Mechanism of Inhibition for BMS-791325, a Novel Non-nucleoside Inhibitor of Hepatitis C Virus NS5B Polymerase

Received for publication, September 26, 2014, and in revised form, October 8, 2014. Published, JBC Papers in Press, October 9, 2014, DOI 10.1074/jbc.M114.613653

Karen L. Rigat1, Hao Lu1, Ying-Kai Wang1, Argyrides Argyrou1,2, Caroline Fanslau3, Brett Beno4, Yi Wang4, 5, Jovita Marcinkewicziene4, 5, Min Ding4, Robert G. Gentles3, Min Gao3, Lynn M. Abell1, and Susan B. Roberts5

From the Departments of 1Virology, 2Computer Assisted Drug Design, and 3Early Discovery Chemistry, Research and Development, Bristol Myers Squibb Co., Wallingford, Connecticut 06492 and the 4Department of Mechanistic Biochemistry, Research and Development, Bristol Myers Squibb Co., Pennington, New Jersey 08534

Background: BMS-791325, a non-nucleoside inhibitor of HCV NS5B, has robust clinical efficacy.

Results: Biochemical and biophysical methods revealed a non-competitive time-dependent inhibition mechanism and permitted complete parameterization of inhibitor binding kinetics.

Conclusion: Thumb and finger variants affect BMS-791325 association rates.

Significance: The impact of NS5B variants on BMS-791325 binding provides insight into the basis of inhibitor resistance and the process of replication complex formation.

HCV infection is an urgent global health problem that has triggered a drive to discover therapies that specifically target the virus. BMS-791325 is a novel direct antiviral agent specifically targeting HCV NS5B, an RNA-dependent RNA polymerase. Robust viral clearance of HCV was observed in infected patients treated with BMS-791325 in combination with other anti-HCV agents in Phase 2 clinical studies. Biochemical and biophysical studies revealed that BMS-791325 is a time-dependent, non-competitive inhibitor of the polymerase. Binding studies with NS5B genetic variants (WT, L30S, and P495L) exposed a two-step, slow binding mechanism, but details of the binding mechanism differed for each of the polymerase variants. For the clinically relevant resistance variant (P495L), the rate of initial complex formation and dissociation is similar to WT, but the kinetics of the second step is significantly faster, showing that this variant impacts the final tight complex. The resulting shortened residence time translates into the observed decrease in inhibitor potency. The L30S variant has a significantly different profile. The rate of initial complex formation and dissociation is 7–10 times faster for the L30S variant compared with WT; however, the forward and reverse rates to form the final complex are not significantly different. The impact of the L30S variant on the inhibition profile and binding kinetics of BMS-791325 provides experimental evidence for the dynamic interaction of fingers and thumb domains in an environment that supports the formation of active replication complexes and the initiation of RNA synthesis.

1 Both authors contributed equally to this work.

2 Present address: Dept. of Biological Sciences, GlaxoSmithKline, Stevenage, Hertfordshire SG1 2NY, United Kingdom.

3 Present address: EZBiolab, 1033 Third Ave. SW, Carmel, IN 46032.

4 Present address: Dept. of Cardiovascular and Metabolic Diseases, Novartis Institute of Biomedical Research, 250 Massachusetts Ave., Cambridge, MA 02139.

5 To whom correspondence should be addressed: Dept. of Virology, Bristol-Myers Squibb Co., 5 Research Pkwy., Wallingford, CT 06492. Tel.: 203-677-6707; Fax: 203-677-6088; E-mail: susan.roberts@bms.com.

6 The abbreviations used are: HCV, hepatitis C virus; NS5B, nonstructural protein 5B; DAA, direct acting agent; GT, genotype; NUC, 2′C-methyl-GTP.
assays (IC\textsubscript{50} and EC\textsubscript{50} values of 0.7–4 nM), selection of significant resistance at a single substitution site, and a robust pharmacokinetic profile in animal models, anticipated the strong antiviral effect observed in patients (10, 11).

Co-crystal structures and resistance selection in HCV replicon cells demonstrated this non-nucleoside inhibitor interacts with a site in the thumb domain of the polymerase (10, 12, 13) (Fig. 1). The site, referred to as thumb site 1, is occupied in the apo-protein by a protein loop (Δ1) within the fingers domain of the polymerase (14–17). Co-crystal structures show that BMS-791325, like other thumb site 1 inhibitors, displaces the finger loop when it binds this pocket (17).

Less potent thumb site 1 inhibitors have been evaluated for mechanism of inhibition, and some of these have also been tested in clinical studies (16–28). Primer-dependent replication model systems were used to characterize 2 thumb site 1 inhibitors with K\textsubscript{i} values of 120–200 nM. The inhibitors were shown to be non-competitive with primer-template and NTP and unable to inhibit preformed replication complexes (18). Resistance selection in the replicon system identified substitutions at proline 495, an amino acid 30 Å from the active site, as responsible for resistance. Based on the mechanistic and resistance selection results, the authors proposed that the allosteric thumb site 1 inhibitors interact with the enzyme-RNA complex and impact a slow conformational transition, preceding nucleotide binding, which is required for the formation of productive initiation complexes. When co-crystal structures of NS5B and two structurally similar inhibitors confirmed the site of binding (17), the authors hypothesized that thumb site 1 inhibitors interfere with enzyme activity by preventing the formation of intramolecular contacts between fingers and thumb, precluding the coordinated movements required for RNA synthesis.

Biochemical and biophysical methods were used to characterize the interaction between BMS-791325 and the HCV NS5B polymerase. The inhibitor delivers potent, specific, and time-dependent inhibition of the isolated enzyme and is non-competitive with respect to both template and nucleotide substrates. The use of wild type (WT) and variant NS5B polymerases (P495L and L30S; Fig. 1 (10, 29, 30)) helped to elaborate details of the inhibition mechanism.

Our studies demonstrate how the inhibitor binding mechanism contributes to the ability of BMS-791325 to deliver potent antiviral activity. In addition, the impact of variants (P495L and L30S) on inhibitor binding to HCV NS5B reveals a detailed mechanism of resistance for a clinically relevant resistance variant and provides experimental evidence for a dynamic interaction between the fingers and thumb that impacts the formation of active replication complexes.

**EXPERIMENTAL PROCEDURES**

**Compound Synthesis**—BMS-791325 was synthesized at Bristol-Myers Squibb Co. (10). Purity was ≥95% as determined by LC-MS. The 2’Me-methyl-GTP (NUC) inhibitor was obtained from Inhibitex, Inc. (Alpharetta, GA).

