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Development and characterization of a replicon-based phenotypic assay for assessing HCV NS4B from clinical isolates

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ABSTRACT

The hepatitis C virus (HCV) NS4B inhibitors have shown potent inhibition of HCV replication in vitro. To assess the effect of viral diversity on the susceptibility to NS4B inhibitors, genotype (GT)-specific GT1a and GT1b replicon shuttle vectors were designed and created for cloning HCV NS4B genes from clinical isolates. For the GT1b NS4B shuttle vector, the S2204I adaptive mutation was introduced in NS5A to improve replication due to the replacement of the K1846T adaptive mutation in NS4B with NS4B from the clinical isolates. In addition to the adaptive mutations, a newly identified Huh-7 cell line, Huh-7-1C, which is highly permissive for both GT1a and GT1b replication, was used to further enhance the replication levels. HCV NS4B gene from clinical isolates was amplified and inserted into the corresponding GT1a and GT1b modified lab strain chimeric replicons. GT1a and GT1b chimeric replicons expressing diverse NS4B genes from corresponding subtypes of clinical isolates replicated at highly efficient levels for phenotypic analysis. Due to natural variation in their amino acid residues in NS4B, these isolates displayed varying drug susceptibilities to an NS4B inhibitor. In mixed populations with wild-type, the sensitivity of resistance detection of NS4B resistant mutants H94R and V105M was between 20% and 80%. The chimeric shuttle vectors can be used to characterize the activity of antiviral drugs targeting NS4B from diverse natural clinical isolates and aid in the development of novel compounds against HCV NS4B.

1. Introduction

Hepatitis C virus (HCV) is among the leading causes of persistent chronic liver disease and liver transplants worldwide. The recent approval of two direct antiviral agents (DAA), the HCV NS3 protease inhibitors telaprevir and boceprevir, for treatment of HCV in combination with peglyated interferon and ribavirin, has improved the sustained virologic response (SVR) rates to 60–70% vs. 40–50% SVR rates in pegylated interferon (peginterferon alfa) and ribavirin treated patients with HCV genotype 1 (Bacon et al., 2011; Jacobson et al., 2011; Sherman et al., 2011). As more DAA combinations are being tested in clinical trials to further improve clinical outcome, overcoming viral resistance remains a challenge. HCV, an RNA virus, has an error-prone polymerase which causes accumulation of nucleotide substitutions within the genomes during replication, resulting in a highly diverse population of quasispecies in infected individuals (Chayama and Hayes, 2011). Among patient quasispecies, preexisting drug-resistant variants are present as minor populations, but can quickly become dominant during antiviral treatment, resulting in treatment failure (Farci, 2011). Polymorphisms, which represent natural variations in HCV sequence, can be associated with resistance and can lead to reduced viral response to antiviral treatment. Polymorphisms affecting drug response have been identified in the NS3 protease (Ferreon et al., 2005; Zhao et al., 2012) and NS5A proteins of HCV (El-Shamy et al., 2008).

The most advanced HCV targets have been the NS3/4A serine protease and NS5B RNA polymerase, with recent advances made to target the non-enzymatic NS5A protein (Park et al., 2009). Another protein of interest is the HCV NS4B. NS4B is a hydrophobic transmembrane protein localized in the endoplasmic reticulum (ER) (Aizaki et al., 2004). It is a key enzyme required for RNA replication and for the assembly of other non-structural proteins in membrane-associated replication complexes (Blight, 2011; Paul et al., 2011; Einav et al., 2004; Gouttenoire et al., 2010). Disrupting NS4B function represents an attractive anti-HCV strategy (Zheng et al., 2005). Biochemical assays and in vitro replicon resistance assays have identified compounds from a pyrazolopyrimidine series that specifically target NS4B as well as key resistance mutations in NS4B, such as H94R and V105M/L that confer resistance to these NS4B inhibitors (Bryson et al., 2010). The residue 94 in NS4B is highly polymorphic, amino acid variations such as histadine (H), serine (S) and asparagine (N) have been observed in clinical isolates (EU HCV database). The frequency of naturally occurring H94R across genotypes is rare. The residue 105 in NS4B is mostly conserved with valine (V). Leucine (L) at 105 has been observed in small percent of clinical isolates (EU HCV database). Other

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antiviral agents effective in disrupting NS4B are amiloride analogues, clemizole hydrochloride, and clemizole-related indazole series (Dvory-Sobol et al., 2010; Rai and Deval, 2011).

HCV subgenomic replicons provide a valuable tool in measuring antiviral effects on replication and compound susceptibility, however, only a few replicating GT1a, GT1b, and GT2a reference lab strains are available. The spectrum of antiviral activity of inhibitors can be better understood by measuring activity against naturally occurring HCV clinical isolates using a phenotypic assay. Phenotypic assays for the NS3 protease, NS5A, and NS5B genes from HCV isolates have been developed by modifying existing replicon strains. However, NS4B phenotypic assays have not been described and are needed for evaluation of NS4B inhibitors.

It has been demonstrated that replication of the GT1a H77 strain of HCV is attenuated compared to the GT1b Con1 strain in both Huh-7 Lunet cells and Huh-7.5 cells (Blight et al., 2003). Consequently, GT1b Con-1 or 1b-N replicons as backbones were used for the replicon-based phenotypic assays for GT1a and GT1b NS3 protease, NS5A, and NS5B genes (Middleton et al., 2007; Qi et al., 2009; Tripathi et al., 2007; Le Pogam et al., 2008). In these phenotypic assays, chimeric GT1b replicons carrying NS3 protease, NS5A, or NS5B from both GT1a and GT1b produced robust replication for drug susceptibility testing. However, this strategy will likely present a challenge for a GT1a NS4B phenotypic assay, as a lethal phenotype in transient replication assays was observed for a GT1b Con1 chimeric replicon containing the NS4B gene from H77 in place of the Con1 NS4B sequence (Paredes and Blight, 2008). To overcome these issues, we have developed subtype specific replicon shuttle vectors for cloning NS4B genes from quasispecies pools of clinical isolates and have used it to investigate how the genetic heterogeneity of NS4B affects HCV replication and determine the sensitivity of patient derived NS4B clinical isolates to HCV inhibitors using a replicon-based transient system.

