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A genotype 2b NS5B polymerase with novel substitutions supports replication of a chimeric HCV 1b:2b replicon containing a genotype 1b NS3-5A background

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Abstract

HCV diversity suggests that evaluation of HCV inhibitors for broad genotypic efficacy is warrented. The replicon system enables cell-culture compound efficacy evaluation against an active replication complex, and a functional replicon dependent upon a genotype 2b polymerase would augment existing cell-culture efficacy studies that are presently limited to genotype 1a, 1b, and 2a replicons. We made a chimeric Neo^r 1b:2b replicon where genotype 2b NS5B was inserted into a genotype 1b NS3-5A background and transfected replicon RNA to generate Neo^r cell lines. All cell lines contained novel substitutions within NS5B which were subsequently engineered into the parental 1b:2b replicon and shown to enhance replication to various degrees. A single NS5B M31I substitution enhanced replication to levels sufficiently robust to quantify sensitivity to HCV inhibitors in a transient replication assay. The M31I 1b:2b replicon was similarly sensitive to an active-site nucleoside inhibitor of NS5B as genotype 1b replicons, but was insensitive to two non-nucleoside inhibitors which were otherwise efficacious against the genotype 1b replicons. This work describes a novel HCV replicon sustained by a genotype 2b polymerase that is sufficiently robust for quantifiable analysis in a transient replication assay, and demonstrates its utility in characterizing anti-HCV compounds for cross-genotypic efficacy.

Keywords: HCV; Replicon; NS5B; Polymerase; Inhibitor

1. Introduction

Persistent infection with hepatitis C virus (HCV) is a primary cause of several debilitating liver diseases including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Colombo, 1998). Approximately, 170 million people worldwide are infected, and more than half are likely to develop severe liver disorders (Wasley and Alter, 2000). Present treatment is limited to pegylated alpha interferon, administered with or without ribavirin. The regimens are poorly tolerated with variable efficacy. Recently, several HCV inhibitors of the NS5B RNA-dependent RNA polymerase (Carroll et al., 2003; Chan et al., 2004; Chun et al., 2005; Dhanak et al., 2002; Hashimoto et al., 2003; Koh et al., 2005; Stuyver et al., 2004; Wang et al., 2003) and NS3/4A protease (Lamarre et al., 2003; Lin et al., 2004) have been identified, and such inhibitors hold the promise of more tolerable, efficacious treatment of HCV infection.

Use of HCV sub-genomic replicons enables measurement of replication in cell-culture (Blight et al., 2000; Lohmann et al., 1999). This versatile system has facilitated a quantitative analysis of replication including the sensitivity of replication to inhibitors, and the selection and characterization of mutants resistant to HCV antivirals (Carroll et al., 2003; Guo et al., 2001; Lu et al., 2004; Migliaccio et al., 2003; Nguyen et al., 2003; Tomei et al., 2003; Trozzi et al., 2003). Ideally, HCV inhibitors should be broadly active against the diverse genotypes and variants encountered in clinical settings, and it would be useful to have cell-based replication assays to assess cross-genotypic efficacy and potential differences in resistance profiles of HCV antivirals. To date efficient sub-genomic replicons have been developed for genotypes 1a, 1b, and 2a (Blight et al., 2000, 2003; Grobler et al., 2003; Gu et al., 2003; Ikeda et al., 2002; Kato et al., 2003; Yi and Lemon, 2004). While these genotypes encompass the majority of clinical HCV infections, ~20% of

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infections are due to other genotypes, with most of these latter infections associated with either genotype 2b or 3a (Farci and Purcell, 2000).