**Experimental Reagents**—Reagents of the highest quality available were purchased from commercial sources as noted.

**Cloning, Expression, and Purification of HCV NS5B Proteins**—The cDNA encoding the open reading frame for HCV NS5B was amplified from viral RNA by RT-PCR and cloned into a pet21b vector for expression (31). Untagged NS5B proteins were expressed and isolated to >90% purity using heparin-Sepharose and poly(U)-Sepharose chromatography (31). Enzymes were stored at −80 °C in buffer containing 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.1 mM EDTA, 2 mM DTT, 0.5% Triton X-100, 50% glycerol.

**Polymerase Activity Assays**—RNA synthesis was measured by detecting the incorporation of radiolabeled nucleotides. Regardless of the assay format used to measure activity, inhibition by BMS-791325 was detected as a decrease in the incorporation of radiolabeled nucleotides compared with an untreated control. In all assay formats, BMS-791325 and control compounds were serially diluted 1:3 in DMSO and transferred to 96-well assay plates (Corning 3365) for a final DMSO concentration of 0.01%.
turation of 2%. In all assay formats, except for the assay performed in the presence of scintillation proximity assay beads, the newly synthesized RNA product was precipitated by 10% TCA and quantified on the Packard Top Count NXT scintillation/luminescence counter.

**De Novo Assay**—NS5B polymerase initiates de novo using a single nucleotide in the absence of a primer. The reaction contains homopolymeric C template (0.35 nM), NS5B enzyme (2.8 nM), 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 2.5 mM KCl, 1 mM DTT, 50 μg/ml BSA (B6917, Sigma), 625 μM GTP, and [³³P]GTP (5 μCi, 3000 Ci/mmol; PerkinElmer Life Sciences NEG-606H). After a 1-h preincubation of NS5B polymerase, template, and compound, de novo RNA synthesis was initiated by the addition of GTP. Reactions were incubated at 30 °C for 15 min.

**Dinucleotide Primer Assay**—NS5B polymerase initiates primer-dependent replication in a reaction containing pGpG primer (8.6 μM), homopolymeric C template (0.35 nM), NS5B enzyme (2.8 nM), 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 2.5 mM KCl, 1 mM DTT, 50 μg/ml BSA (B6917, Sigma), 1 μM GTP, and [³³P]GTP (1 μCi, 3000 Ci/mmol, PerkinElmer Life Sciences NEG-606H). After a 0–24-h preincubation of NS5B polymerase, template, and compound, RNA synthesis was initiated by the addition of primer and GTP. Reactions (total volume of 0.06 ml) were incubated at 30 °C for 15 min.

**12-Mer Primer Assay**—NS5B polymerase initiates primer-dependent RNA synthesis on poly(A) template using biotinylated oligo(dT)₁₂ primer that is precaptured on streptavidin-coupled scintillation proximity assay beads. The assay was performed essentially as described (31, 32).

**Copy-back Assay**—NS5B polymerase initiates RNA synthesis on a heteropolymeric template using a primer-dependent, copy-back mechanism. The reaction contains heteropolymeric template (0.4 nM, 2650 nucleotides), NS5B enzyme (8.4 nM), 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM DTT, 50 mM NaCl, and 50 μg/ml BSA (B6917, Sigma); 1.6 units of RNase inhibitor (N251B, Promega, Madison, WI); and 3 μM cold ATP, CTP, GTP, UTP, and [³³P]UTP substrate (4 μCi, 3000 Ci/mmol; PerkinElmer Life Sciences,NEG-608H). After a 1-h preincubation of NS5B polymerase, template, and compound, primer-dependent RNA synthesis was initiated by the addition of NTP. Reactions were incubated at 30 °C for 3 h.

The half-maximal inhibition values (IC₅₀) were determined using seven different inhibitor concentrations, [I], and calculated using the formula, y = y_min + ((y_max − y_min)/(1 + IC₅₀/x))x, where x is the inhibitor concentration and n is the Hill coefficient.

**Mode of Inhibition**—Inhibition modality was determined using a poly(C) template and dinucleotide (pGpG) primer assay. Reactions contained HCV NS5B enzyme (2.8 nM), poly(C) template (concentration noted), pGpG primer (8.6 μM), and [³³P]GTP (1–3 μCi; 3000 Ci/mmol; PerkinElmer Life Sciences, NEG-606H) supplemented with unlabeled GTP (concentration noted). BMS-791325 was titrated in DMSO with 2-fold dilutions and final concentrations ranging from 0.049 to 50 nM. To determine the inhibition modality with respect to template, NS5B was preincubated for 24 h at room temperature in TRIS buffer (20 mM, pH 7.5) containing BMS-791325 or DMSO, poly(C) template (0.15–4.8 nM), GTP (1 μM), BSA (50 μg/ml), and MgCl₂ (7.5 mM). Reactions were initiated by adding pGpG primer and radiolabeled GTP, and reaction progress was monitored over 15 min. To determine the inhibition modality with respect to GTP, NS5B was preincubated for 24 h at room temperature in TRIS buffer (20 mM, pH 7.5) containing BMS-791325 or DMSO, template (0.35 nM), GTP (0.78–25 μM), BSA (50 μg/ml), and MgCl₂ (7.5 mM). The reaction was initiated by adding pGpG primer and radiolabeled GTP and monitored over 15 min. Reactions were quenched with TCA (10%). The precipitated RNA was washed and detected on a Packard Top Count NXT. Experiments to determine the mode of inhibition were also carried out using a poly(A)dT₁₂·UTP assay, using similar conditions.

Because the potency determined for BMS-791325 was close to the enzyme concentration used in the reactions (2.8 nM), Kᵢ,app values were determined by fitting the inhibition data to the Morrison equation in order to account for the depletion of inhibitor in solution (33).

\[
v_i/v_0 = 1 - \left[\frac{[E] + [I] + K_{app}}{[E] + [I] + K_{app}}\right]^2 - 4([E][I])^{0.5}
\]

(Eq. 1)

The values for Kᵢ,app were plotted as a function of template or GTP concentration to determine inhibition modality and calculate Kᵢ.

