Mechanisms of Activity and Inhibition of the Hepatitis C Virus RNA-dependent RNA Polymerase*\(^3\)

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The RNA-dependent RNA polymerase NS5B is a key enzyme of the replication of hepatitis C virus (HCV) and a major therapeutic target. Applying a novel continuous assay with highly purified protein and a fluorescent RNA-template we provide for the first time a comprehensive mechanistic description of the enzymatic reaction. Using fluorescence spectroscopy, the kinetics of NS5B was confirmed to consist of two half-reactions, namely substrate binding and turnover. Determining the binding constants of the substrates and the rate constants of individual reaction steps, NS5B was shown to bind the template single-stranded RNA with high affinity (nanomolar range) and in a stepwise process that reflects the substrate positioning. As demonstrated by CD, NTP(s) binding caused a tertiary structural change of the enzyme into an active conformation. The second half-reaction was dissected into a sequential polymerization and a subsequent, rate-limiting product release reaction. Taking advantage of these tools, we analyzed the mechanism of action of the NS5B inhibitor HCV-796, which was shown to interfere with the formation of double-stranded RNA by blocking the second half-reaction.

Infections with hepatitis C virus (HCV)\(^3\) represent a major health problem affecting ~2% of the world’s population (1). Most patients remain chronically infected and 15–20% eventually develop liver cirrhosis and hepatocellular carcinoma. Vaccination is not available, and current therapies are associated with limited efficacy and side effects (2, 3). A central issue for developing alternative antiviral strategies entails gaining a better knowledge of the molecular mechanisms governing the HCV life cycle. Among the HCV-encoded proteins, NS5B was characterized as being the viral RNA-dependent RNA polymerase (RdRp), i.e. a key enzyme of the viral RNA replication process and an attractive drug target (4–6). Like other RNA polymerases, NS5B is capable of initiating RNA synthesis in the presence of a primer as well as de novo (7–12). The available structures of NS5B display the typical right-hand architecture of polymerases consisting of “palm,” “thumb,” and “finger” domains (13–15). The NTP substrates are assumed to enter via a defined tunnel conformation of the protein. Modeling studies suggest that the exit of the double-stranded primer/template is blocked by a β-hairpin or flap (8, 13), indicating that NS5B undergoes major conformational changes to accommodate the double-stranded RNA product (16).

Among a huge variety of yet characterized nucleoside and non-nucleoside inhibitors (NNI), the benzofurane derivative NNI HCV-796 was demonstrated to yield significant antiviral effects in mice with chimeric human livers and in patients infected with HCV (17). HCV-796 binds to a hydrophobic binding pocket at the “palm” domain of NS5B (18–21); however, its mode of inhibition remains to be defined.

Although NS5B has been studied in the absence and presence of inhibitors, a firm biophysical characterization of the enzyme is lacking. To address this, we have purified the protein with a high quality and established a new assay system, which has allowed a quantitative characterization of binding and substrate turnover by rapid transient kinetic methods. This system also enabled us to unravel the mechanism of action of HCV-796.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—The gene coding for NS5BΔ21 (HCV1b BK, including a deletion of 21 amino acids at the C terminus) was cloned into the pET SUMO vector and expressed in the *Escherichia coli* strain BL21(DE3) star. Biomass production was carried out using fermentation. Briefly, cells were grown at 30 °C in 6 liters of medium (50 g/liter yeast extract, 0.06 mM KHPO₄, 6 mM MgSO₄, 0.03 mM glucose, 0.01 mM NH₄Cl) with glycerol as carbon source (300 g/liter yeast extract, 3 mM glycerol). Gene expression was induced at A₆00 = 50 by adding 0.8 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested after 4.5 h of induction. After resuspension the cells were lysed using a French press and centrifuged at 4 °C and 4.84 ω² (rotor type 45 Ti, Beckman Coulter) to remove unbroken cells and debris. Ammonium sulfate was added to the supernatant (0.5 g/ml), the resulting precipitate collected by centrifugation as above and re-dissolved. Soluble proteins were loaded on a nickel-nitrioltriacetic acid column, bound proteins were eluted by applying an imidazole gradient. After cleaving off the