In this work, we describe efficient replication of a chimeric HCV replicon dependent upon genotype 2b NS5B polymerase. Stable geneticin resistant cell lines were selected by transfection of a weakly replicating BK 2b chimeric replicon, and characterization of these lines showed that each harbored replicon RNA which encoded a distinct change from the input BK 2b replicon sequence. These changes were engineered into the parental BK 2b replicon and shown to enhance replication to varying degrees. A single M31I substitution within NS5B enhanced replication to a level suitable for quantifying replication in a transient replication assay. This replication was similarly sensitive to the NS5B active site inhibitor 2′*C*-methyladenosine (Carroll et al., 2003) as two genotype 1b replicons. However, replication was insensitive to two non-nucleoside inhibitors which bind at distinct pockets of NS5B away from the active site (Tomei et al., 2003; Wang et al., 2003) and are potent inhibitors of the genotype 1b replicons. This chimeric replicon will be useful in the cell-based evaluation of NS5B inhibitors for cross-genotypic efficacy against the genotype 2b polymerase.

2. Materials and methods

2.1. BK 2b chimeric replicons

The previously described cs2b replicon served as the parental chimera for these studies (Ludmerer et al., 2005). Briefly, the NS5B region from chimpanzee infected with the prototype isolate HC-J8 was cloned from serum (kindly provided by Dr. Jens Buhk, NIH), and the NS5B sequence matched the prototype sequence (GenBank accession number D10988). Total RNA was isolated from sera using the QIAGEN RNeasy Mini Kit according to manufacturer's instructions (QIAGEN Inc., Valencia, CA) and served as a template for the reverse transcription reaction (Superscript II RT, Invitrogen Life Technologies, Carlsbad, CA) with an oligo $(dA)_{34}$ primer. RT reactions were heat inactivated at 65 °C for 15 min and then digested with 1 μl each RNAseH and RNAseT1 (Roche Applied Science, Indianapolis, IN) at 37 °C for 20 min to remove RNA prior to PCR. Nested PCR was performed using Expand High Fidelity PCR System (Roche Applied Science, Indianapolis, IN) and the following primers: PCR1, forward 5′ CTCCGTCGTGTGCTGCGCCATGTC and reverse oligo$(dA)_{34}$ primer; PCR 2, forward 5′ ATACTCCTGGACAGGGGCCCT and reverse 5′ CCGCTCTACCGAGCGGGGAGT. The polymerase region starting from amino acid 10 was cloned into a genotype 1b BK replicon. Within the first 10 NS5B codons, the BK and genotype 2b sequences differ only at the sixth residue (T in BK, S in 2b). The BK replicon encoded previously described BK cell-culture adaptive changes (Grobler et al., 2003). A con1 2b chimeric replicon was generated which is similar to BK 2b but NS3 through NS5A are con1 and include the NS5A S232I adaptive change (Murray et al., 2003). Substitutions identified under geneticin selection in this work were engineered into the BK 2b or con1 2b replicons using site-directed mutagenesis (QuickChangeXL, Stratagene Corp., LaJolla, CA) and standard molecular biology techniques. Codon changes subsequently engineered into the genotype 2b NS5B were: N24S, TCG to TCA; M31I, ATG to ATT; I392L, ATC to CTC.

2.2. Preparation of RNA and transfection of cell lines

RNA was prepared and transfected using published protocols (Murray et al., 2003). For geneticin selections, 300,000 Huh-7 cells were transfected with 1.0 μg RNA. For transient replication analysis, 300,000 MR2 cells (Murray et al., 2003) were transfected with 5.0 μg RNA. MR2 cells are enhanced relative to parental Huh-7 cells in establishment of transient replication.

2.3. Selection and characterization of geneticin resistant replicon colonies

Selections were conducted in Dulbecco's modified complete media (DMEM) containing 10% fetal bovine serum, penicillin/streptomycin, GlutaMAX, non-essential amino acids, and supplemented with 250 μg/ml Geneticin (Invitrogen Corp., Carlsbad, CA). Fresh medium was exchanged twice weekly over the course of the 3 weeks selection, and cloning cylinders were used to isolate individual colonies. RNA was isolated from expanded colonies using Qiagen's RNA Easy Kit (Qiagen Inc., Valencia, CA). The entire NS3-5B region was rescued in five overlapping fragments using RT/PCR and sequenced in entirety. PCR primer sequences are listed in Table 1. RT reactions were performed with Stratascript RT (Stratagene, LaJolla, CA), and PCR reactions were performed using Expand Polymerase (Roche Diagnostics Corp., Indianapolis, IN). For experiments where G418 resistant colonies were counted, the colonies were stained with 0.06% (w/v) Coomassie Brilliant Blue/50% methanol/10% acetic acid. Where colony number exceeded 1000, counts were taken on a 9 cm^2 representative grid of the T150 flask, and multiplied by 16.6 to generate CFU per flask. Colony numbers were derived from a representative set of tranfections/selections performed with a uniform set of reagents. Selections were repeated three times.