Time-dependent inhibition of NS5B activity by BMS-791325 was investigated by measuring the initial reaction velocity after different periods of enzyme-inhibitor preincubation using the template-primer (poly(A)dT₁₂) assay. The reactions contained 14 nM HCV NS5B enzyme, 4 nM poly(A) template, 3000 nM biotinylated oligo(dT)₁₂ primer, and 3 μCi of [³³P]UTP (3000 Ci/mmol; Amersham Biosciences, NEG-607H) supplemented with 25 μM cold UTP in assay buffer (20 mM Hepes, pH 7.5, 2.5 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 1.6 units of RNase inhibitor (Promega, N2515), 0.1 mg/ml BSA (Promega, R3961), and 2% glycerol). After preincubuating inhibitor and NS5B enzyme for various periods of time as indicated (Fig. 4A), reactions were initiated by adding template, primers, and UTP and incubated at room temperature for up to 70 min. At various time intervals, the reactions were stopped with 50 mM EDTA. The newly synthesized RNA product was precipitated by 10% TCA, washed, and detected on a Packard Top Count NXT counter. Fractional velocity (vᵢ/v₀) versus preincubation time (t) was fit to the exponential equation, vᵢ/v₀ = exp(−k_obs t), to obtain k_obs, where vᵢ is the inhibited reaction rate, and v₀ is the uninhibited reaction rate. The k_obs values were plotted versus inhibitor concentration (Fig. 4B). Data were fit to a single step binding model, kᵢ,app = kᵢ(1 + I/Kᵢ,app), to derive kᵢ and Kᵢ,app. The value of kᵢ is derived from the equation, Kᵢ = kᵢ/Kᵢ,app.

**Binding Kinetics**—BMS-791325 binding to WT, L30S, and P495L NS5B polymerases was characterized using solution state and biolayer interferometry methods.

**Solution State**—Binding kinetic constants for BMS-791325 and NS5B polymerases (WT, L30S, and P495L) were measured by stopped flow (SX 20, Applied Photophysics). Binding events were measured in the millisecond time scale by monitoring the
change in fluorescence of BMS-791325 using an excitation wavelength of 320 nm and an emission wavelength of 420 nm upon rapid mixing of the compound with NS5B (WT, L30S, or P495). All solutions were prepared in NS5B binding buffer (20 mM Tris·HCl, pH 7.5, 2.5 mM MgCl2, 2.5 mM KCl, 0.01% Triton X-100, and 1 mM DTT). The instrument was prepared by washing 10 ml of Millipore water through each drive syringe and then equilibrating the syringes with 5 ml of buffer to normalize the voltage. A 10-ml stock of 10 nM BMS-791325 was used for all experiments. 1-ml solutions of NS5B enzyme were prepared at various concentrations (50–1000 nM) as indicated and loaded into disposable 1-ml syringes. The compound syringe contained 5 nM BMS-791325 and 0.1% DMSO (v/v). Background spectra were acquired using the buffer to ensure a stable signal. Starting with the lowest enzyme concentration, an average of 10 spectra were acquired and averaged. The enzyme chamber was washed with 5 ml of buffer before the next concentration was added. When all data were collected, the system was washed with 10 ml of 1 M NaOH, followed by 20 ml of water.

Data were fit, using SigmaPlot (Systat Software, Inc.), to a single-exponential equation, \( Y = A \times \exp(-k_{obst}t) + C \), or a double exponential equation, \( Y = A_1 \times \exp(-k_{obst}t) + A_2 \times \exp(-k_{obs2}t) + C \), where \( A \) is the amplitude, \( k_{obst} \) is the observed rate constant, and \( C \) is the end point.

**Biosensor Assay**—Binding kinetics were also measured using the Octet Red (ForteBio, Menlo Park, CA), an optically based sensor format. WT NS5B enzyme and L30S and P495L variants were chaotically biotinylated using the EZ-Link kit (Pierce, 21338). Briefly, a 5-fold excess of EZ-Link reagent was added to the enzyme in storage buffer. The mixture was incubated for 25 min at room temperature. Tris buffer (20 \( \mu \)l of 1 M, pH 8.0) was added to quench the reaction. The biotinylated enzymes were stored at \(-80^\circ\) C and were used directly without further purification.

Biotinylated NS5B protein was diluted using the loading buffer (20 mM Tris·HCl, pH 7.5, 2.5 mM MgCl2, 2.5 mM KCl, and 0.01% (v/v) Triton X-100) to a final concentration of 0.8, 0.3, and 0.4 \( \mu \)M for WT, L30S, and P495L, respectively. Superstrepavidin sensor (ForteBio part 18-5057) was equilibrated with the diluted enzyme for 1 h at room temperature. Control experiments were conducted in parallel by equilibrating the sensor with loading buffer only. BMS-791325 in solution at various concentrations was prepared by diluting a DMSO stock into the binding buffer (20 mM Tris·HCl, pH 7.5, 2.5 mM MgCl2, 2.5 mM KCl, and 0.02% (v/v) Tween 20). Final DMSO concentration was controlled at 2% (v/v). Compound association curves were obtained by dipping the sensor immobilized with biotinylated enzyme into the compound solution; the sensor equilibrated with loading buffer only was used to measure nonspecific binding, and the signal was subtracted. Once the binding reached equilibrium, the sensor tip was moved to binding buffer containing 2% DMSO for the dissociation measurement.

Association curves were fit using GraphPad Prism to a single-exponential equation, \( Y = C - A \times \exp(-k_{obs}t) \) or a double exponential equation, \( Y = C - A_1 \times \exp(-k_{obs1}t) - A_2 \times \exp(-k_{obs2}t) \), where \( A \) is the amplitude, \( k_{obs} \) is the observed rate constant, and \( C \) is the end point. Dissociation curves were fit into a single decay equation, \( Y = C + A \times \exp(-k_{off}t) \), where \( A \) is the amplitude, \( k_{off} \) is the dissociation rate constant, and \( C \) is the end point.

### RESULTS

**Potent and Specific Inhibition of HCV NS5B Polymerase Activity**—BMS-791325 inhibits GT-1 HCV NS5B polymerase with nanomolar potency. It is also highly selective for NS5B, showing no activity against the closely related RNA-dependent RNA polymerase from the pestivirus, bovine viral diarrhea virus, or human DNA polymerases \( \alpha, \beta, \) and \( \gamma \) (IC\(_{50} \) values >25 \( \mu \)M) (11).

Intrinsic potency was investigated using isolated GT-1b enzyme (Con1 \( \Delta A18 \)) and four different replication model systems (Table 1). The model systems, poly(C) template-GTP, poly(C) template-pGpG dinucleotide, poly(A) template-dT\(_{12} \) primer, and HCV RNA-NTP, support distinct initiation mechanisms, including de novo, primer-dependent, and copy-back, respectively. The highly purified and untagged NS5B that we used for these studies has robust replication activity in each of these in vitro systems; however, published reports suggest that the de novo mechanism is predominant in vivo (34, 35). In each case, the inhibitor was preincubated for 1 h with NS5B enzyme and template, and the assay was initiated with the addition of NTP(s) (and primer, as required by the model system). The potency (IC\(_{50} \)) of BMS-791325 is similar in all four replication systems (Table 1).