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\(^3\)The abbreviations used are: HCV, hepatitis C virus; dsRNA, double-stranded RNA; HCV-RdRp, RNA-dependent RNA polymerase of HCV; NNI, non-nucleoside inhibitor; NS5B, non-structural protein 5B; NS5BΔ21, NS5B with C-terminal deletion of 21 amino acids; NTP(s), nucleotide(s) (mixture containing ATP, GTP, CTP, and UTP at equimolar concentration); ssRNA, single-stranded RNA.
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SUMO-protein by the SUMO-protease, a poly-(U)-Sepharose affinity chromatography step and an additional nickel-nitrilotriacetic acid chromatography step were performed, yielding untagged NNS5B21 with the authentic N terminus. Purified NNS5B21 was dialyzed against 50 mM HEPEs/NaOH, 20% (v/v) glycerol, 6.5 mM MgCl$_2$, 2 mM tris(2-carboxyethyl)phosphine, pH 7.5 (referred to as assay buffer) and stored at $-80^\circ$C. Protein concentration was determined by measuring the absorbance at 280 nm using the extinction coefficient 83770 M$^{-1}$ cm$^{-1}$.

Nucleotides and Oligonucleotides—Lyophilized NTP(s) at a purity > 95% were purchased from Sigma-Aldrich and dissolved in 50 mM HEPEs/NaOH, 20% (v/v) glycerol, 6.5 mM MgCl$_2$, pH 7.5. Concentrations were determined from the absorbance at 260 nm using the following extinction coefficients: $\varepsilon_{260}$ (ATP) = 15400 M$^{-1}$ cm$^{-1}$, $\varepsilon_{260}$ (GTP) = 13,700 M$^{-1}$ cm$^{-1}$, $\varepsilon_{260}$ (CTP) = 9,000 M$^{-1}$ cm$^{-1}$, and $\varepsilon_{260}$ (UTP) = 10,000 M$^{-1}$ cm$^{-1}$.

The 5'-FAM fluorescently labeled template ssRNA with the sequence 5'-CUAGAGUCUCUGCUCG-3' was purchased from IBA (Göttingen, Germany). The concentration was determined from the absorbance at 260 nm using the extinction coefficient $\varepsilon_{260}$ = 148,600 M$^{-1}$ cm$^{-1}$.

Determination of the $K_D$ Value for the Binary Complex Composed of NNS5B21 and Template ssRNA by Equilibrium Fluorescence Measurements—NNS5B21 (HCV1b BK) was titrated to 66 nM 5'-FAM-labeled template ssRNA (16-mer) in assay buffer at 22.5 °C. Fluorescence was monitored on a FluoroMax-4 spectrofluorometer (Jobin Yvon, France). After attaining equilibrium and smoothed adaptively with both convolution width and deviation noise set to 5 (Spectra Manager I, Basco). No significant spectral contributions of the buffer and nucleotides were observed. The change in the amplitude of the CD signal of the polymerase at 240 nm was directly plotted against the concentration of nucleotides or inhibitor and fitted to a sigmoidal binding behavior yielding $S_{0.5}$ and $K_s$ values of nucleotides and HCV-796, as well as the cooperativity in the absence of template ssRNA.

Fast Kinetics of Protein-RNA Complex Formation and Dissociation—The kinetics of both the association of NNS5B21 (HCV1b BK) and the 5'-FAM-labeled template ssRNA (16-mer) and the dissociation of a preformed polymerase-template ssRNA complex on dilution were measured using a stopped-flow machine (SX.20 MV, Applied Photophysics) equipped with a fluorescence detection unit. The excitation of the fluorescent probe was set to 491 nm. Fluorescence emission was monitored using a cut-off filter of >515 nm. Slit widths were set to 2.2 nm each. All experiments were carried out in assay buffer at 22.5 °C. Traces were fitted to a quadruple-exponential first order reaction (Equation 3) with the program KaleidaGraph™, yielding the respective observed rate constants. For association measurements, 78 nM NNS5B21 (HCV1b BK) was rapidly mixed with an equal volume of increasing amounts of the 5'-FAM-labeled template ssRNA. Dissociation rate constants were measured by a 1:2 dilution (mixing ratio 1 + 1) of 78 nM protein and 210 nM 5'-FAM-labeled template ssRNA, preincubated in assay buffer to allow complex formation, where $\Delta F$ is the total change of the relative fluorescence amplitude, $v$, $x$, $y$, and $z$ are signal amplitudes of the respective phases, $k_{r1}$, $k_{r2}$, $k_{s1}$, and $k_{s2}$ are the observed first order rate constants of the respective phases, $t$ is time (seconds), and $n$ is the relative fluorescence intensity at the end point of the reaction (offset).