2. Methods and materials

2.1. Compounds

The imidazo [1,2-a] pyridine NS4B inhibitor GS-546288 and the protease inhibitor GS-9451 used as a control were made by Gilead Sciences. 10 mM stocks were prepared and stored at −20 °C. All compounds were solubilized in DMSO.

2.2. Clinical isolates

Twenty-three HCV NS4B clinical isolates (twelve GT1a and eleven GT1b) were obtained from serum samples of untreated HCV infected individuals.

2.3. Cell lines

Huh-7-1C cell line is a clone of Huh7-Lunet, which was isolated and identified by Gilead from drug resistance replicons cured with multiple, distinct classes of HCV inhibitors (Butkiewicz et al., 2000). The transfected replicon cells were plated in Dulbeccos's modified Eagle's medium (DMEM) with GlutaMAX-I (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 1 U/mL penicillin (Invitrogen), 1 μg/mL streptomycin (Invitrogen), and 0.1 mM nonessential amino acids (Invitrogen).

2.4. Design and construction of subtype specific NS4B shuttle vectors

The parental replicon vector genotype GT1b Con1, designated as GT1b PiRluc, was used for the construction of the GT1b NS4B shuttle vector as depicted in Fig. 1A (Friebe et al., 2001). GT1b PiRluc has three adaptive mutations, two in NS3 (E1202G + T1280I) and one in NS4B (K1846T). The parental replicon vector GT1a H77, designated as GT1a PiRluc, was used for the construction of the GT1a NS4B shuttle vector. GT1a PiRluc has five adaptive mutations, two in the NS3 (Q1067R + V1655I), one in the NS4A (K1691R), two in the NS5A (K2040R + S2204I). The firefly luciferase reporter in these vectors was replaced with renilla luciferase, which results in higher luciferase reading.

To create the GT1b Con1 NS4B shuttle vector, three unique restriction sites (XbaI, BsrGI and ClaI) were introduced into the parental GT1b PiRluc vector. XbaI was introduced in NS3 helicase, 3 amino acid upstream from the NS3–NS4A junction. BsrGI was introduced in NS5A, 11 amino acid downstream from the NS4B–NS5A junction. Both the restriction sites did not change amino acid sequence. ClaI was introduced at the 3′ end of NS4A, seven amino acids upstream from the NS4A-4B junction and caused a conservative amino acid from phenylalanine (Phe) to isoleucine (Ile). The pre-existing BsrGI site in NS3 at position 3019, was removed prior to addition of BsrGI in NS5A. To generate a replication-defective shuttle vector, another XbaI site was introduced at position 5363

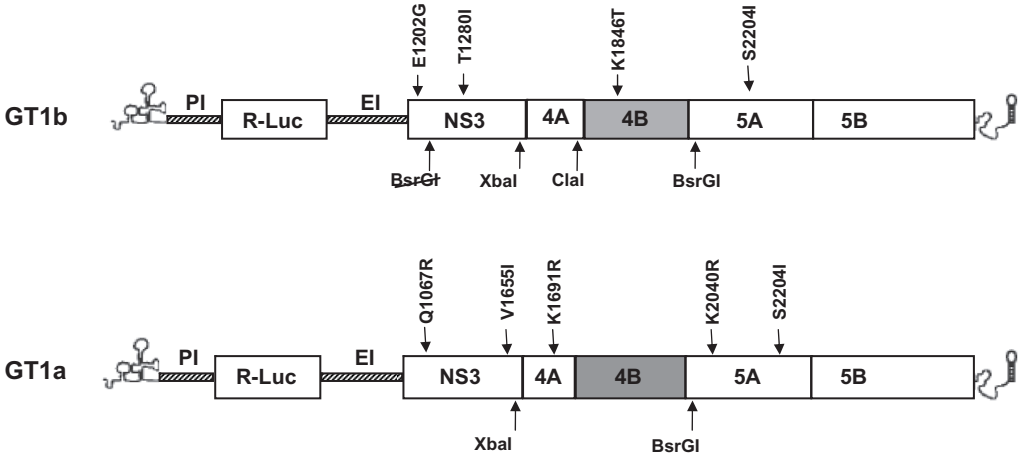

Fig. 1A. HCV replicons from reference strains genotype 1b Con1 or genotype 1a H77 were modified to create NS4B shuttle vector. Cloning sites and adaptive mutations are indicated.

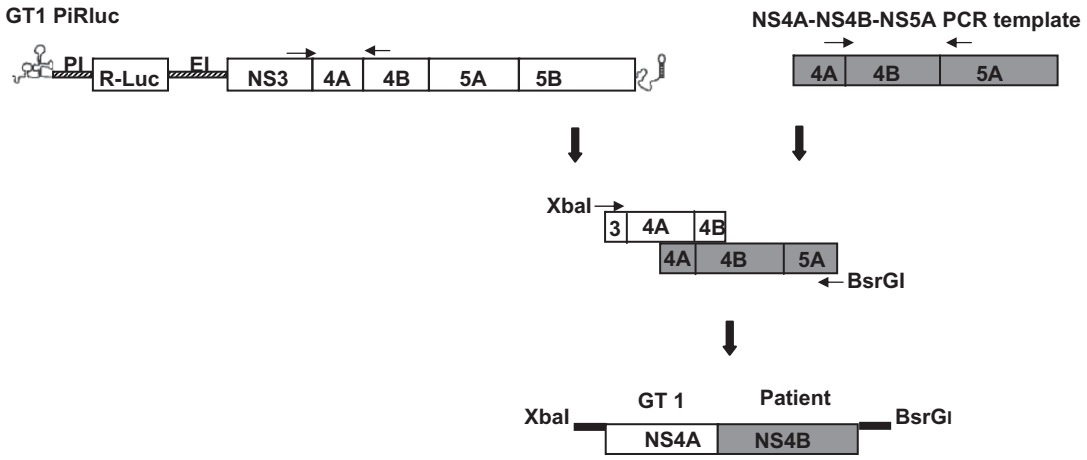

Fig. 1B. Overlap PCR strategy using NS4A–NS4B–NS5A PCR template from untreated HCV infected serum. NS4B was amplified and fused with NS4A from the corresponding replicon genotype to generate overlapped fragment with the cloning sites XbaI–BsrGI.

in the NS4B. The region between two XbaI sites was deleted to create a replication defective vector, which was used for cloning of NS4A–NS4B gene.