Because each of these systems requires replication complex formation and incorporation of a first NTP, each system models these aspects of replication initiation; however, NS5B complexed with an RNA duplex (poly(A) template-dT\(_{12} \) primer or copy-back template) is likely to have a conformation required specifically for elongation (36–38) and not a conformation required for de novo initiation.

**Mode of Inhibition**—Previous reports showed that compounds binding at thumb site 1 inhibit the initiation of RNA replication (16, 18) and not elongation; however, BMS-791325 appears to be 5–75 times more potent than previously studied compounds and inhibits equally in model systems of de novo and primer-dependent synthesis. Because two of the four replication systems are assumed to model elongation (poly(A) template-dT\(_{12} \) and HCV RNA), a trapping experiment was performed to confirm that BMS-791325 has no impact on the elongation phase of replication.

A time course of inhibition was generated in the presence and absence of heparin, using the poly(C)-pGpG assay (Fig. 2). Heparin traps polymerase molecules that are not engaged in stable replication complexes, permitting replication from complexes engaged at the time of heparin addition and preventing

| TABLE 1 | Inhibition of WT HCV NS5B polymerase in assays using multiple modes of initiation |
|---------|---------------------------------------------|
| Assay   | IC\(_{50} \) (\( \mu \)M) ± S.D. |
| Poly(C)-GTP, de novo | 3.5 ± 0.5 |
| Poly(C)-pGpG, primer-dependent | 2.9 ± 0.9 |
| Poly(A)-dT\(_{12} \), primer-dependent | 4.5 ± 2.3 |
| HCV RNA-NTP, copy-back | 3.9 ± 0.4 |
the formation of new replication complexes (39). The progress curve for the poly(C) template-pGpG dinucleotide reaction with BMS-791325 added (10 μM) parallels the progress curve with heparin added (10 μg/ml). Inhibition was not enhanced by the combination of BMS-791325 and heparin. On the contrary, the inhibition curves (BMS-791325 alone, heparin alone, and BMS-791325 plus heparin) are essentially superimposed. A nucleoside analog, NUC, reported to inhibit both initiation and elongation, was included as a positive control (40). The presence of the NUC (1 μM) blocked all replication, generating progress curves (NUC alone or the NUC plus heparin) that overlap and lie along the x axis.

These results confirm that BMS-791325, like the previous thumb site 1 inhibitors tested, has no impact on elongation (16, 18). The inhibition in systems that model elongation suggests that BMS-791325 blocks a step before the incorporation of nucleotides.

Inhibition modality was further investigated by preincubating BMS-791325 with NS5B in the presence of template and GTP before initiating the reaction. These studies were initially performed with a 1-h preincubation (not shown); however, based on the slow binding kinetics observed in binding studies and described below, the studies were repeated with a 24-h preincubation. The 24-h preincubation ensures binding equilibrium between inhibitor and the potential competitors (template or GTP). Essentially identical results were observed, regardless of the preincubation time (1 or 24 h). The initial velocities were fit to the Morrison equation (Fig. 3, A and B). A plot of apparent $K_i$ versus concentration of template or GTP confirmed the modality as non-competitive (Fig. 3, C and D), with $K_i$ values of 1.1 ± 0.1 and 2.6 ± 0.4 nM for template and GTP, respectively. Similar results were observed (3.9 ± 0.43 nM and 1.6 ± 0.09 nM for template and UTP, respectively) using a poly(A)-dT$_2$-UTP assay (data not shown).

**Time-dependent Inhibition of HCV NS5B Polymerase Activity**—To determine whether the inhibition of NS5B by BMS-791325 is time-dependent, the IC$_{50}$ was measured with and without preincubation of inhibitor with NS5B before replication was initiated by adding NTP. The potency of BMS-791325 was enhanced by increasing the time of preincubation (Table 2). The enhanced potency that was observed with WT enzyme after preincubation was the first indication that BMS-791325 could be a slow binding inhibitor; the small but repro-
ducible difference in potency between 1 and 5 h indicates that a preincubation time of >1 h is required to reach equilibrium (Table 2). The enhanced potency with preincubation is not due to instability of the enzyme or RNA template because no differences in the amount of replication product formed (cpm incorporated/ng of protein) in the absence of BMS-791325, were observed after 0-, 1-, 5-, and 24-h preincubation (data not shown).

Two genetic variants (L30S and P495L) were also examined, based on a hypothesis that these amino acid variants could impact the time to reach equilibrium (Table 2). The specific activity (cpm incorporated/ng of protein) of the L30S polymerase is reduced (~100-fold) compared with WT (data not shown) but the potency of BMS-791325 on L30S, after preincubation, is equal to that on WT polymerase. The preincubation time required to achieve maximum inhibition of L30S appears to be shorter than that for WT (similar IC50 values for L30S after 1-, 5-, and 24-h preincubation). The preincubation effect was detected (Table 2).

To explore the mechanism of time-dependent inhibition for BMS-791325, the initial reaction velocity was measured after different periods of WT NS5B inhibitor preincubation, using a template-primer (poly(A)-dT12) assay. Rates of incorporation of radiolabeled nucleotide were determined. The initial velocity decreases as enzyme-inhibitor preincubation time increases at various inhibitor concentrations (Fig. 4A).

These data confirm that BMS-791325 is a time-dependent inhibitor of WT NS5B.

The kobs values were calculated by fitting the initial reaction rate decreases versus time of preincubation. A second plot of kobs versus inhibitor concentration was fit to either a linear or hyperbolic curve to compare one- and two-step binding models. Based on Akaike criteria (Prism GraphPad), the one-step binding model is favored (82.8% versus 17.2%) (Fig. 4B). Using a one-step binding model, the k1 and k2 values were 2.1 ± 0.2 × 10^4 M^-1 s^-1 and 5.0 ± 0.1 × 10^5 s^-1, respectively (Scheme 1).

Binding Kinetics—To fully characterize the inhibition mechanism, binding studies were conducted with WT NS5B and two NS5B genetic variants (L30S and P495L) using two different methods. BMS-791325 has intrinsic fluorescence properties that permit detection of changes in the ligand environment upon binding to the enzyme in stopped-flow studies. Kinetic measurements, monitoring fluorescence intensity decreases at 420 nm, yielded binding curves at different enzyme concentrations for each NS5B variant (WT, L30S, and P495L). The curves were fit to a single-exponential equation for the WT and L30S variants to calculate kobs values. For the P495L variant, the binding curves follow a double exponential equation, characterized by a fast rate constant (kobs1) and a slower rate constant (kobs2) that does not vary with different enzyme concentrations. Accordingly, we consider the higher kobs1 values to define the rate of enzyme-inhibitor binding. Plots of kobs versus NS5B enzyme concentration showed linear correlations for all three enzymes, implying a single step binding mechanism (Fig. 5). The forward rates (k1) of BMS-791325 binding for WT, L30S,

| Preincubation time | WT | L30S | P495L |
|--------------------|----|------|-------|
| h                  |    |      |       |
| 0                  | 784 ± 213 | 20.4 ± 9.1 | ~1270 |
| 1                  | 2.9 ± 0.9 | 0.6 ± 0.1 | 497 ± 147 |
| 5                  | 0.7 ± 0.1 | 0.7 ± 0.1 | ND    |
| 24                 | 0.7 ± 0.3 | 0.7 ± 0.2 | 431 ± 110 |

TABLE 2
Effect of BMS-791325-NS5B polymerase preincubation time on inhibition (IC50).