Fast Kinetics of RNA-dependent RNA Polymerization and Product Release Reaction—To measure the incorporation of nucleotides and subsequent release of the RNA products by the HCV-RdRp, 78 nM NNS5B21 (HCV1b BK) and 210 nM 5'-FAM-labeled template ssRNA (16-mer) were preincubated in assay buffer to allow the binary complex to form. Polymerase reactions were started by the addition and rapid mixing of an equal volume of increasing concentrations of NTP(s) in the stopped-flow machine. When investigating the effect of the HCV-796 on the RdRp reaction, the inhibitor was first preincubated to the
preformed protein-template ssRNA complex. Afterward the reaction was started by adding 3 mM NTP(s). Because the inhibitor was dissolved in DMSO, all experiments were carried out in 1.1% (v/v) DMSO. Kinetics were monitored as fluorescence change on excitation of the FAM probe at 491 nm using a cut-off filter of >515 nm for emission. Slit widths were set to 2.2 nm each. Traces were fitted to a quadruple-exponential first order reaction according to Equation 3 considering the positive amplitude sign. Four rate constants were obtained. For data investigation using the program KaleidaGraph™ the rate constants were plotted either against the concentration of nucleotides or the inhibitor. All experiments were carried out at 22.5 °C.

**HCV-NSSB Polymerase Assay**—The assay to determine HCV-RdRp activity was performed in a total volume of 40 μL containing 0.04 μM NSSBΔ21 (HCV1b BK), 3 mM each of ATP, GTP, and UTP, and 0.0825 μM of [α-32P]CTP (3000 Ci mmol⁻¹, Hartmann Analytic GmbH, Braunschweig, Germany) and 0.2 μM of 5’FAM-labeled template ssRNA in the assay buffer established. HCV-796 at concentrations of 5 μM and 10 μM was added to the sample in the inhibition test. The samples were incubated for 2 h at 22 °C. Recovery of the radioactively labeled RNA was performed by using phenol/chloroform extraction followed by ethanol precipitation. RNA was re-suspended in formamide buffer containing 50% formamide, 5 mM EDTA, and dye marker (0.05%), pH 8.0, and separated on a denaturing polyacrylamide gel (6.5 M urea, 12% polyacrylamide in 89 mM Tris base, 89 mM boric acid, 2 mM EDTA). Autoradiography analysis was performed by phosphorimaging.

**RESULTS**

**Preparation of HCV-NSSBΔ21**—Based on earlier reports, we used in the current study the NSSBΔ21 variant lacking the C-terminal amphipathic helix that is involved in membrane anchoring but is not essential for RdRp activity (22). For quantitative mechanistic studies it was crucial to generate the protein in a well defined association state and devoid of any contaminating nucleic acids or nucleotides. Accordingly, a new optimized expression and purification scheme was established purifying NSSBΔ21 to homogeneity (supplemental Fig. S1; see “Experimental Procedures”).

The absorbance spectrum of the purified recombinant NSSBΔ21 displayed a ratio of absorbance 280 nm/260 nm of ~3, clearly indicating the absence of any contaminating nucleic acids or nucleotides (supplemental Fig. S2). Analytical ultracentrifugation of NSSBΔ21 demonstrated its initial monomer state in the assay buffer used for all measurements (supplemental Fig. S3).