To create the GT1a H77 NS4B shuttle vector, two unique sites XbaI and BsrGI were introduced into the parental GT1a PiRluc vector. XbaI was introduced in the NS3 helicase, 3 amino acids upstream from the NS3–NS4A junction. BsrGI was introduced in NS5A, 12 amino acids downstream from the NS4B–NS5A. To generate a replication defective shuttle vector, two ClaI sites was introduced at position 4672 and 5336, the region (4672–5336) was deleted to create a deletion shuttle vector, which was used for cloning in of NS4A–NS4B gene.

2.5. Amplification of NS4B genes from patient sera

Individual NS4B genes from clinical isolates were amplified from a PCR template which consisted of partial NS4A and complete NS4B regions. The nested PCR was performed using the AccuPrime Pfx Super Mix (Invitrogen, Cat. No. 12344-040) as directed by the manufacturer. The amplified PCR products consisting of NS4B from clinical isolates served as a template for the overlap PCR with the NS4A amplified from the corresponding parental plasmid vector (Fig. 1B). Using the nested primers consisting of the cloning sites, the two products were combined to generate an overlapped NS4A-4B cassette with cloning sites incorporated at either ends. The NS4A-4B cassette was cloned into the shuttle vector using XbaI and BsrGI cloning sites. The ligated products were transformed in *E. coli* XL-Gold Competent Cells (Stragene) and grown under ampicillin antibiotic selection.

2.6. Transient replication assay and luciferase assay

The cloned chimeric replicon plasmids were linearized with ScaI for GT1b or HpaI for GT1a and purified using Qiagen PCR purification kit following the manufacturer's instructions. RNA transcripts were prepared by using T7 RiboMAX™ Express Large Scale RNA Production System (Promega, P1320), as directed by the manufacturer. 2 μg of RNA was transfected into 4×10^6 Huh-7-1C cells by electroporation at 270 V/950uF in a Gene Pulsar II System (Bio-Rad, Hercules, CA). The electroporated cells were plated in 96-well white solid bottom plates at a density of 2×10^4 cells/well in growth medium Dulbecco's modified Eagle medium (Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1 IU/mL of penicillin, 1 μg/mL of streptomycin and non-essential amino acids. Cells were harvested at 4 h and 96 h after transfection and assayed for luciferase activity. For EC_{50} determination, the compounds were serially diluted in DMSO and were added to the assay plates one day after transfection at a final DMSO concentration of 0.5%. The cells were harvested 3 days after compound addition and lysed for renilla luciferase assay as specified by the manufacturer's instructions (Promega, Madison, MI). The luciferase was measured with a Victor luminometer (Perkin Elmer, Waltham, MA). EC_{50} values and dose response curves were generated using the XLFit5 program (ID Business Solutions Ltd., Surrey, UK). Signal to noise window was determined as a ratio of luciferase activity from cells treated with 0.5% DMSO versus activity of cells treated with compound.

3. Results

3.1. Construction and replication of modified parental vector containing restriction sites

GT1a- and GT1b-specific NS4B shuttle vectors were constructed for cloning NS4B genes from GT1 clinical isolates as shown in Fig. 1A. The parental vector GT1b PiRuc has higher replication levels in the transient assay due to the presence of three adaptive mutations and hence was selected for construction of the GT1b NS4B shuttle vector. Higher replication levels of the parental vector ensures that the introduction of the heterogeneous clinical isolates in the shuttle vector results in replication levels that can be measured and used to assess the drug susceptibility of the cloned clinical isolates. The presence of renilla luciferase reporter in place of the firefly luciferase reporter also allows for the more robust replication and efficient detection of low replicating clinical isolates cloned in the shuttle vector.

For the construction of the GT1b NS4B shuttle, two cloning sites XbaI in the NS3 helicase and BsrGI in the NS5A, were created in the parental GT1b PiRluc for the cloning of the overlapped NS4A-4B fragment. The third restriction site ClaI, at 3′ end of NS4A was created for cloning of only NS4B gene. The ClaI site was located outside the NS4A–NS4B cleavage recognition site. The ClaI site caused a conservative change in the amino acid from phenylalanine (Phe) to isoleucine (Ile), both being hydrophobic amino acids. Replication levels of the parental GT1b PiRluc containing the individual cloning sites and combined cloning sites were determined as shown in Fig. 2A. Introduction of the individual XbaI restriction site in the NS3 helicase and the BsrGI restriction site in the NS5A, did not affect the replication capacity of the GT1b PiRluc replicon. Combination of the two cloning sites XbaI and BsrGI also resulted

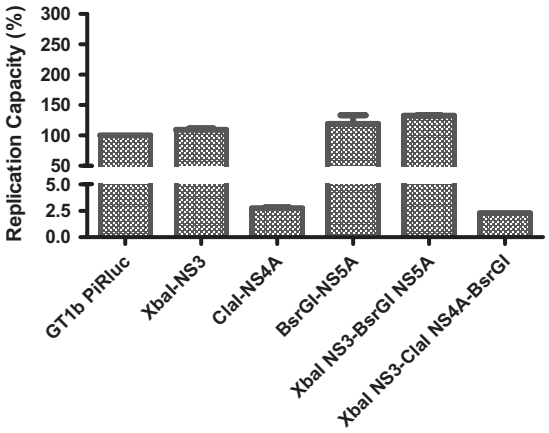

Fig. 2A. Comparison of replication capacity of GT1b parental vector consisting of individual cloning sites (XbaI–NS3, ClaI–NS4A and BsrGI–NS5A) and combined cloning sites (XbaI NS3–BsrGI NS5A and XbaI NS3–ClaI NS4A–BsrGI NS5A) to the wild-type unmodified replicon vector (GT1b PiRluc). Plotted values represent the means ± standard deviations (SD) of at least three independent experiments.

