



HCV-1b intra-subtype variability: Impact on genetic barrier to protease inhibitors



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ABSTRACT

Due to error-prone RNA polymerase and the lack of proofreading mechanisms, to the spread worldwide and probable long-term presence in human population, HCV showed a high degree of inter- and intra-subtype genetic variability.

Protease inhibitors (PIs), a new class of drugs, have been designed specifically on the HCV genotype 1 NS3 protease three-dimensional structure. The viral genetic barrier limits the efficacy of PIs, and fourteen loci in the HCV NS3 gene are involved in resistance to PIs. A sensitive method (15 UI/ml) for study the HCV genetic profile of 125 strains from patients naïve to PIs, was developed through the use of new degenerate primers for subtype 1b.

We observed the presence of naturally resistance-associated variants in 14% of the HCV strains (V36L, F43S, T54S, I153V, R155Q, D168A/G). T54S was the most common mutation (4%) detected.

We investigated, through minimal score (*m.s.*) calculating, how the HCV intra-subtype 1b variability modifies the genetic barrier to PIs. For >60% of strains a single transition (*m.s.* of 1) was required for selection of low to medium resistance mutations, while more than one transition/transversion (*m.s.* ≥ 2.5) or one transition plus one transversion (*m.s.* ≥ 3.5) was necessary for most of the high level PI-resistant-associated mutations, except for A156V, for which a single transition was sufficient (*m.s.* of 1). However, the presence at locus 36 of the amino acid polymorphism S36 in one case and the wild type V36 in 6 isolates, encoded by unusual GTA or GTG codons, might determined a higher probability of V36L/M mutations because of the reduction of the genetic barrier. Instead, the presence of the CGA and CGT codons in the 155th position increases the genetic barrier for R155M or R155Q/M.

The large intra-subtype variability, suggests that a routine baseline resistance test must be used before PIs-treatment.

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1. Introduction

Hepatitis C virus (HCV) is an enveloped positive single-stranded RNA virus from the family *Flaviviridae* and the genus *Hepacivirus*. HCV genome carries a single, long open reading frame (ORF) encoding a polyprotein that is cleaved co- and post-translationally into 10 different polypeptides. The serine protease encoded by the NS3 gene, has an essential role in the viral replication and is therefore the target for a new class of direct acting antivirals (DAA) compounds named protease inhibitors (PIs) (Pawlotsky et al., 2007). The high genetic diversity of HCV (genotypes, subtypes and quasispecies) is linked to the high viral production rates (10^{11} – 10^{12} virions/day), to error-prone RNA polymerase, and to

the lack of proof-reading mechanisms, which generate a high mutation rate ($>1/10^5$ nucleotides/replication cycle). All possible single and double nucleotide mutations are generated daily (Powdrill et al., 2011).

The genetic variability affects the response to old and new therapies. Indeed, the HCV genotype 1, the most widespread in the world, is associated with a minor rate of sustained virological response to standard of care therapy (pegIFN plus RBV) (Mchutchison et al., 2009). Although, the PIs-drugs has been designed specifically on HCV-1 NS3 protease three-dimensional structure, the different HCV-1 subtypes showed a diverse probability of development of resistance variants because of a diverse genetic barrier to PIs, defined as the number and the type (transition/transversion) of nucleotide substitution required to select mutations (Götte, 2012). Two linear peptidomimetics ketoamides, boceprevir (BOC) and telaprevir (TVR), have been approved in triple combination regimes with the standard of care for the treatment