2.4. Establishment of transiently replicating cell lines and analysis of replication

Replication was quantified with a recently described transient replication assay using β-lactamase activity for detection as determined by emission at 460 nm (Ludmerer et al., 2005). The values of the emission at 460 nm for the BK replicon were assigned a fitness value of 1, and the values for all experimental replicons were assessed in relationship to the value for BK. The background emission value was defined as emission at 460 nm in the presence of 10 μM 2′*C*-methyladenosine (a concentration $\geq 10\times$ EC_{50} of the BK replicon and resulting in <5% residual activity) and was subtracted from all values. A control transfection with the non-replicating GAA replicon was included in every experiment to verify that residual β-lactamase activity was reduced to background. GAA is non-functional due to

Table 1
Primer pairs used to rescue NS3-5B from Neo^r cell lines

1. EMCV IRES 3′ end to 3′ end of NS3	
Outer	
5′ CTCTCCTCAAGCGTATTCAACAAGG	Forward
5′ CCGTGCAGCGTAGGTTTCAGCCGTA	Reverse
Inner	
5′ CCCATTGTATGGGATCTGATCTGG	Forward
5′ CAAGCTGAAGTCGACTGTCTGGGTGACA	Reverse
2. NS3 internal to 3′ end of NS4B	
Outer	
5′ TACTTGGTCACGAGACATGCTGACGTCAT	Forward
5′ GGAGAGGATCTGAGTAACA	Reverse
Inner	
5′ CGTATATGTCTAAGGCACACGGTATTGAC	Forward
5′ GGCTGGTGATAGAGGCTGTGAATGCCAT	Reverse
3. NS3 3′ end to 5′ end of NS5B	
Outer	
5′ GGATCAAATGTGGAAGTGTCTCATACGG	Forward
5′ TCGAGGTTGTGGAGTACAC	Reverse
Inner	
5′ GCAATAGCATCATTGATGGCATTCACAGC	Forward
5′ GGCCTCGATGAGGTCAGCGT	Reverse
4. NS5A internal to internal site of NS5B	
Outer	
5′ GTAAAGTGCCCATGCCAGGT	Forward
5′ CATGATAGTTGTGTCAATTG	Reverse
Inner	
5′ GTGCGGTTGCACAGGTACGCTCC	Forward
5′ TCGAGGTTGTGGAGTACAC	Reverse
5. NS5A 3′ end to 3′ UTR	
Outer	
5′ GTCTACCGTGAGCGAGGAA	Forward
5′ oligo(dA)$_{34}$	Reverse
Innner	
5′ CTACACATGGACAGGCGCCTT	Forward
5′ CATCGATCGGGGAGTAAAAAGATGCCTAC	Reverse

Asp-to-Ala substitutions within the active site of NS5B (Murray et al., 2003). EC$_{50}$ determinations were calculated as a percent of the DMSO control by fitting the data to a four parameter fit function (minimum, maximum, slope, and inflection point) using Kaleidagraph software (Synergy Software, Reading, PA) as previously described (Ludmerer et al., 2005). Titrations were performed in triplicate and the values averaged. Cytotoxicity was not observed with any of the inhibitors at concentrations evaluated in this work. All titrations were repeated at least once in entirety with new transfections to further verify reproducibility.

2.5. NS5B inhibitors

2′*C*-methyladenosine was obtained from the Merck chemical collection. The non-nucleoside benzimidazole (Tomei et al., 2003) and thiophene (Wang et al., 2003) inhibitors were obtained from Dr. Frank Narjes (Istituto di Ricerche di Biolgia Molecolare, Rome, Italy).