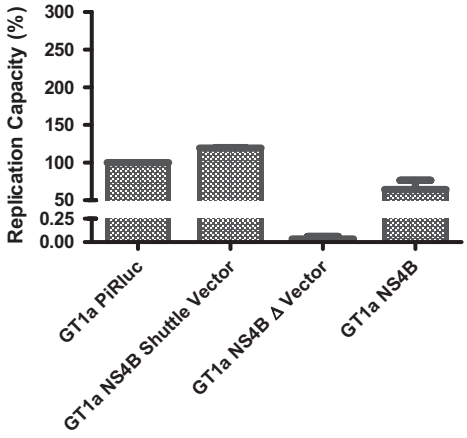

Fig. 2B. Comparison of the replication capacity of GT1a NS4B shuttle vector consisting of XbaI–BsrGI cloning sites to the wild-type unmodified replicon vector (GT1a PiRluc). Replication of the GT1a NS4B deletion (Δ) shuttle vector is restored after subcloning of NS4B from replicon (GT1a NS4B). Plotted values represent the means ± standard deviations (SD) of two independent experiments.

in replication levels that were comparable to the parental GT1b PiRluc. In contrast, introduction of ClaI site in the NS4A drastically lowered the replication levels to 2.5% of the parental replicon. The GT1b NS4B shuttle vector with restriction sites XbaI–BsrGI had robust replication and was selected as the NS4B shuttle vector for further characterization.

The GT1a PiRluc parental vector containing five adaptive mutations for enhanced replication was selected for creating the GT1a NS4B shuttle vector. For the design of the GT1a NS4B shuttle vector, similar cloning sites used for the GT1b NS4B shuttle vector were used to construct the vector. Incorporation of the BsrGI site in the region resulted in the change in amino acid from threonine to glutamic acid. The replication level of the GT1a NS4B shuttle vector with XbaI and BsrGI sites (GT1a NS4B shuttle vector) was comparable to the wild-type replicon as shown in Fig. 2B. A ClaI site was not constructed in GT1a NS4A due to the failure of the ClaI site to replicate in the GT1b parental vector. The ClaI insertion caused the same amino acid change as in GT1b replicon. The replication competent GT1a NS4B shuttle vector was further modified to create a deletion shuttle vector, (GT1a NS4B Δ shuttle vector), that was replication incompetent, to ensure no background

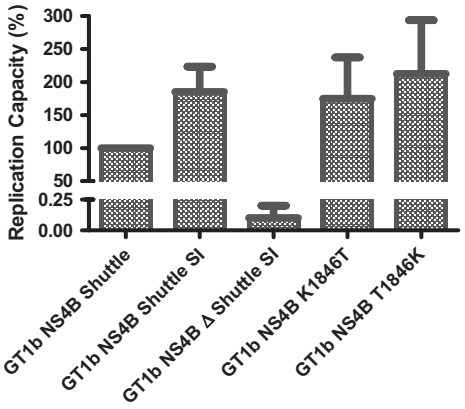

Fig. 2C. Comparison of the replication capacity of the GT1b NS4B shuttle vector, with NS4B K1846T mutation, and GT1b NS4B shuttle vector containing NS4B K1846T and NS5A S2204I adaptive mutation (SI). GT1a NS4B deletion (Δ) shuttle vector SI replication is restored when GT1b NS4B with NS4B K1846T or NS4B wild-type T1846K is shuttled in. Plotted values represent the means ± standard deviations (SD) of two independent experiments.

replication of the shuttle vector and that the replication levels were measured only from insertion of NS4B from clinical isolates. No replication was observed with the GT1a NS4B deletion vector.

3.2. Construction and replication of the GT1b NS4B shuttle vector containing S2204I

Replication of the GT1b NS4B shuttle vector can be greatly hindered when the replicon NS4B gene, which contains K1846T, an adaptive mutation, is replaced with the entire NS4B gene from clinical isolates. K1846T is not prevalent in NS4B from clinical isolates. Therefore, the replication of the shuttle replicon can be enhanced by introduction of another adaptive mutation: S2204I in the NS5A (Lemon et al., 2010). A modified GT1b shuttle replicon containing two adaptive mutations, K1846T in NS4B and S2204I in NS5A, (GT1b NS4B shuttle SI), was constructed and the replication of this GT1b NS4B shuttle vector was assessed to be robust (Fig. 2C). The replication of the deletion shuttle vector (GT1b NS4B Δ shuttle vector SI) was found to be fully replication defective (background luciferase activity).

3.3. Cloning and replication levels of GT1a and GT1b NS4B from replicon and clinical isolates

To re-establish the replication of the NS4B deletion shuttle vectors, NS4B genes from GT1a or GT1b PiRluc were amplified and fused with the corresponding GT NS4A by overlapping PCR. Using the cloning sites XbaI and BsrGI, the NS4A–NS4B overlapped fragment was cloned back into the corresponding deletion shuttle vector. The replication of the GT1a deletion vector was enhanced to 77% of the parental replication competent shuttle vector (Fig. 2B). The replication of the GT1b NS4B deletion shuttle vector was enhanced to 91% by cloning GT1b NS4B containing K1846T and to 110% by cloning in GT1b NS4B containing wild-type reverted T1846K (Fig. 2C). The replication of the GT1b NS4B shuttle vector in the absence of adaptive mutation K1846T was rescued by the presence of S2204I in the NS5A.

To investigate the genetic heterogeneity of HCV NS4B in untreated HCV infected patients, NS4B gene from twelve untreated GT1a and eleven untreated GT1b infected patients was cloned into the corresponding GT1a and GT1b NS4B shuttle vectors, respectively (Fig. 1B). The replication capacities of the GT1a clinical isolates were significantly higher than GT1b clinical isolates, suggesting better compatibility with the replicon backbone. The

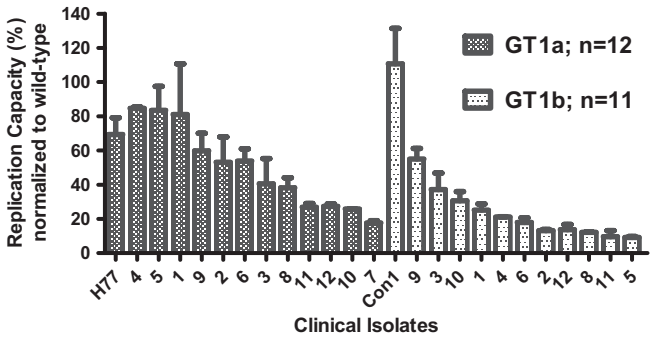

Fig. 3. Replication capacity of GT1a and GT1b NS4B clinical isolates compared to the control replicon NS4B from the corresponding genotype. Plotted values represent the means ± standard deviations (SD) of two independent experiments.