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of HCV 1 chronic infections. Several linear and macrocyclic inhibitors, including BI-201335 (faldaprevir), TMC-435 (simeprevir), ITMN-191 (danoprevir), BMS-650032 (asunaprevir) and MK7009 (vaniprevir), are in various phases of clinical trials. Fourteen loci in the HCV NS3 gene are currently involved in resistance to PIs as demonstrated by replicon systems. The mutations are classified as low (0–10 fold), medium (10–100 fold), and high (>100 fold) according to the level of resistance caused *in vitro*, defined as the fold of change in the concentration of drug at which the HCV RNA level is reduced by 50% (EC₅₀) compared with wild type (Bartels et al., 2008). The mutations R155K/T/Q/M and A156M/S/T/V reduce the susceptibility to the most PIs. V36A/M/L, Q41R, F43S, T54A/S, V55A, R109K, I153V, and V170A/T are associated with resistance to the linear PIs, while the mutations Q80R/K, S122R, S138T, and D168A/V/T/H/N/G are selected only by macrocyclic PIs (Gaudieri et al., 2009; Lenz et al., 2010).

In the perspective of future IFN-free PIs therapy, we evaluated in HCV subtype 1b strains, from patients with chronic infection naïve to PIs, the intra-subtype variability to identify the frequency of resistance associated-variants (RAVs) and the presence of natural nucleotide and amino acid polymorphisms able to modify the genetic barrier to PIs.

2. Materials and methods

2.1. Samples

We studied 125 strains from Sicilian patients with HCV subtype 1b infection and chronic liver disease assessed by liver biopsy observed at the Liver Unit of the University of Palermo between November, 2008 and February, 2010. All patients (M/F: 75/50, mean age: 60 years) were HCV-RNA positive (mean level of serum HCV RNA: 4×10^6 UI/ml) and naïve for antiviral therapies. None were co-infected by HBV and/or HIV.

2.2. Methods

Viral RNA was extracted from 140 µl of serum using the QIA-amp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. A fragment of 495 bp encoding the HCV NS3 protease catalytic domain was obtained with an in-house RT nested PCR using new degenerate primers (NS3-3131F 5'-GG YTGATCATYACYAGYCT-3' and NS3-3675R 5'-AATGWCTCCGG YACRGC SG-3', NS3-3154F 5'-GGCCGGGAYAAACCAGGT-3' and NS3-3649R 5'-GARTTRTCCGTRAAACCGGG-3') designed on some Sicilian HCV-1b NS3 sequences obtained from patients with high viral load (HCV RNA >100000 UI/ml) by Sarrazin et al., 2007 protocols. The first round of PCR was done with the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. RT-PCR cycling conditions consisted of an initial incubation at 50 °C for 60 min, a hot-start at 94 °C for 2 min, 40 cycles, each at 94 °C for 45 s, at 52 °C for 45 s, and at 68 °C for 45 s, and a final step at 68 °C for 5 min. 5 µl of first-round PCR product were added to 45 µl of reaction mixtures containing the AccuPrime Pfx DNA polymerase system (Invitrogen, Carlsbad, USA). The nested PCR thermal cycle was characterized by a hot-start at 94 °C for 3 min, followed by 40 cycles, each at 94 °C for 30 s, at 55 °C for 30 s, and at 68 °C for 45 s, and by a final extension at 68 °C for 7 min. Standard procedures for reducing contamination were strictly followed. The sensitivity of the RT-PCR was tested using serial dilutions of Italian reference HCV-1b positive serum (HCV RNA ISS/1005, Pisani et al., 2007) containing 1000 IU/ml.

The nucleotide sequence of the sense and antisense strands was determined by direct sequencing, using the ABI Prism Big

Dye Terminator v 1.1 Cycle Sequencing Kit and an automated sequencer ABI Prism 3100 instrument (Applied Biosystems). HCV sequences found in GenBank (GenBank accession Nos.: AF009606, JN704295, AF369247, AF483269, AJ132996, AJ132997, AJ238799, AJ238800, EU155381, EU255382, EU482833, EU781832, JN120912, JN704289, JN704297, JN704299, U45476, AY651061, HM568433, AM910652, AF369216, AY051292) were used to confirm the HCV 1b subtype. All sequences were aligned with the Clustal W algorithm integrated into the BioEdit software, and the phylogenetic tree was constructed with the Mega 4.1 program using the Kimura two-parameter system and the Neighbor-joining method (bootstrap 1000 replicates). One consensus sequence, was constructed by Bioedit software using the 125 sequences obtained and choosing 60% of threshold frequency. Primary resistance mutations and/or natural polymorphisms were identified by visual inspection. The HCV genetic barrier was evaluated using the minimal score calculation: a score of 1 was assigned to each transition (A–G and C–T), and a score of 2.5 to each transversion (A–C, A–T, G–C and C–T) (van de Vijver et al., 2006; Svicher et al., 2011).