3. Results

3.1. Characterization of stable Neo^r BK 2b cell lines for substitutions within replicon sequences

To date efforts to generate a homologous genotype 2b replicon have been unsuccessful. We previously demonstrated that chimeric replicons encoding patient-derived NS5B sequences of genotypes 1a or 1b in a genotype 1b NS3-5A background were competent for replication and suitable for NS5B inhibitor efficacy studies using transient β-lactamase expression (Ludmerer et al., 2005). When we extended this approach to NS5B sequences of other genotypes, we observed at best low levels of replication insufficient for quantifiable evaluation. To utilize geneticin selection to select for amino acid substitutions which increase the replication efficiency (Yi and Lemon, 2004) of a poorly replicating 1b:2b chimeric replicon BK 2b, we replaced the co-encoded β-lactamase gene with the Neo^r gene, transfected replicon RNA into the Huh-7 cell line, and selected stable Neo^r colonies.

Three Neo^r colonies were isolated, RNA was rescued from each, and the sequence across the entire NS3-5B region determined. Each colony harbored a single homogenous substitution within NS5B, N24S, M31I, and I392L. When engineered into the parental Neo^r BK 2b replicon, these substitutions increased the number of geneticin resistant colonies >100-fold over that obtained with the parental BK 2b replicon (Fig. 1). The M31I substitution produced the greatest enhancement, yielding ∼16% the number of Neo^r colonies as the homologous BK replicon. The N24S and I392L substitutions produced near equivalent numbers of Neo^r colonies, both ∼0 3% that of BK. A non-functional GAA replicon harboring a mutation in the NS5B active site produced no colonies (data not shown).

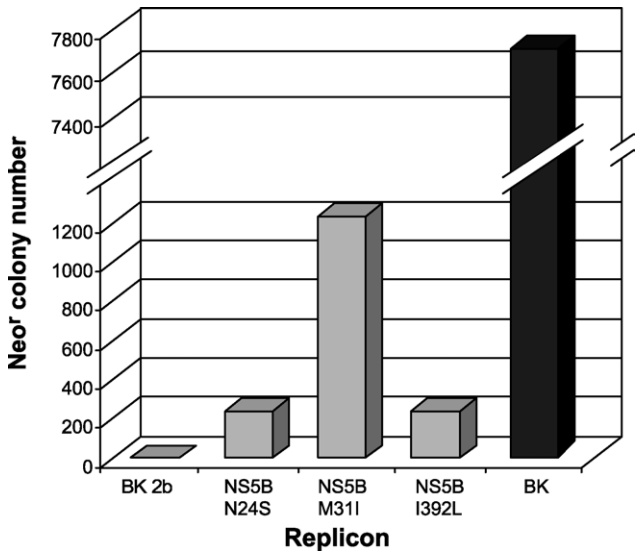

Fig. 1. Single substitutions enhance BK 2b Neo^r colony formation. 300,000 Huh-7 cells were transfected with 1.0 μg of BK (shown in dark shading) or BK 2b replicon RNA encoding the indicated substitution, and the number of geneticin resistant colonies isolated is shown.

Two cell lines each encoded a second heterogenous substitution within the NS3-5A portion of the replicon. The NS5B N24S cell line encoded both N and S at NS5A residue 268 (N is parental), while the NS5B M31I cell line encoded both M and R at NS3 residue 470 (M is parental). Direct comparisons in Neo^r colony formation demonstrated that the NS5B substituted replicons with NS3 R470 or NS5A S268 produced substantially fewer colonies than when encoding the parental NS3 M470 and NS5A N268 residues (data not shown). All replicons utilized for subsequent studies encoded the parental NS3 R470 and NS5A N268 residues.