IC50 values represent the mean ± S.D. from more than two independent experiments. The NO preincubation reaction was performed by assembling the reaction (poly(C) template, pGpG primer, [32P]GTP, BMS-791325) in buffer and initiating the reaction with NNSB. The preincubation reactions were performed by incubating NNSB with BMS-791325 in the presence of poly(C) template for 1, 5, or 24 h before initiating with the addition of pGpG primer and [32P]GTP. ND, not determined.

FIGURE 4. Time dependence of inhibition. A, dependence of the fractional velocity (v/v0) on the time of preincubation at varying concentrations of BMS-791325. Data were fit to an exponential dissociation equation, v/v0 = exp(−kobs1t) to derive kobs1 values. B, plot of kobs as a function of inhibitor concentration, generated by fitting data to a single-step binding model, kobs = k2(1 + IC50/app), to derive k2 and IC50. The value of k1 is derived from the equation, k1 = k2/k4, where Kd = Kd,app for the non-competitive inhibitor BMS-791325. Data indicate a single step slow binding inhibition mode. Error bars, S.D.
followed a double exponential equation (similar to the results and L30S enzyme; however, kinetic curves for the P495L variant curves were fit to a single-exponential equation for both WT enzymes could be measured using this method. Association


dissociation of BMS-791325 to/from WT, L30S, and P495L NS5B measured in real time (43, 44). Both association and dissociation of BMS-791325 to/from WT, L30S, and P495L NS5B enzymes could be measured using this method. Association curves were fit to a single-exponential equation for both WT and L30S enzyme; however, kinetic curves for the P495L variant followed a double exponential equation (similar to the results from stopped-flow experiments). The observed rate constant from the fast phase (k_{obs}) was used to define enzyme-inhibitor binding (Fig. 6). Values for k_{obs} were plotted as a function of inhibitor concentration. The data yielded hyperbolic curves for all three enzymes, indicating a two-step, slow binding mechanism (Scheme 1).

The forward rate constants to form the final tight complex (k_{f}) were similar for both WT and L30S (0.043 ± 0.001 and 0.035 ± 0.006 s⁻¹, respectively); however, the value for P495L (0.25 ± 0.01 s⁻¹) increased ~6-fold compared with WT (Table 4). The reverse rate constant for the second slow step (k_{r}) could not be calculated from curve fitting, probably due to the small value compared with the forward rate (k_{f}).

Dissociation of BMS-791325 from each enzyme variant was also measured and fit to a single-exponential decay equation (data not shown). The data showed that binding of BMS-791325 to NS5B is reversible. Residence times for WT and the L30S variant are 98 ± 23 and 167 ± 17 min, respectively. Dissociation of BMS-791325 from the P495L variant was much faster, with a residence time of 2 ± 0.6 min (Table 4).

The equilibrium dissociation constant (K_d) to form the initial complex is 151 ± 49 nM for WT enzyme. This value is similar for the L30S variant (115 ± 10 nM) and about 2-fold weaker for P495L (322 ± 100 nM).

**DISCUSSION**

BMS-791325 is the most potent thumb site 1 inhibitor of GT-1 NS5B polymerase to reach clinical trials (Tables 1 and 2) (45). *In vitro*, it inhibits replication, regardless of the mode of initiation (de novo, primed, copy-back) but has no impact on elongation (Fig. 2). The equal potency observed in systems that model *de novo* and primer-dependent replication and the failure to inhibit elongation support a model of inhibition that was
proposed on the basis of crystal structure studies with thumb site 1 inhibitors (17); the binding of BMS-791325 blocks the formation of active replication complexes, regardless of whether the complexes initiate de novo or, once formed, model elongation. Our experiments show that BMS-791325 is non-competitive with respect to both template and the nucleotide substrate, and the inhibition of NS5B activity by BMS-791325 is clearly time-dependent (Fig. 4 and Table 2). The limitations of the activity assays, with readouts that include signal from elongation and inhibitor. Leucine 30 is located in a loop (Δ1) of the fingers domain that interacts with a pocket in the thumb domain that contains the binding site for BMS-791325 (Fig. 1). The substitution of serine for leucine at position 30 (L30S) reduces the affinity of the Δ1 loop for the hydrophobic thumb pocket and weakens the finger-thumb interaction (42). The L30S variant has dramatically decreased polymerase activity, suggesting that the finger and thumb interaction is important for replication activity (41, 42). Substitutions at Leu-30 have not been observed after inhibitor selection in the replicon system, but characterization of the L30S variant has been reported (41, 42). Although the overall activity of L30S polymerase is reduced (~100-fold) compared with WT (42), inhibition of the variant by BMS-791325 (IC_{50} = 0.7 ± 0.2 nM) is equal to the inhibition of WT polymerase (IC_{50} = 0.7 ± 0.3 nM) when the inhibitor-NS5B interaction is allowed to reach equilibrium before polymerization is initiated (Table 2). The activity assays show that the NS5B-inhibitor complex reaches equilibrium faster with L30S compared with WT but that the variant has no impact on inhibitor potency. X-ray co-crystals show that the same finger loop is displaced by the binding of BMS-791325 in the thumb pocket (10). The structural data combined with our inhibition data suggested that the finger loop “competes” with BMS-791325 for binding to the thumb pocket, and thus, the L30S variant was used to probe the impact of the finger-thumb interaction on BMS-791325 binding kinetics.

Another variant (P495L) used to probe the interactions between NS5B and BMS-791325 is located in the thumb pocket, which is occupied by the Δ1 finger loop in the apo-protein structure and by BMS-791325 in co-crystal structures (10). The activity of the P495L variant is ~2-fold lower than WT and is highly resistant to BMS-791325 inhibition (EC_{50} >500 nM and IC_{50} = 431 ± 110 nM; Table 2) (10, 11). It was observed in replicons after selection in vitro and in clinical specimens of GT-1 patients treated with BMS-791325 (7, 11). The P495L and L30S variants were used in this study to confirm that the inhibition and binding observed with WT NS5B is specific and to probe the impact of the weakened interaction between the thumb and finger on the inhibitor-enzyme interactions.