3. Results

Analysis of the amino acid sequences from 36 to 176 residues of the NS3 protein of HCV subtype 1b isolates obtained from 125 patients was done by an in-house direct sequencing method characterized by a sensibility of 15 UI/ml. The phylogenetic tree (Fig. 1) contained the 125 NS3 sequences and 22 HCV subtype 1 sequences found in GenBank, confirmed the 1b subtype for all isolates as determined by reverse dot-blot INNOLiPa (Siemens Healthcare Diagnostic, NY, USA).

HCV strains with mutations able to confer resistance to linear and macrocyclic PIs were identified in 18 of the 125 strains (14%). In particular, 1.6% isolates displayed a V36L mutation, 3.2% a F43S, 4% a T54S, 3.2% an I153V, 0.8% an R155Q, 0.8% a D168A, and 0.8% a D168G. These RAVs were detected as single mutations, except for one isolate in which two mutations were found (F43S plus T54S).

Amino acid polymorphisms V36G, V36S, Q80L, I153L, A156P, V170L, V170S, and E176Q were observed in 8 isolates, V55I in 2 isolates, and V170I in 62 isolates. Double and triple polymorphisms were detected in 3 different HCV strains (V36D + E176Q, Q80L + V170I and A156P + V170S + E176R).

The presence of these amino acid polymorphisms (Table 1) usually increased the genetic barrier to PIs, as evaluated by minimal score (*m.s.*) calculation, with 3 exceptions (S36, P156 and I170), which instead reduced it. The TCT codon encoding for serine in position 36 showed a *m.s.* of 1 for the S36L mutation versus a *m.s.* of 2.5 for GTT of the wild type valine. The P156 polymorphism reduced the *m.s.* from 2.5 to 1, and from 3.5 to 2 for P156S and P156G mutations, respectively. Polymorphism I170 showed a *m.s.* of 1 versus 2 for the consensus sequence for the V170T mutation.

Synonymous mutations in the nucleotide triplets for a wild-type amino acid change the genetic barrier at various levels of the *m.s.* In most HCV isolates the presence of polymorphic codons did not affect the genetic barrier to PIs, except for the 36th and 155th positions of the NS3 domain of 13 strains. The wild-type amino acid valine at position 36 was encoded by a GTA codon in 4 isolates, and by a GTG codon in 2 strains, thus determining a reduction of the *m.s.* for the V36 M from 3.5, calculated for the consensus sequence, to 2 for GTA, and to 1 for GTG. The CGA codon in the 155th position observed in 6 cases increased the *m.s.*, from 5 to 6, only for the R155 M mutation, and to 7.5 when the CGT codon was present (1 strain). The CGT codon also increased the *m.s.*, from 1 to 3.5, for the R155Q mutation (Table 2).

Table 2
Synonymous mutations able to modify the genetic barrier to PIs in 125 isolates of HCV-1b.