GT1b NS4B clinical isolates were replicating on average at 22% of the wild-type Con1 replicon. The signal to noise ratio was 800, which allowed the efficient measurement of the replication capacity and phenotypic analysis of the clinical isolates. GT1a NS4B clinical isolates replicated on average at 49% of the wild-type H77 replicon (Fig. 3).

3.4. Phenotypic analysis of GT1a and GT1b clinical isolates

The drug susceptibility of the GT1a and GT1b NS4B clinical isolates against the HCV NS4B imidazo [1,2-a] pyridine inhibitor GS-546288, structurally related to anguizole (Bryson et al., 2010; Florese et al., 2002), and the control HCV NS3 protease inhibitor GS-9451 (Waris et al., 2004) was determined using the transient assay (Fig. 4). The NS4B inhibitor GS-548288 EC_{50} values against the twelve GT1a NS4B isolates, were in the low nanomolar range (3–18 nM) and varied up to 6-fold among the isolates (Table 2), with the exception of clinical isolate 7, which showed an increase in EC_{50} of 29-fold relative to the wild-type 1a-H77 control. In contrast, the mean EC_{50} value of the GT1a clinical isolates against the control NS3 inhibitor GS-9451 was 17.8 ± 6.8 nM and was within 2-fold of the GT1a 1a-H77 wild-type control, demonstrating limited assay variation. The assay specificity for NS4B as the vector backbone for the clinical isolates is similar. The drug susceptibility of the GT1b NS4B clinical isolates against HCV NS4B inhibitor GS-548288 varied significantly among the eleven isolates, with GT1b clinical isolates 1, 2, 4, and 5 showing reduced susceptibility with increases in EC_{50} values ranging from 17- to 184-fold, compared to the control GT1b Con-1. The mean EC_{50} value of the GT1b clinical isolates against the control NS3 inhibitor GS-9451 was 8.6 ± 1.8 nM and was within 2-fold of the wild-type EC_{50} value, suggesting the variation of the drug susceptibility is specific to NS4B inhibition.

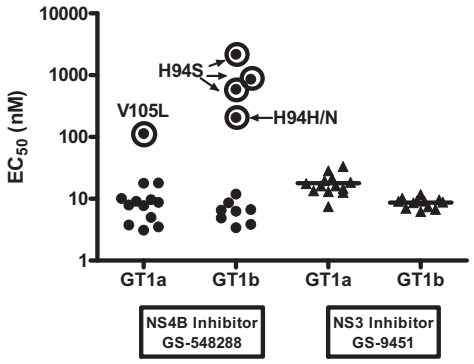

Fig. 4. EC_{50} values (nM) of GT1a and GT1b NS4B clinical isolates against NS4B inhibitor GS-548288 (●) and NS3 inhibitor GS-9451 (▲). Circled dots are clinical isolates with reduced susceptibility to GS-548288. NS4B polymorphism associated with reduced drug susceptibility is indicated. Plotted values represent the means ± standard deviations (SD) of two independent experiments.

3.5. Sequence analysis of GT1a and GT1b clinical isolates

Sequence analysis revealed natural variations present in the NS4B amino acids among these clinical isolates (Table 1 and Fig. 8). These natural variations in sequence may contribute to the small differences in drug susceptibility to the NS4B inhibitor GS-548288.

Analysis of GT1a clinical isolate 7 NS4B sequence, which demonstrated reduced susceptibility to NS4B inhibitor GS-548288, revealed a natural variation at amino acid residue 105 from valine (V) to leucine (L), which has been associated with NS4B inhibitor resistance (Bryson et al., 2010). Sequence analysis of the NS4B from the GT1b clinical isolates revealed variation in the amino acid residues compared to the GT1b Con1 reference strain with polymorphisms at residue 94 for clinical isolates 1, 2, 4, and 5 (Table 1). Polymorphisms at NS4B amino acid residue 94 have been shown to confer a reduced susceptibility to NS4B inhibitors (Bryson et al., 2010 and additional). Clinical isolates 1, 2, and 4 contain H94S which may be contributing to the observed NS4B inhibitor resistance phenotype (50, 184 and 73-fold shift in EC_{50}, respectively) while clinical isolate 5 contains an H94H/N mixture and a phenotypic shift of 17-fold against NS4B inhibitor GS-548288. GT1b clinical isolate 2, exhibiting reduced susceptibility of 184-fold in EC_{50}, contains a novel change at K18R in addition to H94S. Further evaluation of the clinical isolate 2 was done to determine if the combination of H94S and K18R may be causing a greater shift in EC_{50} compared to H94S alone. Two clones (A and B) from clinical isolate 2 NS4B pooled population were selected to revert K18R and/or H94S to wild-type (Fig. 5). Reverting K18R to wild-type with the H94S backbone did not change the EC_{50} fold shift of the two clones. In contrast, reverting the H94S to wild-type H94H, irrespective of the K18R mutation, increased inhibitor susceptibility from >843-fold to a 34 to 74-fold shift in EC_{50} to GS-548288 for the two clones, suggesting additional determinants such as I22L, T45A, and F90L in combination with H94S may be responsible for reduced NS4B inhibitor susceptibility.