Binding curves from stopped-flow studies indicated that the initial binding of BMS-791325 to NS5B is fast, reaching equi-

### TABLE 4

| Constant   | WT (μM) | L30S | P495L |
|------------|---------|------|-------|
| k_{on} (μM) | 151 ± 49 | 115 ± 10 | 322 ± 100 |
| k_{off} (s^{-1}) | 0.043 ± 0.001 | 0.035 ± 0.006 | 0.25 ± 0.01 |
| k_{cat} (s^{-1}) | (1.7 ± 0.4) × 10^{-4} | (1.0 ± 0.1) × 10^{-4} | (8.3 ± 2.5) × 10^{-3} |
| τ (min) | 98 ± 23 | 167 ± 17 | 2.0 ± 0.6 |

**FIGURE 6.** Binding of BMS-791325 to NS5B enzymes analyzed by biolayer interferometry (Octet Red). A–C, association binding curves of BMT-791325 at different concentrations to WT, L30S, or P495L NS5B enzyme, respectively. For WT (A) and the L30S variant (B) enzymes, each trace was fit to a single-exponential equation by nonlinear regression. For the P495L variant (C), each trace was fit to a double-exponential equation by nonlinear regression. The fast phase defined the rate of binding, whereas the slow phase corrected for a small shift in intensity. k_{on}, from the fast phase was used for the plot versus [I] for the P495L variant. D–F show that the pseudo-first order rate constants (k_{cat}) have a hyperbolic relationship with the inhibitor concentration for WT, L30S, and P495L NS5B enzyme, respectively. Error bars, S.D.

| WT | L30S | P495L |
|----|------|-------|
| Constant | 1/m | 1/m | 1/m |
| k_{on} (μM) | 151 ± 49 | 115 ± 10 | 322 ± 100 |
| k_{off} (s^{-1}) | 0.043 ± 0.001 | 0.035 ± 0.006 | 0.25 ± 0.01 |
| k_{cat} (s^{-1}) | (1.7 ± 0.4) × 10^{-4} | (1.0 ± 0.1) × 10^{-4} | (8.3 ± 2.5) × 10^{-3} |
| τ (min) | 98 ± 23 | 167 ± 17 | 2.0 ± 0.6 |

| A | B | C |
|---|---|---|
| D | E | F |
librium in seconds (Fig. 5). These results clearly deviated from the time-dependent inhibition experiments, which showed that more than 1 h of preincubation between enzyme and inhibitor is needed to reach steady-state inhibition of WT NS5B (Table 2). In addition, although a linear correlation between $k_{obs}$ and inhibitor concentration was detected for WT, L30S, and P495L enzymes, suggesting a one-step binding mechanism (Fig. 5 and Scheme 1), the on-rate ($k_1$) calculated for WT NS5B from stopped-flow data ($6.0 \pm 0.3 \times 10^6 \text{M}^{-1} \text{s}^{-1}$) is about 300 times faster than the on-rate calculated from activity data ($2.1 \pm 0.2 \times 10^5 \text{M}^{-1} \text{s}^{-1}$). The discrepancy in the on-rates suggested the existence of a slow step that was not detected by stopped flow.

In contrast to the results from stopped flow, results from Octet showed a much longer time to reach equilibrium for all three NS5B enzymes. In addition, plots of Octet showed a much longer time to reach equilibrium for all existence of a slow step that was not detected by stopped flow.

The inhibition of NS5B by BMS-791325 (Scheme 1, Table 2, and Octet (Fig. 6) supported a two-step slow binding mechanism for which measure the intrinsic fluorescence changes of BMS-791325 and NS5B polymerase. The stopped flow experiments, of the mechanism that characterize the interaction of BMS-791325, a Novel Non-nucleoside Inhibitor of HCV NS5B complementarity, the detection methods exposed different steps

104 M $f$ faster than the on-rate calculated from activity data ($6.0 \pm 0.3 \times 10^6 \text{M}^{-1} \text{s}^{-1}$), although the kinetics for the formation of final complex ($k_2$ and $k_3$) were unaffected by the finger loop substitution. The kinetic data suggest that a more exposed pocket is available for the initial binding of BMS-791325 to L30S.

The kinetic data are consistent with the observation that the preincubation time needed to reach maximum inhibition in the activity assay is less for L30S than for WT enzyme (Table 2). The data are also consistent with the observation that the L30S variant has a trypsin digest pattern identical to WT NS5B bound to a site 1 inhibitor (42). The on-rate for L30S is essentially diffusion-controlled. The faster on- and off-rates of the first binding step for L30S suggest that the opening and closing of the $\Delta{1}$ finger loop of WT NS5B is slower than the initial binding of BMS-791325 to the thumb domain. This difference in the initial on-rate supports the hypothesis that the finger loop “competes” with the inhibitor for binding. The difference in on-rates of BMS-791325 for WT and L30S provides experimental evidence, in an assay environment conducive to polymerase replication, for a dynamic interaction between the fingers and thumb that can, in the case of WT, reduce the rate of inhibitor association below the rate of diffusion. Despite the fact that the NS5B apoenzyme is observed in a closed conformation in crystal structures, a dynamic interaction between fingers and thumb has been hypothesized based on single-molecule reconstruction transitions observed with FRET for DNA polymerases and the observation that NS5B can utilize a circular template (49–53). The dynamic interaction is also supported by rapid changes observed in binary NS5B-template complexes detected with surface-immobilized FRET-labeled RNA templates (54).

The L30S substitution enhances the association and dissociation of NS5B with BMS-791325; however, L30S has little impact on the formation of the final tight complex (Table 5). The slow isomerization step does not appear to be affected directly by the weaker finger-thumb interaction. The inability of L30S to substantially impact the affinity of the thumb site 1 binding pocket for BMS-791325 is not surprising in light of structural studies showing that displacement of the finger loop by thumb site 1 inhibitors causes no change in the structure of the thumb domain itself (17).