3.2. Single substitutions enhanced replication of the BK 2b replicon in a transient replication assay

To develop a transient assay for cross-genotypic analysis of polymerase inhibitors, NS5B substitutions N24S, M31I, and I392L were engineered into the parental β-lactamase BK 2b replicon, transfected into the MR2 cell line, and replication fitness assessed using a transient replication assay (Fig. 2A). For quantitative analysis of fitness we compared replication of the experimental replicons to that of the homologous BK replicon. The most robust replication from a singly substituted replicon was observed with M31I substituted BK 2b, which produced a signal ∼75% that of BK. N24S and I392L substitutions also enhanced replication, but the signals were lower than that of M31I (Fig. 2A). The enhancement level among the three substitutions paralleled their comparative strength in Neo^r colony selection. Relative to BK, the magnitude of enhancement was greater in the transient replication assay, which may be due in part to the use of an enhanced cell line in this assay.

The various combinations of doubly and triply NS5B substituted replicons were also generated to ask whether the enhancement conferred by any of these substitutions is additive (Fig. 2A). Whereas the combination of either N24S or I392L with M31I produced at most nominally higher replication than M31I, the triply substituted replicon replicated to a level comparable to the homologous BK replicon. In addition, the doubly substituted N24S, I392L replicon produced a signal ∼65% that of BK, slightly higher than the additive contributions of N24S (∼25%) and I392L (∼20%). The molecular basis for the increased fitness of the N24S I392L substituted replicon was not further investigated.

To evaluate context dependence, M31I was engineered into a con1 2b replicon and fitness assessed using the transient replication assay (Fig. 2B). Replication was enhanced approximately four-fold over the parental con1 2b replicon, demonstrating that the effect of this NS5B substitution was not restricted to a BK specific interaction. The magnitude of enhancement was not as pronounced as within the BK background, showing context dependence and consistent with an earlier observation that BK 2b replicated weakly but con1 2b replication could not be demonstrated at all (Ludmerer et al., 2005). Replication of N24S or I392L con1 2b replicons could not be quantified above the parental con1 2b replicon (data not shown).

3.3. BK 2b M31I replication is insensitive to non-nucleoside NS5B inhibitors potent against genotype 1b NS5B

M31I substituted BK 2b replication was sufficiently robust to quantify sensitivity to NS5B inhibitors. For this model study