**Binding the Template ssRNA to NSSBΔ21**—Development of the RNA polymerase activity of NSSB requires binding of the two substrates, namely the template ssRNA and nucleotides. To characterize enzyme-template ssRNA binding and polymerase activity, we established a novel assay system applying 5’FAM fluorescently labeled ssRNA oligonucleotides as templates. Importantly, all tested RNAs enabled effective binding and de novo initiation of RNA synthesis. That is, we found no significant differences when testing RNAs corresponding to the 3’-end of the HCV negative-strand intermediate, which was utilized as a preferred substrate of the polymerase (18), and a randomly composed 16-nucleotide RNA that was primarily used in this study. The affinity of NSSBΔ21 to fluorescently labeled substrate polymers was determined by monitoring the FAM fluorescence quenching upon binding to the protein (Fig. 1A). Thus, data analysis assuming a single binding site yielded Kᵦ values in the nanomolar range; for example, with the RNA 16-mer, it was determined to be 0.01 μM (Table 1). The fact that a low Kᵦ value was measured irrespective of whether the template contained virus-specific sequence elements demonstrated that binding of NSSBΔ21 to the RNA occurred at high affinity without template specificity. Apparent discrepancies between these results with those in earlier reports that measured different Kᵦ values of NSSBΔ21 and short template ssRNA might be explained by differences in the mode of protein preparation as well as interfering ionic effects. To investigate further these discrepancies, the Kᵦ value was measured at increasing concentrations of NaCl in the assay buffer. Starting...
we interpreted the observed changes of NS5B structural properties of proteins are reflected by near-UV CD, decreased at 240 nm and at in the absorbance range of the aromatic amino acids. Upon CD spectra of NS5B binding of the NTP(s) to the enzyme (see “Discussion”).

Binding parameters of NTP(s) and inhibitor using an enzyme concentration of 17.5 μM. The corresponding concentrations of the NTP(s) and of HCV-796 are given in the insets of the figures. The arrow in B indicates the bathochromic shift of the local negative maximum. The experiments were performed as described under “Experimental Procedures.”

Binding the Nucleotides to NS5BΔ21—Nucleotides are the other substrate of NS5B necessary to perform the polymerization reaction. Analyzing the NTP(s) binding by CD, near-UV CD spectra of NS5BΔ21 displayed a characteristic positive band in the absorbance range of the aromatic amino acids. Upon binding the NTP(s), the dichroic intensity of the protein decreased at 240 nm and at ~260 nm (Fig. 2A). Because tertiary structural properties of proteins are reflected by near-UV CD, we interpreted the observed changes of NS5BΔ21 upon NTP binding as conformational changes in the protein. The change in dichroic signals on titration of NTP(s) to the enzyme in the absence of template ssRNA revealed the affinity of the respective nucleotides as being in the micromolar range (Fig. 3 and Table 1). It should be noted that the data displayed a sigmoidal binding behavior reflecting a positive cooperativity throughout binding of the NTP(s) to the enzyme (see “Discussion”).

The Kinetics of NS5B Comprises Two Half-reactions—The overall enzymatic reaction of NS5B proceeds as a two-substrate reaction that involves binding of the template ssRNA and the NTP(s) followed by an interaction of the two bound substrates. Substrate turnover then results in the formation of dsRNA and inorganic diphosphate as products.

In contrast to classic discontinuous assays that measure incorporation of radioactively labeled NTP(s) into dsRNA (4), here we used a continuous fluorometric assay, which enabled detailed mechanistic studies of the polymerase. Binding of template ssRNA to NS5BΔ21 led to a fluorescence decrease (quenching), whereas release of either ssRNA and/or dsRNA caused a fluorescence increase (de-quenching) (Fig. 4).

As equilibrium measurements could not be performed with the ternary complex consisting of NS5BΔ21 and the two substrates, kinetic analysis was used to determine the $K_m$ value of NTP(s). Incubation of the preformed enzyme-template ssRNA complex with nucleotides resulted in an increase of the fluoros-
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Kinetic parameters of NS5BΔ21-polymer binding and release

The rate constants listed and indicated with a prime represent observed rate constants (according to Scheme 1) determined by fitting progress curves of quenching and de-quenching the FAM fluorescence in NS5BΔ21 reactions according to a quadruple-exponential first order reaction, respectively. The reactions were initiated by a rapid 1 + 1 mixing of the reactants and stopped-flow measurements performed as described under “Experimental Procedures.” Errors of the kinetic measurements were in the range of 10%.

| Experiment                                      | $k_{+1}$ | $k_{-1}$ | $k_{+2}$ | $k_{-2}$ | $k_{+3}$ | $k_{-3}$ | $k_{+4}$ | $k_{-4}$ |
|------------------------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|
| NS5BΔ21 + 5’FAM-RNA                             | 22        | 8.8      | 0.59     | 0.69     | 0.05     | 0.08     | 0.0022   | 0.0044   |
| NS5BΔ21 + 5’FAM-RNA + NTP(s)                   | 3.7       | 37       | 0.37     | 0.036    | ND       | 0.036    | ND       | ND       |
| NS5BΔ21 + 5’FAM-RNA + HCV-796 + NTP(s)         | ND       | 19       | ND       | 2.3      | 0.45     | ND       | ND       | 0.044    |