3.6. Sensitivity of the NS4B phenotypic assay in the detection of resistance in a mixed population

Mutations H94R and V105M in GT1b NS4B have been identified as conferring resistance to the NS4B inhibitors. The sensitivity of the NS4B phenotypic assay was characterized in detecting the level of resistance of these two NS4B mutant replicons in a mixed population with wild-type GT1b. Resistance mutations H94R and V105M were created in the GT1b Con1 replicon and after in vitro transcription, the mutants were mixed in varying proportions with wild-type GT1b Con1 replicon. Changes in NS4B inhibitor GS-548288 sensitivity for different percentages of mutant/wild-type replicon mixtures are presented in Fig. 6. Significant fold shifts in EC_{50} (>3-fold) were observed for the NS4B mutant H94R at a ratio of 80% mutant/20% wild-type and for the NS4B mutant V105M at a ratio of 20% mutant/80% wild-type. In comparing the two resistant mutants at 50% mutant/50% wild-type, significantly higher levels of resistance were detected for V105M, with a 193-fold shift in EC_{50} for GS-548288 compared to a 4-fold shift in EC_{50} values for H94R. It has been shown that the levels of resistance detected are partially dependent on the replication capacity of the mutant in a mixed population (Qi et al., 2009). Therefore, we assessed the time course replication kinetics of the two NS4B mutants H94R and V105M in relation with GT1b wildtype replicon. The

Table 1
Polymorphisms present in the NS4B gene of GT1a and GT1b clinical isolates.

GT1a Subject	S 1	Q 2	Q 9	M 12	L 22	Q 26	R 30	T 36	A 38	V 39	N 42	V 48	T 91	G 93	I 100	V 105	A 117	A 121	L 123	A 124	I 128	E 161	V 162	T 165	P 225	A 231	A 235	I 253	S 255	C 257	T 258	T 259
1	–	–	–	L	–	–	–	A	T	–	–	T	–	S	–	–	–	–	–	T	I/V	–	–	–	–	–	–	V	–	–	–	–
2	–	–	–	–	–	–	–	A	A/T	–	–	A	–	S	–	–	–	S	I/L	–	–	D	–	M	-	A/T	A/T	–	–	–	V	–
3	–	–	–	–	–	–	–	A	V	A	S	A	S	–	–	–	–	–	–	–	–	–	–	–	Q	–	–	–	T	–	–	–
4	–	–	–	L/M	I	H	–	–	–	–	–	T	–	S	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	H	–	V	–	–	A	–	S	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	–	–	–	V	–	–	–	A	–	–	–	A	–	S	–	–	–	–	–	–	V	–	–	–	–	–	–	–	–	–	–	–
7	–	S	–	–	–	–	–	A	–	–	–	A	–	S	V	L	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–
8	–	–	–	–	–	–	–	A	–	–	–	A	–	S	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	–	–	H/Q	–	–	–	–	A/T	–	–	–	A	–	S	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	T	–	–
10	–	–	–	–	–	–	–	A/T	–	–	–	A	–	S	–	–	A/S	–	–	A/T	–	–	–	–	–	–	–	V	–	–	V	A
11	–	–	–	A/M/T/V	–	–	–	–	–	–	–	A	–	S	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	S	–	–
12	A	–	–	V	–	–	–	A	–	–	–	T	–	S	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

GT1b Subject	K 18	I 22	K 30	A 34	A 35	A 36	T 45	A 48	L 90	H 94	S 116	A 117	A 124	V 128	V 157	M 158	S 159	G 160	E 161	M 162	P 163	S 164	V 169	I 242	K 247	S 258
1	–	L	–	–	–	–	–	–	–	S	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	R	L	–	–	–	–	A	–	F/L	S	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	L	–	V	V	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	L	–	–	–	–	A	T	–	S	T	S	–	–	–	–	–	–	–	–	–	–	I	–	–	–
5	–	L	–	–	–	–	A	T	–	H/N	–	–	–	–	–	–	–	–	–	V	–	S/T	–	–	–	–
6	–	L	–	–	–	–	A	T	–	–	–	–	A/V	I/V	I/V	–	–	–	–	M/V	–	T	–	V	K/R	-
8	–	L	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–	–	D	–	–	–	–	–	–	–
9	–	L	–	–	–	A/V	A	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	–	L	R	–	–	–	A	T	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–
11	–	L	–	–	–	–	A	T	–	H/N/R/S	–	–	–	–	I/V	–	–	–	–	–	–	S/T	–	–	–	S/T
12	–	L	–	–	–	–	A	T	–	–	–	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–

Table 2
Susceptibility of NS4B sequences derived from clinical isolates to NS4B inhibitor GS-548288 and NS3 inhibitor GS-9451. EC50 values represent the means ± standard deviations (SD) of two independent experiments.

NS4B clinical isolate	Genotype	NS4B Inhibitor EC_{50} (nM) GS-548288	NS3 Inhibitor EC_{50} (nM) GS-9451
Con 1	1b	11.8 ± 2.6	11.7 ± 0.4
1	1b	589.4 ± 109.6	9.2 ± 1.9
2	1b	2176.4 ± 34.7	7.4 ± 0.5
3	1b	6.7 ± 2.4	7.0 ± 0.5
4	1b	859.8 ± 39.2	8.8 ± 0.9
5	1b	206.2 ± 71.7	6.8 ± 2.1
6	1b	6.5 ± 2.6	6.2 ± 0.02
8	1b	8.6 ± 3.5	9.1 ± 1.6
9	1b	6.3 ± 2.2	10.2 ± 0.48
10	1b	3.3 ± 1.3	9.1 ± 1.6
11	1b	3.8 ± 1.2	9.6 ± 1.3
12	1b	4.9 ± 1.9	8.6 ± 1.1
H77	1a	5.0 ± 1.0	19.5 ± 5.4
1	1a	3.5 ± 0.6	21.3 ± 5.2
2	1a	3.1 ± 0.7	16.1 ± 4.5
3	1a	9.6 ± 2.2	17.5 ± 3.8
4	1a	7.7 ± 2.0	33.1 ± 6.6
5	1a	17.9 ± 5.2	28.2 ± 8.7
6	1a	9.1 ± 2.1	13.1 ± 2.2
7	1a	113.4 ± 14.5	18.4 ± 2.2
8	1a	17.9 ± 2.1	13.5 ± 0.5
9	1a	10.1 ± 1.7	16.1 ± 2.0
10	1a	8.7 ± 1.1	7.5 ± 0.3
11	1a	7.9 ± 1.8	12.6 ± 1.4
12	1a	3.8 ± 0.4	14.6 ± 0.7

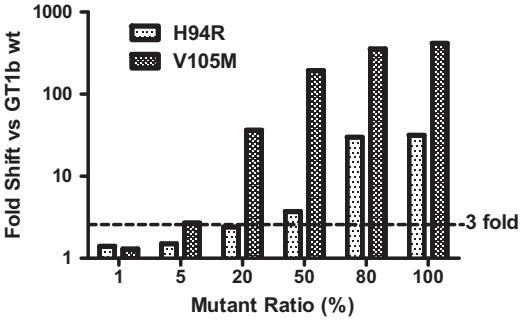

Fig. 6. Fold shift in EC_{50} values of NS4B inhibitor GS-548288 to mixtures of wild-type with either H94R or V105M mutant.