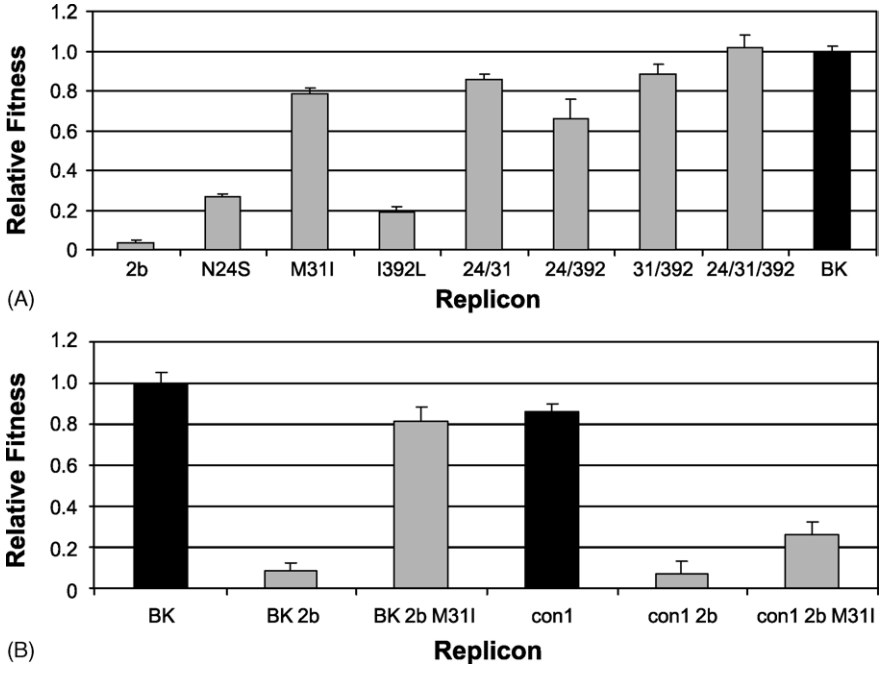

Fig. 2. Replication fitness and context dependence of NS5B substitutions. Substitutions within NS5B were engineered as indicated into the β-lactamase BK 2b or con1 2b replicons. Replication was measured as described in Section 2, and replication levels were normalized to that of BK. Homologous genotype 1b replicons con1 and BK are indicated in dark shading. Chimeric BK:2b replicons are shown in lighter shading. (A) Replication fitness of NS5B substituted replicons, with the parental BK 2b replicon shown as 2b. (B) M31I enhancement in the BK vs. con1 background.

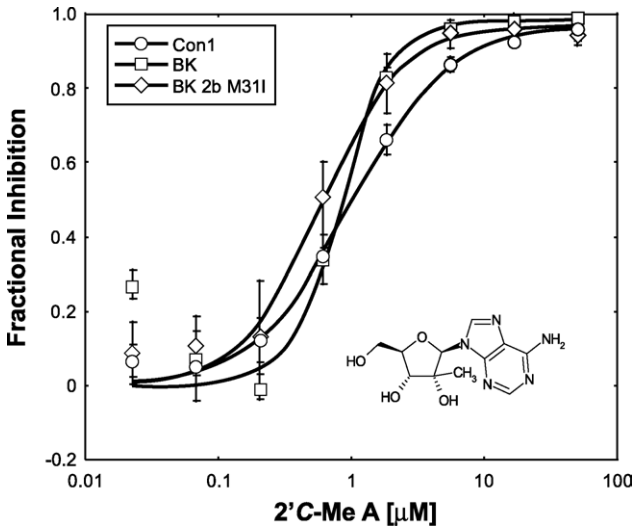

Fig. 3. The nucleoside inhibitor 2′*C*-methyladenosine is similarly potent against M31I substituted BK 2b as genotype 1b replicons. The enhanced Huh-7 cell line MR2 was transfected with replicon RNA and expanded as described in Section 2. On day 4, the cells were collected, plated in the presence of compound, and cultured for 2 days. For each replicon, replication in the absence of compound (DMSO control) was normalized to 1 following a background subtraction as described in Section 2. Titrations of the nucleoside inhibitor 2′*C*-methyladenosine are shown for con1, BK, and M31I substituted BK 2b replicons.

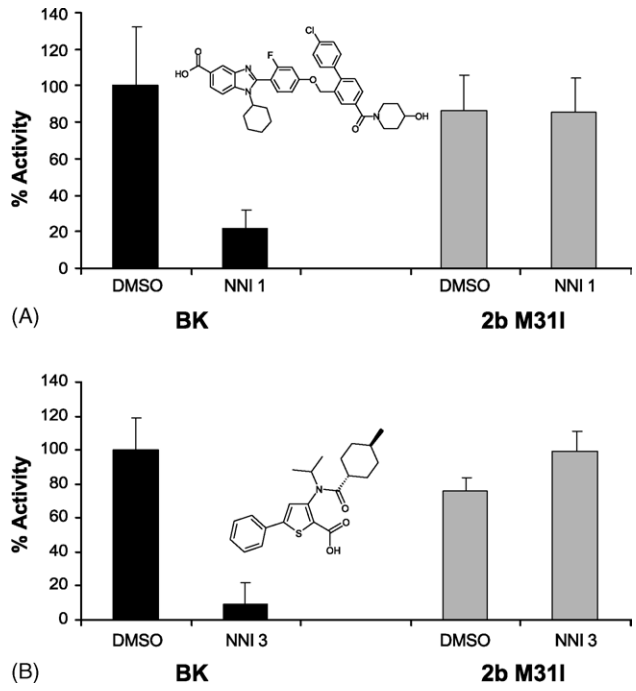

Fig. 4. M31I substituted BK 2b replicon is insensitive to two non-nucleoside inhibitors potent against the BK replicon. The enhanced Huh-7 cell line MR2 was transfected with replicon RNA and expanded as described in Section 2. On day 4, cells were collected and plated in the presence of DMSO or compound (at 10× BK EC_{50}) and cultured for 2 days. Replication of BK (DMSO) was set to 100%, and replication of BK (compound) and M31I substituted BK 2b (DMSO or compound) were normalized to the BK (DMSO) control. (A) Benzimidazole NNI 1 inhibitor at 6.7 μM (BK EC_{50} of 600 nM). (B) Thiophene NNI 3 inhibitor at 50 μM (BK EC_{50} of 4 μM).