	Codon	No. of isolates	Mutation	<i>m.s.</i>	Mutation	<i>m.s.</i>	Mutation	<i>m.s.</i>	Mutation	<i>m.s.</i>
V36	GTC	42	V36M	3.5	V36L	2.5	V36A	1.0		
	GTT	72		3.5		2.5		1.0		
	GTA	4		2.0		2.5		1.0		
	GTG	2		1.0		2.5		1.0		
R155	CGG	117	R155K	3.5	R155T	5.0	R155Q	1.0	R155M	5.0
	CGA	6		3.5		5.0		1.0		6.0
	CGT	1		3.5		5.0		3.5		7.5

m.s.: minimal score, a score of 1 was assigned to each transition, and a score of 2.5 to each transversion.

the A156V mutation, able to increase 110–200 fold the resistance to telaprevir, faldaprevir and simeprevir. One transversion or one/two transversions plus one transition (*m.s.* of 2.5–5) were necessary for the selection of the main low to high primary mutations, such as the low-medium R155 G/M/T mutations, which showed and *m.s.* of 5, and the high resistance mutations F43V, R155K, A156T and D168A/H/Y/T/V with a *m.s.* of 2.5–3.5.

4. Discussion

The high genetic diversity of HCV, due to its high viral production rate and to the error-prone RNA polymerase, allows the virus to adapt rapidly to DAA drugs through the selection of RAVs, as demonstrated in both replicon and clinical studies (Bartenschlager and Lohmann, 2000; Lin et al., 2004). The first generation of PI drugs was designed specifically on the HCV genotype 1 NS3 protease three-dimensional structure. Mutations at positions V36, T54, R155 or A156 are the principal RAVs selected under PI therapy (Kieffer et al., 2007; Sarrazin and Zeuzem, 2010). Reductions in PIs efficacy were found in patients infected by different subtypes of genotype 1 or by non-genotype 1 (Cento et al., 2012).

Clinical studies of telaprevir alone or in combination with PEG IFN, the viral breakthrough and selection of RAVs have been observed more frequently in patients infected with HCV-1a than HCV-1b (McHutchison et al., 2009; Kieffer et al., 2007) because a single nucleotide change was required for the selection of V36M and R155K resistance mutations in HCV-1a while two changes in HCV-1b (Sarrazin and Zeuzem, 2010). In 2013 Cento et al. identified the uncommon HCV subtype 1g, in a non-responder to boceprevir-based therapy. HCV-1g NS3 sequences showed the resistance substitution T54S, plus P131S and L135F changes, located in the highly conserved NS3 positions within the boceprevir-binding site.

Because of HCV-1b variability of NS3 gene, to obtain a very sensitive protocol for HCV RNA detection (>15 UI/ml) and for assessing the resistance profile of 125 strains from PIs-naïve patients, we used new degenerated primers specifically designed on Sicilian HCV subtype 1b sequences. The phylogenetic analysis of these sequences confirmed the subtype 1b, previously assessed by LiPA, and ruled out uncommon HCV subtypes (1c–1m) (Nakano et al., 2012; Cento et al., 2013).

Primary mutations associated with low and medium level of resistance to PIs (V36L, F43S, T54S, I153V, R155Q, D168G) were found in 14% of the HCV strains, as reported in other studies of PIs-naïve patients (Vicenti et al., 2012; Paolucci et al., 2012; Bartels et al., 2013). D168A, a high resistance mutation, was identified in only one viral isolate (0.8%). T54S was the most common mutation (4%) detected in our study. Recently, an Italian study (Vicenti et al., 2012) found mutations V36L in 7% and T54S in 3.5% of 28 HCV subtype 1b isolates. The T54S mutation was associated *in vitro* with a low level of resistance to BOC and TVR, increasing the EC50 by 8.5 and 4.2-fold, respectively (Lenz et al., 2010). T54S was found, at the