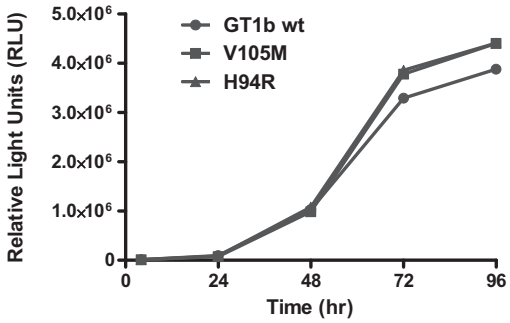

Fig. 7. Time course kinetics of NS4B mutants H94R or V105M in a mixed population with wild-type GT1b Con1 replicon. In vitro transcribed RNA of GT1b PiRluc wild-type and H94R or V105M PiRluc were mixed at a 50% ratio with GT1b PiFluc wild-type serving as internal control.

mutants with PiRluc reporters were mixed at a 50% ratio with wild-type replicon GT1b PiFluc which served as an internal control (Fig. 7). By 96 h, both the V105M and H94R mutants, when present at a 50% ratio in the mixed population, were as replication competent as the GT1b Con1 wild-type.

4. Discussion

In recent years, the cell culture adapted replicon and virus systems have proven to be advantageous in evaluating the antiviral activity of HCV inhibitors. However, these techniques have limitations since the evaluation of antivirals has been done against only a small subset of the HCV reference strains. Effective phenotypic systems that evaluate the antiviral activity of HCV inhibitors against naturally occurring HCV isolates have recently been established for NS3 protease, NS5A, and NS5B (Qi et al., 2009; Tripathi et al., 2007; Middleton et al., 2007; Li et al., 2012). The advantage of such chimeric shuttle vectors in a transient assay system include the shorter duration of time required to evaluate the ability of antivirals to inhibit diverse heterogeneous HCV natural isolates compared to establishing stable replicon systems over long periods. Importantly, these systems allow baseline and treatment induced heterogeneity in patient viral populations and their drug susceptibility to antivirals to be effectively studied using the chimeric replicons.

Studies describing the characterization of NS4B from clinical isolates have not been published. In this study, we describe the development of a chimeric replicon system for phenotyping

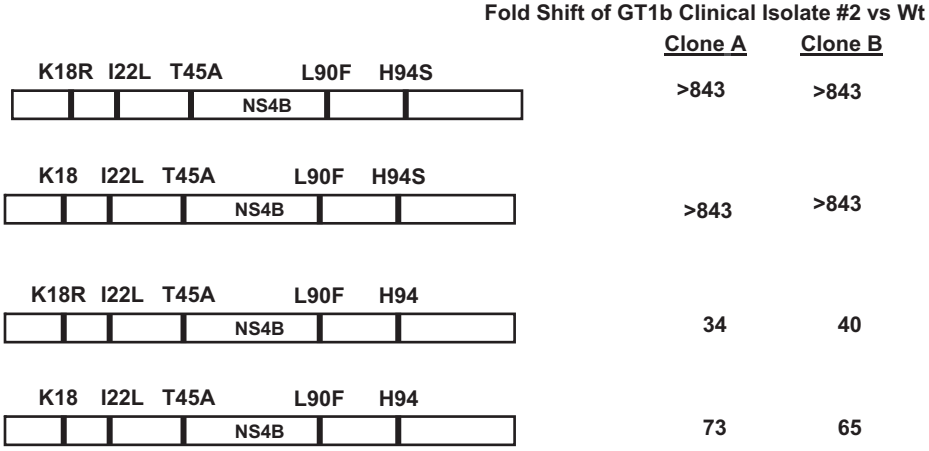

Fig. 5. Fold shift in EC_{50} of GT1b NS4B clinical isolate #2 representative clones after reversion of residues 18 and 94 to wild-type.

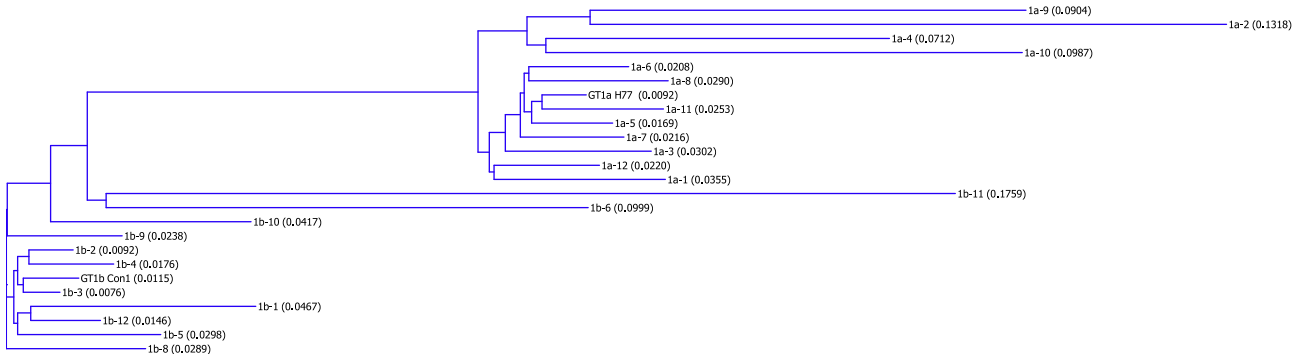

Fig. 8. A phylogenetic tree showing relatedness of the HCV NS4B clinical sequences is depicted.