we chose a nucleoside analogue directed at the active site and two non-nucleoside inhibitors targeted to distinct sites near the NS5B surface. All three compounds are potent inhibitors of con1 and BK replicons.

The nucleoside analogue 2′*C*-methyladenosine is an active-site inhibitor of NS5B which, when incorporated into nascent RNA, results in pre-mature chain termination (Carroll et al., 2003). Both the con1 and BK replicons are sensitive to this

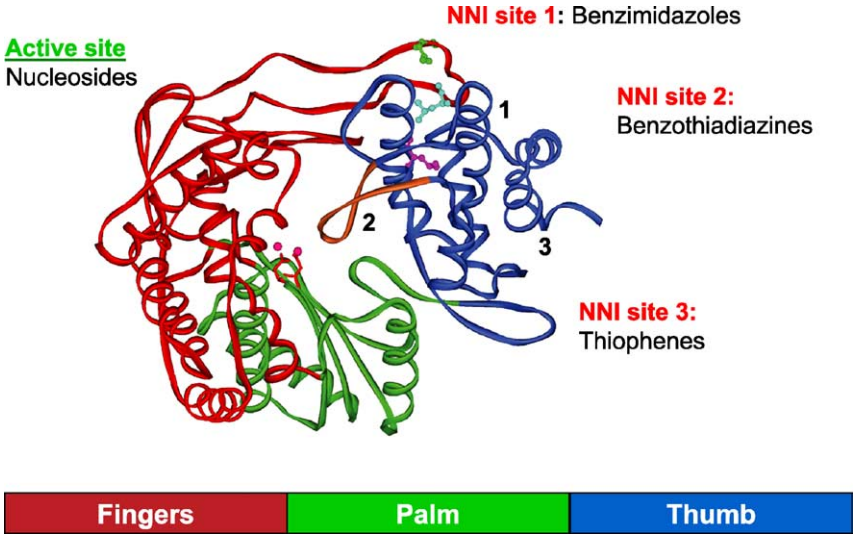

Fig. 5. NS5B adaptive substitutions reside outside the active site towards the polymerase surface. The polymerase model is based upon the published crystal structure of the BK Δ55 enzyme. The three genotype 2b NS5B amino acid substitutions which enhance replication of the BK:2b replicon are shown as 'ball and stick' appendages. N24S (green) and M31I (blue) are located in the finger–thumb connecting loop, while I392L (magenta) is within an α-helix on the thumb. The catalytic aspartate (red ball and stick) at the active site is shown toward the center of the figure. The location of known inhibitor binding sites is indicated and numbered according to an earlier description, with the inclusion of the benzodiathiazene NNI 2 site shown for completeness. N24S and M31I are in the proximity of non-nucleoside binding site 1, while I392L is near but outside the active site.

compound with EC_{50}s of ~1 μM (Ludmerer et al., 2005). This compound was equally potent against the NS5B M31I substituted BK 2b replicon (Fig. 3). The M31I con1 2b replicon was similarly sensitive to this inhibitor (data not shown).