time of treatment failure with TVR, in approximately 7% of HCV subtype 1b infected patients (Kieffer et al., 2012) and in a patient infected by HCV subtype 1g and not responsive to triple therapy with BOC (Cento et al., 2013). Two isolates with a T54S mutation were also characterized by the simultaneous presence of the polymorphism V55I. The T54S and V55I double variants have shown, in a replicon system, a clinically significant 7.9-fold increase in the TVR EC50. The V55I alone determined no significant increase in EC50 for BOC and only a 1.4-fold increase for TVR (Welsch et al., 2012). Q80K, which is expected to confer resistance to simeprevir and to a second generation PI-inhibitor ACH-1625 when found in HCV subtype 1a (Palanisamy et al., 2013; Fabrycki et al., 2012), was not detected in our population of 1b infected patients. V36M and R155K, the most common RAVs identified in HCV-1a isolates, were not found in our HCV-1b isolates according to the higher genetic barrier to generation them (Sarrazin and Zeuzem, 2010). Various amino acid polymorphisms were detected in RAV-associated positions, and the impact of the most of these unproven resistance mutations on the PIs susceptibility and on the HCV fitness has never been studied directly. Only V36G and Q80L polymorphisms have been partially characterized *in vitro*. The rare variant V36G determined an 11.2-fold decrease in sensitivity to TVR (Zhou et al., 2008), while the Q80L polymorphism was characterized by a 2.1-fold decrease in simeprevir susceptibility (Lenz et al., 2010).

An important viral factor affecting the outcome of drug therapy is the intrinsic viral genetic barrier to generating resistance mutations, which may also affect the likelihood of having RAVs.

With an HIV and HBV model (van de Vijver et al., 2006; Svicher et al., 2011), we analyzed the genetic barrier with the *m.s.* calculation, assigning different values for the type of nucleotide substitutions, because transitions, rather than transversions, were more likely to occur in HCV (Powdrill et al., 2011). HCV strains of different genotype and subtype are prone differently to the development of PI-resistant mutations (Cento et al., 2012), but little is known about how the intra-subtype variability influences the genetic barrier. Within subtype 1b, some viral isolates may have a lower genetic barrier as isolates belonging to 1a subtype.

In this study, for >60% of strains a single transition (*m.s.* of 1) was required for selection of low to medium resistance mutations, while more than one transition/transversion (*m.s.* ≥ 2.5) or one transition plus one transversion (*m.s.* ≥ 3.5) was necessary for most of the high level PI-resistant-associated mutations, except for A156V, for which a single transition was sufficient (*m.s.* of 1). Selection of this mutation, despite the very low genetic barrier observed, has rarely been found at the time of treatment failure, due to the reduction of replicative fitness, 80% less than wild type, associated with it (Lenz et al., 2010). In our preliminary study of HCV strains from patients under triple therapy with TVR, A156V variant was identified, after two weeks of treatment, in two patients with sustained virological response.

Some HCV subtype 1b isolates show an altered genetic barrier due to the presence of amino acid and/or nucleotide polymorphisms in resistance-related positions. Presence at locus 36 of

Table 3
Genetic barrier of >60% of HCV-1b isolates to low, medium and high resistance mutations.