$^a$ The reaction of template binding was initiated by mixing NS5BΔ21 and 5’FAM-RNA at final concentrations of 0.04 μM enzyme and 0.05 μM to 1.53 μM substrate-polymer. Observed rate constants with a positive sign indicate binding of the substrate-polymer (template ssRNA) and formation of the binary complex at saturating conditions of the reactants occurring in the first half-reaction. Conversely, observed rate constants with a negative sign indicate the release reaction of the substrate-polymer (template ssRNA) from the enzyme initiated by 1 + 1 dilution of a preformed binary complex composed of 0.08 μM NS5BΔ21 and 0.21 μM 5’FAM-RNA.

$^b$ The product formation (dsRNA) was initiated by mixing the preformed binary complex, composed of 0.04 μM NS5BΔ21 and 0.11 μM 5’FAM-RNA, with NTP(s) at saturating concentrations, respectively. Observed rate constants with a negative sign indicate the release reaction of the substrate-polymer (dsRNA) from the ternary complex as a result of nucleotide turnover occurring in the second half-reaction.

$^c$ Template ssRNA release was initiated by mixing the preformed complex, composed of 0.04 μM NS5BΔ21, 0.11 μM 5’FAM-RNA, and HCV-796 in a concentration range up to 55 μM, with NTP(s) (3 mM of each nucleotide), respectively.

$^d$ ND, not determined.
Inhibitor HCV-796: Mechanism of Action on Substrate Turnover—Taking advantage of the tools established here, we wanted to examine next the action of the potent NNI inhibitor HCV-796 to understand whether this compound affects the binding kinetics of the RNA-template and/or the release kinetics of the product during NTP turnover. First, by titrating the inhibitor to NS5B/H900421, we determined an IC₅₀ value of 0.03 mM (Fig. 3 and Table 1). No spectral contribution of HCV-796 itself in the CD analyses was detected. Performing binding assays with template ssRNA and NS5B/H900421 at saturating concentrations of HCV-796 we found that the compound did not interfere with template binding in the absence of NTP(s) (Table 1).

For kinetic measurements of product formation and release, we applied a preformed complex composed of NS5BΔ21, template ssRNA, and varying concentrations of HCV-796. By adding NTP(s) at saturating concentrations, again four phases of release were monitored, and, at increasing inhibitor concentrations, all phases were accelerated in a hyperbolic manner (Fig. 5B and Table 2). It is important to note that, at saturating concentrations of HCV-796 and NTP(s), the discontinuous radioactive assay revealed no dsRNA formation (Fig. 6). Thus, acceleration of the observed phases was concluded to reflect an augmented template ssRNA release. Fitting the rate constants in dependence of the inhibitor concentration yielded a $K_i$ value of $7 \mu M$ for the inhibitor. This result was congruent with data obtained when testing the inhibitor in the radioactive discontinuous assay performed at concentrations of 5 $\mu M$ and 10 $\mu M$, respectively (Fig. 6). Hence, it can be stated that HCV-796 interferes with dsRNA formation by blocking the second half-reaction. Importantly, no binding of the template ssRNA to NS5BΔ21 was measured when preincubated with HCV-796 and NTP(s) at saturating concentrations.

DISCUSSION

Mechanistic Aspects of NSSB Action—The main aim of this work was to characterize the HCV-RdRp in terms of substrate binding, turnover, and product release. As with other polymerases (24–27), the entire enzymatic reaction can be characterized as a two-substrate reaction, namely the incorporation of...
nucleotides to a substrate-polymer on the basis of complementary base pairing. Using a fluorescently labeled template ssRNA and monitoring the course (kinetics) of the enzymatic reaction in a continuous assay and by CD spectroscopy we demonstrated that the template ssRNA and the NTP(s) are capable of binding independently to the enzyme. Importantly, the binding constants for both substrates turned out to be rather different. The $K_D$ values for the applied template ssRNAs were in the nanomolar range, whereas the $K_D$ values of the respective NTP(s) turned out to be in the micromolar range (Table 1). Interestingly, the $K_m$ value of the NTPs indicated that nucleotide binding improved as a result of template ssRNA binding during the course of the entire enzymatic reaction (Table 1). These data are reasonable in view of the physiological situation of the cell where nucleotides are present at saturating concentrations. We therefore concluded that substrate turnover proceeds according to a two-substrate reaction composed of two half-reactions. The binding reaction of both substrates covers the first half-reaction of the overall sequential reaction mechanism (formation of the ternary complex).