The non-nucleoside benzimidazole and thiophene inhibitors interact with non-nucleoside inhibitor sites NNI 1 (Hashimoto et al., 2003; Tomei et al., 2003; Wang et al., 2003) and NNI 3, respectively (Chan et al., 2004; Wang et al., 2003). Both are active with variable potencies against genotype 1a and 1b replicons (Ludmerer et al., 2005). Fig. 4 shows replication in the presence of inhibitor at concentrations approximately 10× EC_{50} of the BK replicon. M31I substituted BK 2b replication was insensitive to both compounds at concentrations which inhibited >80% BK replication. The M31I con1 2b replicon was similarly insensitive to these inhibitors at these concentrations (data not shown). The lack of sensitivity of M31I substituted BK 2b replication to these non-nucleoside inhibitors at concentrations efficacious against genotype 1b replication demonstrates that sensitivity to inhibitors characterized against one genotype does not necessarily translate to efficacy across genotypes.

4. Discussion

The genetic diversity among NS5B sequences suggests difficulty in developing broad-spectrum non-active site inhibitors. To facilitate inhibitor analysis against the genotype 2b polymerase in a cell-based replication assay, we identified single amino acid substitutions which increased the replication fitness of a poorly active intergenotypic 1b:2b replicon. A single M31I substitution within NS5B enhanced replication to a level suitable for inhibitor efficacy studies in a transient, quantitative replication assay. Replication of M31I substituted BK 2b was sensitive to an NS5B active site nucleoside analogue with an EC_{50} similar to that of two genotype 1b replicons, but was insensitive to two non-nucleoside compounds that are potent inhibitors of the genotype 1b replicons. These results demonstrate the utility of the M31I substituted BK 2b replicon in evaluating replication sustained by genotype 2b NS5B in an active replication complex, and underscore the need to address cross-genotypic potency in the pre-clinical development of HCV antivirals. These results extend earlier observations showing that the efficacy of non-nucleoside inhibitors has greater variability against diverse NS5B sequences than that of a nucleoside inhibitor targeting the active site (Ludmerer et al., 2005).

The three NS5B 'enhanced' substitutions are predicted to reside outside the active site and near the polymerase surface (Fig. 5). Residues 24 and 31 lie towards the thumb region on a polypeptide stretch which connects the finger and thumb domains, while residue 392 lies on an α-helix coil within the thumb (Bressanelli et al., 1999). All three of the enhancing substitutions are outside of non-nucleoside binding pockets previously defined by binding or resistance studies (Bressanelli et al., 1999; Dhanak et al., 2002; Love et al., 2003; Tomei et al., 2003; Wang et al., 2003). The general proximity of the M31I substitution to the benzimidazole binding site NNI 1 may render caution in the use of this replicon to evaluate sensitivity to compounds which bind this site. However, drug inhibition studies with bacterially expressed and purified genotype 2b NS5b enzyme using the parental genotype 2b sequence demonstrated a similar lack of sensitivity to the benzimidazole inhibitor (Steven Carroll, unpublished observations). Thus lack of sensitivity to this NNI 1 inhibitor is an intrinsic property of the genotype 2b NS5B sequence.

A comparison of our genotype 2b NS5B sequence with NS5B from 23 full-length genotype 2b sequences listed in GenBank showed only a single isolate (AB030907) with a variation among residues 24, 31, and 392, an S24 encoded within that sequence. Furthermore, our NS5B sequence differs from a consensus derived from these 23 sequences at a total of only six positions. Thus the substitutions described here may be similarly efficacious in enhancing replication using other genotype 2b sequences, a property useful in the evaluation of diverse clinical genotype 2b isolates. Such substitutions into more genetically distant NS5B sequences, however, may not be as efficacious. We note, for example, that I31 was encoded within chmeric BK 6a replicons which failed to replicate (Ludmerer et al., 2005). This and related observations underscore the role of context dependence in the activity of chimeric replicons (Gates et al., 2004; Lemm et al., 2005), and should be considered when evaluating such a strategy to functionally characterize HCV sequence diversity.

The clinical development of HCV inhibitors is aided by ability to assess the efficacy of HCV inhibitors against a broad array of diverse sequences. These studies generated a chimeric replicon capable of quantifying replication sustained by genotype 2b polymerase and demonstrated its utility in the evaluation of inhibitor efficacy against this genotype. This tool should facilitate analysis of diverse genotype 2b NS5B sequences.

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