NS4B from GT1a and GT1b HCV clinical isolates. Further characterization of the NS4B allowed us to assess the impact of natural variation in HCV NS4B from clinical isolates on the susceptibility to an NS4B inhibitor. The decision to construct subtype specific NS4B shuttle vectors was based on previous studies which have shown that GT1a replicon containing GT1b NS4B do replicate. However, GT1a NS4B does not replicate in a GT1b replicon (Blight, 2007). Hence, genotype compatibility of the NS4B from clinical isolates to the corresponding replicon backbone will likely increase the replication efficiency of NS4B clinical isolates. Both GT1a and GT1b NS4B shuttle vectors developed in these studies were efficient and reproducible in the replicon system. The cloning sites XbaI and BsrGI did not affect the replication levels of the GT1a and GT1b parental vectors. Although the BsrGI site in NS5A in the GT1a shuttle vector did cause an amino acid change, this change in this NS5A region was well tolerated. The introduction of a ClaI cloning site in NS4A provided the insight that the 3' region of NS4A does not tolerate amino acid change, as the ClaI site in NS4A led to an amino acid alteration and strongly decreased replication levels, but did not disrupt the NS3 cleavage recognition site.

Previous studies have shown the importance of the adaptive mutation K1846T in NS4B, in combination with two adaptive mutations in NS3, in the GT1b replicon (Lemon et al., 2010). Studies, however, have not demonstrated whether the two adaptive mutations K1846T and S2204I in the same GT1b replicon backbone act synergistically or antagonistically. In the present study, the GT1b NS4B shuttle replicon replication was robust in the presence of the adaptive mutations K1846T and S2204I either individually or in combination. Replacement of K1846T in NS4B with S2204I in NS5A rescues the replication of the GT1b NS4B shuttle replicon.

The design of the chimeric replicon allowed the study of individual NS4B genes from diverse clinical isolates. Cloning of NS4B from clinical isolates into the shuttle vector allowed the assessment of replication of diverse clinical isolates and their susceptibility to HCV inhibitors. Efficient replication levels were noted for the GT1a NS4B clinical isolates, which replicated at a mean of 49% of the wild-type GT1a replicon. Replication levels of GT1b NS4B isolates were approximately 22% of the wild-type. This reduction from wild type may be due to decreased compatibility to the replicon backbone and/or heterogeneity of NS4B viral sequences.

Alignment of the population sequences of NS4B from clinical isolates revealed naturally occurring polymorphic variation at various amino acid residues compared to the laboratory reference strain. Interestingly, the replication levels of clinical isolates can vary, however, the EC_{50} for the control NS3 inhibitor had minimal variation suggesting that the EC_{50} values are not dependent on the replication levels of clinical isolates. In contrast to the NS3 inhibitor, significant variation in susceptibility to the NS4B inhibitor was observed among the chimeric replicons carrying NS4B from the GT1a and GT1b clinical isolates. In NS4B, certain polymorphic amino acid residues can confer resistance to NS4B inhibitors (Rai and Deval, 2011). In GT1b NS4B clinical isolates 1, 2, 4, and 5, the presence of asparagine (N) and serine (S) at amino acid 94 may have contributed to reduced drug susceptibility to the NS4B inhibitor when combined with other polymorphic changes. In addition, a novel change observed at amino acid 18 from lysine (K) to arginine (R) was observed in clinical isolate 2, a rare change occurring at low frequency in ~3% of HCV-infected population (EU HCV database). Reversion to wild-type R at amino acid 18 did not restore the NS4B drug susceptibility in clinical isolate 2, while reversion to wild-type H at amino acid position 94 resulted in decreased resistance. This suggests that K18R alone does not confer reduced susceptibility to NS4B inhibitors, and other amino acid changes in combination with H94S might be responsible for the decreased susceptibility. The differences in susceptibilities for GT1a isolates were generally modest against the NS4B inhibitor, except for GT1a clinical isolate 7. Polymorphisms at residue 105, from valine (V) to methionine (M) and leucine (L), although highly conserved, have been associated with NS4B inhibitor resistance (Rai and Deval, 2011) and may be affecting the susceptibility of isolate 7. The effect of natural variation in NS4B on treatment outcome remains to be determined.

Drug resistance mutations can arise in HCV-infected individuals during an antiviral treatment that render the HCV quasispecies less susceptible. In this study, the sensitivity of the phenotypic assay to detect NS4B resistant mutants H94R and V105M was assessed in a mixed replicon population. The sensitivity of the assay is highly dependent on the mutant fitness and level of resistance conferred. The phenotypic assay was more sensitive in detecting resistance conferred by NS4B mutant V105M due to greater fold resistance observed to the NS4B inhibitor compared to H94R, even though both mutants display similar levels of replication. An interesting observation was that the EC_{50} fold shift for the NS4B mutant in a 50% mixed population, is significantly lower than 100% mutant suggesting that the presence of wild-type replicon contributes to the lower shift in EC_{50}. The sensitivity of the phenotypic assays is comparable to population sequencing in detecting mutant populations which is approximately 20%; in contrast the sensitivity of phenotypic assays in detecting mutants in mixtures can vary from 25% for NS3 R155K (Zhao et al., 2012), 25% for NS5B L419M (Li et al., 2012), 50% for NS3 A156T (Qi et al., 2009).

In summary, efficient HCV NS4B chimeric shuttle vector for phenotyping GT1a and GT1b clinical isolates were developed and characterized. The HCV NS4B assay was specific for determining the drug susceptibilities of HCV NS4B from HCV-infected patients against an NS4B inhibitor. The drug susceptibilities of clinical isolates to the NS4B inhibitor varied among isolates due to natural variation in NS4B, most notably for GT1b isolates. The HCV NS4B phenotypic assay can be used to detect phenotypic changes for NS4B inhibitors if the resistant mutant is present at >20–80% in a

mixed population with wild-type replicon. As potential NS4B inhibitors could become part of combination therapy for HCV, the use of a simple cell-based phenotypic assay may be beneficial and further aid in understanding the activity of the antivirals against naturally occurring HCV variants.

5. Conflict of interest

All authors are currently employees and stockholders of Gilead Sciences.

6. Submission declaration

Submission of this article implies that the work described has not been published previously and that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

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