A structural interpretation consistent with these observations is depicted in Fig. 7. Distinct protein conformations are invoked for $E + I$, $E_I$, and $E_{1}^{*}$ (Scheme 1). The NS5B “apo” conformation, in which the $\Delta{1}$ loop is bound to the thumb

### Table 5: Binding constants for BMS-791325; two-step mechanism

| Constant | WT | L30S | P495L |
|----------|----|------|-------|
| $k_1$ ($10^6 \text{M}^{-1} \text{s}^{-1}$) | 6.0 ± 0.3 | 4.4 ± 0.1 | 1.2 ± 0.1 |
| $k_2$ ($10^6 \text{M}^{-1} \text{s}^{-1}$) | 0.90 ± 0.29 | 5.1 ± 0.4 | 0.40 ± 0.12 |
| $K_d$ (nM) | 151 ± 49 | 115 ± 10 | 322 ± 100 |
| $k_1$ | 0.043 ± 0.001 | 0.035 ± 0.006 | 0.25 ± 0.01 |
| $k_2$ ($10^4 \text{s}^{-1}$) | 1.8 ± 0.1 | 1.0 ± 0.1 | 1.4 ± 0.4 |
| $K_d$ (nM) | 0.62 ± 0.18 | 0.34 ± 0.03 | 17 ± 5 |
| $k_3$ ($10^5 \text{s}^{-1}$) | 1.7 ± 0.4 | 1.0 ± 0.1 | 8.3 ± 2.5 |
| $K_d$ (nM) | 98 ± 23 | 167 ± 17 | 2.0 ± 0.6 |

*a Data measured from stopped flow. 
*b Data measured from Octet.
domain and Leu-30 occupies thumb site 1, corresponds to the unliganded (E + 1) state (Fig. 7A). It is plausible that L30S mutant NS5B would have a somewhat different conformation, where the serine side chain is solvent-exposed and not buried in the hydrophobic cavity of thumb site 1. The E1 state is poised to involve a protein conformation where the Δ1 loop has dissociated partially from the thumb domain, allowing BMS-791325 to bind (Fig. 7B). The data presented earlier suggest that this is a rapid process and is essentially diffusion-controlled for the L30S mutant protein. There is structural precedent for multiple conformations of the Δ1 loop “fingertip” binding to thumb site 1 without complete dissociation of the loop from the thumb domain in GT-2a NS5B (15), although these would not be completely representative of the NS5B conformation suggested here for GT-1, because thumb site 1 is still partially occluded in the GT-2a structures. Slow conformational rearrangement could result in a third protein conformation (E1I, Fig. 7C) that may be similar to the structure observed in the NS5B GT-1b-BMS-791325-palm site inhibitor ternary complex x-ray structure (10).

The binding profile of P495L is significantly different from that of L30S. The kinetic interaction of the initial complex formation is similar for P495L and WT; however, formation and dissociation of the final complex, the slow isomerization step (k3 and k4), is significantly faster than for WT (Table 5). Formation of the final complex, k3 (0.25 ± 0.01 s⁻¹), increases about 6-fold compared with WT NS5B (0.043 ± 0.001 s⁻¹), and the reverse rate, k4, increases 78-fold (1.4 ± 0.4 × 10⁻² versus 1.8 ± 0.1 × 10⁻⁴ s⁻¹). It is very clear that the formation of the final complex is thermodynamically less favorable for P495L, delivering a much shorter residence time (2.0 ± 0.6 min) than WT (98 ± 23 min) for BMS-791325. The thumb site 1 binding pocket occupied by BMS-791325 is largely hydrophobic in nature and composed of residues Leu-392, Ala-393, Ala-396, Thr-399, Ile-424, Leu-425, His-428, Phe-429, Leu-492, Gly-493, Val-494, Pro-495, Trp-500, and Arg-503. Pro-495 makes multiple contacts with the indole phenyl ring and the sulfamide moiety of BMS-791325, and substitution of leucine at 495 is predicted to reduce binding affinity (10). The drastically shortened residence time with P495L translates into the decreased potency of BMS-791325 against this resistance variant and the ability of the variant to escape inhibition in vivo.

Thus, the mutation that reduced the affinity of the thumb-finger interaction (L30S) resulted in increased association kinetics but did not affect final tight complex formation. In contrast, mutations that make contacts with the inhibitor itself (P495L) did not affect the kinetics of initial enzyme-inhibitor association but did affect the overall affinity of the final complex.

Binding results for BMS-791325 are consistent with results for another thumb site 1 inhibitor studied. The binding of TMC647055 to NS5B WT and P495L was studied by SPR (23). Although this compound is less potent than BMS-791325 (Kd of 4 nM against WT compared with 0.62 ± 0.18 nM for BMS-791325), it showed a residence time of 188 min against WT and a residence time of 0.7 min against P495L. The surface plasmon resonance measurements do not indicate whether this compound has a one- or two-step binding mechanism.

Differences between NS5B WT and L30S with respect to 1) kinetic interactions with BMS-791325 in binding studies and 2) preincubation behavior of BMS-791325 in activity assays provide insight into the formation of active replication complexes. For WT NS5B, the interaction between fingers and thumb, in an assay environment that supports the initiation of RNA replication, is dynamic and slower than the rate of diffusion. The dynamic nature of the interaction supports previously pub-
lished studies demonstrating that RNA binding to NS5B enhances an open conformation similar to the conformation of the L30S variant and similar to NS5B bound to a thumb site 1 inhibitor (42); the template-bound open conformation, in the absence of BMS-791325, closes with the addition of NTP or pGpG dinucleotide. A dynamic finger-thumb interaction would facilitate the association of substrates with NS5B to form productive replication complexes. BMS-791325 blocks the finger-thumb association, inhibiting the initiation of replication. Experiments using surface-immobilized FRET-labeled RNA to detect NS5B-template interactions indicated that a thumb site I analog reduces binding and sliding of NS5B along the template (54). The ability of BMS-791325 to block the initiation of RNA replication, regardless of whether synthesis is primed or de novo, indicates that the finger thumb interaction is essential in both cases. The fact that BMS-791325 is unable to block elongation suggests that the interaction between the fingers and thumb that occurs during the formation of active replication complexes is fundamentally different during elongation. It is possible that the fingers domain is less flexible during elongation and blocks the binding of BMS-791325 or that the shape of the thumb binding pocket is different and cannot bind inhibitor (17, 38). Experiments with non-nucleoside inhibitors that bind to different polymerase structural domains are ongoing to further investigate the impact of the finger-thumb interaction on HCV NS5B replication.

Acknowledgments—We thank our colleagues Dr. Robert Fridell, Dike Quí, and Dr. Julie Lemm (Virology) for constructs used to prepare isolated NS5B variants; Dr. Mark Witmer, Jeffrey Tredup, and Changhong Wan for providing some of the isolated proteins; and Dr. David Langley for insightful comments.