Equilibrium binding of NTP(s) results in conformational changes of the enzyme. This became apparent in far- and near-UV CD by measuring chirality changes (Fig. 3). By fitting the observed sigmoidal binding behavior, we obtained Hill coefficients indicating a positive cooperativity of nucleotide binding. One explanation for this interesting observation is the existence of an additional binding site for nucleotides to NS5B. Such a scenario has been discussed previously in the context of an allosteric regulation or additional docking site of NTP(s) existence of an additional binding site for nucleotides to NS5B. Importantly, the binding of GTP as well as of other nucleotides to HCV-NS5B revealed a Hill coefficient of $\sim 2$. Thus, association of a nucleotide to the NTP site is accompanied by nucleotide binding to the substrate-polymer site.

Interestingly, as measured by phased fluorescence quenching curves (Fig. 4), attachment and incorporation of the template ssRNA to the active site of NS5B were found to proceed in a stepwise manner. We interpreted this observation as a positioning process of the template ssRNA within the active site that is trapped via several intermediates. The same behavior was observed in the release reaction of the template where we measured a phased dye fluorescence de-quenching (Scheme 1).

Binding of the two substrates by NS5BΔ21 in the first half-reaction occurs in a random manner (random bi-bi mechanism). Thus, considering again the difference in the $K_D$ values of the template ssRNA and NTP(s) by some orders of magnitude, a fairly ordered mechanism is conceivable. With the ternary complex as a starting point, the second half-reaction then covers the formation and release of dsRNA. Along this line, the complex kinetics involving at least four kinetically detectable phases (Table 2) reflect a stepwise formation/positioning of the product within the active site and a final release, respectively. Moreover, product formation by NS5BΔ21 acting on the fluorescent template ssRNA was verified by a classic polymerase assay measuring the incorporation of radioactively labeled nucleotides (Fig. 6).

As already outlined, at increasing nucleotide concentrations all apparent rate constants of the first, second, and third phase decreased, whereas the rate constant of the fourth phase increased in a hyperbolic manner (Fig. 5A). Thus, at saturating concentrations, the reaction sequence of product formation/positioning catalyzed by the enzyme is decelerated in comparison to the substrate-polymer dissociation reaction (Table 2). Apparently, at non-saturating nucleotide concentrations a partitioning occurs between product-polymer and substrate-polymer release. In sum, we conclude that the three faster phases in the RdRp reaction sequence represent reaction steps that are directly involved in RNA-dependent RNA polymerization. In other words we suggest the decrease in re-positioning rates along with increasing concentrations of NTP(s) corresponding to the actual incorporation of bound nucleotides into the dsRNA. Conversely, the fourth phase, which is accelerated in effective enzyme catalysis, is assumed to represent the product release and may thus operate as a "gatekeeper" function.

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sites (see the introduction) (31). Applying NS5B of HCV strain Con1 and measuring the change of the intrinsic fluorescence of the enzyme upon compound binding, the potent NNI HCV-796 was recently indicated to develop “slow binding kinetics” (18). Here, by monitoring the amplitude change in CD of NS5BΔ21 when exposed to the inhibitor, a slow binding mode of action of HCV-796 could not be confirmed (Fig. 2B). Discrepancies, which may relate to different applied HCV strains, were also obtained in terms of the determined $K_i$ values. Using NS5BΔ21 of HCV1b strain BK, we established a $K_i$ value of 7 $\mu$M for HCV-796, whereas the previously reported $K_i$ value of 0.07 $\mu$M obtained with NS5BΔ21 of HCV1b strain Con1 was two orders of magnitude lower. It was recently reported that the potential of HCV-796 was significantly reduced with HCV species in which residue Cys-316 in close proximity to the active gatekeeper function by impairing the ability of the enzyme to modulate viral and cellular proteins as well as for the further evaluation of anti-HCV compounds.

Taken together, the procedures established in this study to measure the kinetics of the HCV-RdRp proved to be valuable tools for a further analysis of the activity of modulating viral and cellular proteins as well as for the further evaluation of anti-HCV compounds.

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