral life cycle, is poorly understood. The coincidence of dimeric
structures for both the inhibitor and the NS5A protein, as well as the exceptional potency
of the inhibitors, provides an opportunity to speculate on and test different working
models. Given that the resistance substitutions for BMS-346 lie in the vicinity of the
dimer interface, it is possible that these inhibitors interfere with NS5A dimer formation.
The exceptional potency of NS5A inhibitors would suggest the anti-HCV effect may be
amplified. It is conceivable that NS5A proteins form multimers during formation of the
replication complex and active viral RNA replication. A single inhibitor may not only
disrupt formation of a single NS5A dimer, but may also affect adjacent NS5A dimers,
thereby inactivating the function of an entire replication complex. Alternatively, the
compounds may disrupt the proximal α-helix that promotes essential membrane
association or interaction of NS5A with an unknown host or viral factor(s) required for
HCV RNA replication. Studies are in progress to gain a better understanding of the
multiple functions of NS5A and the mode of inhibition of these NS5A inhibitors.
Efforts to identify the active component of compound BMS-824 revealed dimeric molecules generated from an intermolecular dimerization reaction, leading to the synthesis of a novel class of symmetrical molecules that demonstrate excellent potency against HCV and which target the NS5A protein. These discoveries provided the catalyst for an extensive investigation of further structural modifications to BMS-346 in which optimization efforts focused on broadening genotype coverage and incorporating pharmacokinetic properties suitable for oral administration. BMS-346 formed the foundation for the discovery of BMS-790052, a HCV NS5A inhibitor that exhibits picomolar EC₅₀’s toward a broad range of HCV genotypes \textit{in vitro} and has shown excellent clinical efficacy following oral dosing to subjects chronically infected with HCV genotype 1 (6).

\textbf{Acknowledgements}

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References


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Table 1. Anti-HCV activity of HPLC fractions

<table>
<thead>
<tr>
<th>Sample</th>
<th>EC$_{50}$ (µM)$^a$</th>
<th>Fold resistance$^b$</th>
<th>CC$_{50}$ (µM)$^c$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>wt</td>
<td>Y93H</td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>1</td>
</tr>
<tr>
<td>P2</td>
<td>4.8</td>
<td>&gt;5</td>
<td>1</td>
</tr>
<tr>
<td>P3</td>
<td>0.005</td>
<td>5</td>
<td>1000</td>
</tr>
<tr>
<td>P4</td>
<td>0.043</td>
<td>1.3</td>
<td>30</td>
</tr>
<tr>
<td>P5</td>
<td>2.8</td>
<td>&gt;5</td>
<td>&gt;1.8</td>
</tr>
<tr>
<td>P6</td>
<td>0.0006</td>
<td>2.3</td>
<td>3833</td>
</tr>
<tr>
<td>BMS-824</td>
<td>0.061</td>
<td>6.1</td>
<td>100</td>
</tr>
<tr>
<td>PI</td>
<td>0.200</td>
<td>0.209</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$Major peak fractions identified by HPLC fractionation were titrated on wild-type (wt) and resistant (Y93H) replicon cells and tested for antiviral activity and toxicity. Non-fractionated BMS-824 and an HCV protease inhibitor (PI) were included as controls.

$^b$Fold resistance = Y93H EC$_{50}$/ wt EC$_{50}$

$^c$Due to the amount of P1-P6, the highest concentration used for CC$_{50}$ determination was 5 µM.
Table 2. Biological activity of BMS-346a

<table>
<thead>
<tr>
<th>Replicon</th>
<th>EC$_{50}$ (µM)</th>
<th>CC$_{50}$ (µM)</th>
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<tr>
<td>wt HCV</td>
<td>0.000086</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Y93H</td>
<td>4</td>
<td>&gt;10</td>
</tr>
<tr>
<td>BVDV</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

aEC$_{50}$ values were derived from this single experiment. In multiple test occasions, the EC$_{50}$ values and standard deviations of BMS-346 are: wt: $0.000052 \pm 0.0000076$ µM; Y93H: $3.5 \pm 3.1$ µM.
FIG. 1. Structures of BMS-824 (1) and media-induced products (2, 3, 4-6).
FIG. 2. Biogram analysis to identify regions containing active components. BMS-824 was incubated in media for 48 h then subjected to extraction and HPLC fractionation. Eighty fractions were collected and tested for anti-HCV activity with the region containing the peak inhibitory activity indicated by arrows. (Solid line = HPLC trace; dotted line = Percent inhibition of HCV replicon activity) mAU: milli absorbance units
**FIG. 3.** Fractionation of BMS-824 incubated in media. BMS-824 was incubated in media for 48 h then subjected to extraction with acetonitrile and chloroform followed by preparative HPLC. The six key peaks identified are labelled P1 – P6. Assignment of peak components indicated: P1 = thiourea, P2 = oxidized BMS-824, P3 = BMS-824. Other peaks did not contain enough material for structure determination.
FIG 4. $^1$H- and $^{13}$C-NMR chemical shifts for BMS-824 (1)$^a$ and Dimer (4)$^b$. NMR solvents: (a) acetonitrile-d$_3$, (b) acetone-d$_6$. 

1. Figure 4.

BMS-824 (1)$^a$

Dimer (4)$^b$
FIG. 5. Mass spectrometry analysis of dimers 4 and 6 (positive ion electrospray LC/MS and LC/MS/MS).
Figure 6.

A) 

B)
FIG. 6. Dimeric structures. (A) Proposed structure of the active component in peaks 4 and 6 based on NMR and MS. (B) Structure of dimeric compound BMS-346.

FIG. 7. Resistance analysis of BMS-346 selected substitutions. Huh-7 cells were transfected with wild-type or mutant replicon RNAs and incubated in the presence or absence of various concentrations BMS-346. Luciferase activities were determined in lysates of cells harvested 72 h after transfection and EC50 values determined. The fold-resistance of the mutant RNAs relative to wild-type (mutant EC50/wild-type EC50) are depicted in the graph.