Drug	Low resistance mutation (0–10-fold resistance)				Medium resistance mutation (10–100 fold resistance)				High resistance mutation (>100 fold resistance)			
	Mutation	FR	<i>m.s.</i>	RL (%)	Mutation	FR	<i>m.s.</i>	RL (%)	Mutation	FR	<i>m.s.</i>	RL (%)
Boceprevir	V36M	2.9	3.5	43	A156S	24	2.5	71	A156T	128	2.5	30
	F43S	3.5	2.5	63	V170A	19	1	30				
	R155K	3.6	3.5	40								
	T54A	6.7	1	61								
Telaprevir	V36L	2.2	2.5	56	V36G	11	2.5	ND	A156T	105	2.5	30
	T54S	4.2	2.5	ND	R155T	20	5	7	A156V	112	1	18
	R155M	5.6	5	33	A156F	65	3.5	ND				
	T54A	6.3	1	61								
	V36M	7	3.5	43								
	V36A	7.4	1	22								
	R155G	7.4	5	4								
	R155K	7.4	3.5	40								
	A156S	9.6	2.5	71								
Faldaprevir	V36M	2.1	3.5	43	R155Q	60	1	10	A156V	150	1	18
	Q80K	2.2	2.5	98	D168G	80	1	ND	A156T	270	2.5	30
	T54S	3.5	2.5	ND					R155K	350	3.5	40
									D168A	690	2.5	90
Simeprevir	Q41R	1.9	1	ND	F43I	89	2.5	ND	D168V	970	2.5	94
	Q80H	3.4	2.5	ND	F43V	99	2.5	ND	F43V	100	2.5	ND
	D168N	5.5	1	67	A156G	19	2.5	66	A156V	196	1	18
	Q80R	6.4	1	40	A156T	37	2.5	30	D168T	334	3.5	192
	Q80K	7.8	2.5	98					D168H	401	2.5	104–12
									D168Y	651	2.5	30
									D168A	784	2.5	90
									D168V	3100	2.5	94
									A156T	125	2.5	30
Vaniprevir	Q41R	3.3	1	ND					R155K	219	3.5	40
	F43S	4.9	1	63					D168Y	269	2.5	30
Danoprevir	Q80K	2.3	2.5	98					D168H	133	2.5	104
	Q80R	3.5	1	40					D168A	153	2.5	90
									D168T	300	3.5	192
									D168Y	391	2.5	30
Asunaprevir	V36A	2	1	22					R155K	410	3.5	40
	V36M	2	3.5	43					D168H	100	2.5	104
	F43S	4	2.5	63					D168A	127	2.5	90
	Q80R	4	1	40					D168Y	238	2.5	30
	A156T	6	2.5	30					D168V	280	2.5	94
	Q80K	6.5	2.5	98					D168H	100	2.5	104
	A156S	7	2.5	71					D168A	127	2.5	90
									D168Y	238	2.5	30

FR: fold resistance referred to HCV-1b (Lenz, Antimicrobial Agents and Chemotherapy 2010, McPhee, Antimicrobial Agents and Chemotherapy 2012, Mani, Ann Forum Collab HIV Res 2012). *m.s.*: minimal score, a score of 1 was assigned to each transition, and a score of 2.5 to each transversion. RL: replication level % of that for HCV-1b wild type (Lenz, Antimicrobial Agents and Chemotherapy 2010).

the NS3 protease of the amino acid polymorphism S36 in one case and the wild type V36 in 2 isolates, encoded by GTG codon as in HCV-1a, and in 4 isolates by GTA codon. The presence of these unusual codons might determined a higher probability of V36L/M mutations because of the reduction in the *m.s.* from 3.5 to 1 for GTG and to 2 for GTA. In these 6 isolates the presence, in 155 position of the common CGG codon (*m.s.* 3.5), reduced the probability of selection of double mutation (V36M + R155K) than HCV-1a. Instead, the presence in the 155th position of the CGA in 6 and CGT codons in 1 isolates, increases the genetic barrier for R155 M or R155Q/M.

The large NS3 genetic intra-subtype variability, associated with the selection of RAVs, suggests, as just reported (Poveda and Soriano, 2012), that a routine baseline resistance test must be used before PIs-treatment.

Due to the spread worldwide, and probable long-term presence in human population (Simmonds, 2004), HCV subtype 1b strains showed a high degree of genetic variability.

In Sicily, a southern Italian region of high prevalence for HCV, after introduction during the Second World War (Ferraro et al., 2008) the endemic subtype 1b infection (prevalence of 76.7%) (Pizzillo et al., 2009) evolved determining the selection of RAVs

(14% of cases) and strains with natural amino acid and nucleotide polymorphisms able to influenced the genetic barrier to DAA.

A very sensitive direct sequencing method may be a useful tools for the molecular characterization of HCV strains from patients prior and under therapy, from the identification of common and uncommon subtype to the study of genetic barrier and the kinetic evolution of RAVs. This could be important for the best use of combination regimens of HCV PI-inhibitors in future IFN-free regimens.

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