REFERENCES

1. World Health Organization (2012) Prevention and Control of Viral Hepatitis Infection: Framework for Global Action. World Health Organization, Geneva
2. Ditah, I., Ditah, F., Devaki, P., Ewelukwa, O., Ditah, C., Njie, B., Luma, H. N., and Charlton, M. (2014) The changing epidemiology of hepatitis C virus infection in the United States: National Health and Nutrition Examination Survey 2001 through 2010. J. Hepatol. 60, 691–698
3. El-Serag, H. B. (2007) Epidemiology of hepatocellular carcinoma in U.S.A. Hepatol. Res. 37, S88–S94
4. Averhoff, F. M., Glass, N., and Holtzman, D. (2012) Global burden of hepatitis C: considerations for healthcare providers in the United States. Clin. Infect. Dis. 55, S10–S15
5. Ly, K. N., Xing, J., Klevens, R. M., Jiles, R. B., Ward, J. W., and Holmberg, S. D. (2012) The increasing burden of mortality from viral hepatitis in the United States between 1999 and 2007. Ann. Intern. Med. 156, 271–278
6. Ward, J. W., Rein, D. B., and Smith, B. D. (2012) Data to guide the "test and treat era" of hepatitis C virus infection: Framework for Global Action
7. Biswal, B. K., Cherney, M. M., Wang, M., Chan, L., Yannopoulos, C. G., Bilimoria, D., Nicolas, O., Bedard, J., and James, M. N. (2005) Crystal Structures of the RNA-dependent RNA polymerase genotype 2a of hepatitis C virus reveal two conformations and suggest mechanisms of inhibition by non-nucleoside inhibitors. J. Biol. Chem. 280, 18202–18210
8. McKercher, G., Beaulieu, P. L., Lamarre, D., LaPlante, S., Lefebvre, S., Pellerin, C., Thauvette, L., and Kokol, G. (2004) Binding site characterization and resistance to a class of non-nucleoside inhibitors of the hepatitis C virus 5’-triphosphate. Antimicrob. Agents Chemother. 48, 3496–3505
9. Evans, G. N., Xing, J., Klevens, R. M., Jiles, R. B., Ward, J. W., and Holmberg, S. D. (2012) The increasing burden of mortality from viral hepatitis in the United States between 1999 and 2007. Ann. Intern. Med. 156, 271–278
10. Ward, J. W., Rein, D. B., and Smith, B. D. (2012) Data to guide the "test and treat era" of hepatitis C virus infection: Framework for Global Action
11. Erhardt, A., Deterding, K., Benhamou, Y., Reiser, M., Forns, X., Pol, S., Calleja, J. L., Ross, S., Spangenberg, H. C., Garcia-Samaniego, J., Fuchs, M., Enríquez, J., Wiegand, J., Stern, J., Wu, K., Kukol, G., Marquis, M., Beau lieu, P., Nehmiz, G., Steffen, J., and PILB 1941 Study Group (2009) Safety, pharmacokinetics and antiviral effect of BILB 1941, a novel hepatitis C virus RNA polymerase inhibitor after 5 days oral treatment. Antivir. Ther. 14, 23–32
12. Gentles, R. G., Ding, M., Bender, J. A., Bergstrom, C. P., Grant-Young, K., Hewawasam, P., Hudyma, T., Martin, S., Nickel, A., Regueiro-Ren, A., Tu, Y., Yang, Z., Yeung, K.S., Zheng, X., Chao, S., Sun, J., Beno, B. R., Camac, D. M., Chang, C. H., Gao, M., Morin, P. E., Sheriff, S., Tredup, J., W., Witmer, M. R., Xie, D., Hanumegowda, U., Knipe, J., Masure, K., Santone, K. S., Parker, D. D., Zhuo, X., Lemm, J., Liu, M., Pelosi, L., Rigat, K., Voss, S., Wang, Y., Wang, Y. K., Colonnio, R. J., Gao, M., Roberts, S. B., Gao, Q., Ng, A., Meanwell, N. A., and Kadow, J. F. (2014) Discovery and preclinical characterization of the cyclopropylindolobenzazepine BMS-791325, a potent allosteric inhibitor of the hepatitis C virus NS5B polymerase. J. Med. Chem. 57, 1855–1879
13. Ago, H., Adachi, T., Yoshida, A., Yamamoto, M., Habuka, N., Yatsunami, K., and Miyano, M. (1999) Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Structure 7, 1417–1426
14. Lesburg, C. A., Cable, M. B., Ferrari, E., Hong, Z., Mannarino, A. F., and Weber, P. C. (1999) Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. Nat. Struct. Biol. 6, 937–943
15. Di Marco, S., Volpari, C., Tomei, L., Altmuhr, S., Harper, S., Nijars, F., Koch, U., Rowley, M., De Francesco, R., Migliaccio, G., and Carli, A. (2005) Interdomain communication in hepatitis C virus polymerase abolished by small molecule inhibitors bound to a novel allosteric site. J. Biol. Chem. 280, 29765–29770
16. Bressanelli, S., Tomei, L., Roussel, A., Incitti, I., Vitale, R. L., Mathieu, M., De Francesco, R., and Rey, F. A. (1999) Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Proc. Natl. Acad. Sci. U.S.A. 96, 13034–13039
17. Tatum, H., Thuluvath, P., Lawitz, E., Martorell, C., Cohen, S. M., Rustgi, V., Ravendhran, N., Ghalib, R., Hansson, J., Zamparo, J., Zhao, J., Treitel, M., and Hughes, E. (2013) Safety and efficacy of BMS-791325, a non-nucleoside NS5B polymerase inhibitor, combined with peginterferon α-2a and ribavirin in treatment-naïve patients infected with hepatitis C virus genotype 1. J. Hepatol. 58, Suppl. 1, S182. Poster 1126
indole-diamide inhibitors of the hepatitis C virus NS5B. J. Am. Chem. Soc. 132, 15204–15212
51. Ranjith-Kumar, C. T., and Kao, C. C. (2006) Recombinant viral RdRps can initiate RNA synthesis from circular template. RNA 12, 303–312
52. Santoso, Y., Joyce, C. M., Potapova, O., Le Reste, L., Hohlbein, J., Torella, J. P., Grindley, N. D. F., and Kapanidis, A. N. (2010) Conformational transitions in DNA polymerase I revealed by single-molecule FRET. Proc. Natl. Acad. Sci. U.S.A. 107, 715–720
53. Rothwell, P. J., and Waksman, G. (2007) A pre-equilibrium before nucleotide binding limits fingers subdomain closure by Klentaq. J. Biol. Chem. 282, 28884–28892
54. Karam, P., Powdrill, M. H., Liu, H.-W., Vasquez, C., Mah, W., Bernatchez, J., Götte, M., and Cosa, G. (2014) Dynamics of hepatitis C virus (HCV) RNA-dependent RNA polymerase NS5B in complex with RNA. J. Biol. Chem. 289, 14399–14411