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ORIGINAL ARTICLE

Analysis of sequences of hepatitis C virus NS5A genotype 1 in HIV-coinfected patients with a null response to nitazoxanide or peg-interferon plus ribavirin

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Abstract Even though new drugs have been approved for treatment of hepatitis C virus (HCV) infection, the risk of drug-drug interactions and concern about overlapping toxicities has hindered the development of studies in HIV/HCV-coinfected individuals. Traditional treatment with pegylated interferon plus ribavirin (peg-IFN + RBV) is very expensive and has a low rate of sustained virological response in coinfected patients, especially if they are infected with HCV genotype 1. Nitazoxanide (NTZ) is a drug that is being evaluated for the treatment of chronic HCV infection, both in HCV-monoinfected and HIV/HCV-coinfected patients. Understanding the NTZ resistance mechanism could allow the development of resistance to be minimized and would expand the treatment options, mainly in special populations such as HIV/HCV-coinfected patients. Similarly to IFN, NTZ increases the activity of the cellular protein kinase activated by double-stranded RNA (PKR), a key kinase in the innate antiviral response. In order to elucidate whether sequence heterogeneity in the PKR-binding domain of HCV NS5A genotype 1 could influence the antiviral activity of either NTZ monotherapy or peg-IFN + RBV, baseline and end-of-therapy plasma samples from two groups of eleven non-responder HIV/HCV-coinfected patients that had received NTZ or peg-IFN + RBV were studied. Most of the HCV NS5A sequences examined at the end of therapy did not change from the baseline, even after 30 days course of antiviral therapy. An extensive comparison of HCV NS5A genotype 1 and 4 sequences from the database with reported IFN therapy outcome was performed in order to infer their phylogenetic relationships. The HCV genotype 1 NS5A nucleotide sequences from therapy-non-responder patients were intermingled amongst those from the database, irrespective of their IFN-therapy outcome. When comparing NS5A-PKRBD amino acid sequences, significant differences were observed in genotype 4, but not in genotype 1 ($p < 0.0001$ and $p > 0.05$, respectively). In conclusion, despite IFN and NTZ sharing the protein kinase activated by double-stranded RNA as their cellular target, the HCV genotype 1 strategy to counteract the IFN action mediated by NS5A ISDR/PKRBD does not explain drug resistance in HIV/HCV-coinfected patients. Other viral factors that are possibly involved are discussed as well.

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Introduction

Among patients parenterally infected with HIV, chronic hepatitis C is the most frequent cause of liver complications. The hepatitis C virus (HCV) genome consists of one 9.6-kb single-stranded RNA molecule with positive polarity. Like other positive-strand RNA viruses, the viral genome encodes an approximately 3000-amino-acid polyprotein that is proteolytically processed by cellular and viral proteases into structural and nonstructural (NS) proteins [1].

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Failure of interferon (IFN)-based HCV therapy is associated with high HCV-RNA plasma levels, infection by HCV genotypes 1 or 4 [2], unfavorable IL28B alleles, and/or advanced liver fibrosis [3]. Therapeutic options for this population are limited, and many coinfected patients with advanced liver fibrosis have already died and/or entered liver transplant lists [4]. Liver transplantation is not the ultimate solution for HIV/HCV-coinfected patients, given that HCV re-infection of the allograft is almost universal, and progression to cirrhosis is further accelerated in HIV/HCV-coinfected transplanted patients, with survival rates below 50 % at 5 years after transplantation [5]. New therapies for HCV include the development of agents that affect virus-specific targets such as the viral protease (NS3) and polymerase (NS5B) [6, 7]. Although these drugs show promising efficacy, rapid development of resistance is observed in many cases [8–12]. Therefore, it is necessary to develop agents with novel mechanisms of action that can both increase response rates and decrease the likelihood of resistance [13, 14].

Nitazoxanide (NTZ) is a thiazolide oral prodrug approved for the treatment of protozoal infections [15], and it has been reported to inhibit HCV replication *in vitro* [16]. Although NTZ is inactive against the enzymatic activity of HCV [17], it does mediate host-cell antiviral defenses by increasing the phosphorylation of eukaryotic initiation factor-2α (eIF2α), as well as by augmenting the phosphorylation of protein kinase activated by double-stranded RNA (PKR) [18]. These antiviral actions are shared with IFN-based therapy; consequently, it is plausible that the same NS5A-related mechanisms postulated for IFN resistance might also be responsible, at least in part, for NTZ failure. Such HCV strategies involve a well-defined carboxy-terminal NS5A domain: the PKR binding domain (PKRBD, amino acids 2209-2274), including the IFN-sensitivity-determining region (ISDR, amino acids 2209-2248), which is able to interact with and inhibit cellular PKR. This ISDR/PKRBD-mediated disruption of PKR dimerization results in the repression of the antiviral function of PKR by inhibiting PKR-mediated eIF-2α phosphorylation, and subsequently, protein synthesis. However, variations in these HCV NS5A domains could influence such mechanisms [19–23].

We previously reported that a thirty-day course of oral monotherapy with 500 mg NTZ twice daily (bid) did not produce changes in the HCV viral load in HIV/HCV-coinfected patients [24].

Although the PKR appears to be a shared cellular target for both NTZ- and IFN-based therapies, analysis of the relationship between ISDR/PKRBD sequence variations in HCV NS5A genotype 1 and NTZ and IFN resistance in HIV-coinfected patients did not *per se* provide an explanation for the treatment failure, and it suggested that the primary HCV resistance to both therapies is associated with host factors.

Materials and methods

Study samples

Forty-four HCV isolates from 22 HIV/HCV-coinfected patients were characterized at the NS5A-PKRBD/ISDR sequence level using paired plasma samples collected at baseline and after one month of therapy. The samples came from patients treated with two different therapies: (i) NTZ monotherapy (500 mg bid for 30 days, Roemmers Laboratories, Argentina) and (ii) pegylated IFN plus weight-adjusted ribavirin (peg-IFN + RBV) treatment (1.5 mcg/kg week Peg-Intron-A plus Rebetol 800-1200 every day, Schering Corp., Kenilworth, NJ). The latter was planned for 48 weeks, but it was discontinued if HCV RNA titers at week 12 did not drop by 2 logs compared with baseline values. All patients were adherent to peg-IFN/RBV and to NTZ.

HCV genotyping (Versant HCV Genotyping 2.0 Assay [LiPA]) revealed that all of the isolates were of genotype 1 (38 HCV-1a, 6 HCV-1b), thus minimizing the functional differences recently reported in NS5A according to the HCV genotype [25]. No significant changes ($p > 0.05$) were observed between baseline and end-of-therapy mean HCV RNA plasma levels (Bayer VERSANT HCV RNA 3.0 Assay as log10 IU/mL ± SD) for both groups of patients treated with either NTZ (5.98 ± 0.79 vs. 6.26 ± 0.40) or peg-IFN + RBV (5.5 ± 0.5 vs. 5.0 ± 0.8) [24] (Table 1). All of the subjects ($n = 22$) exhibited an undetectable plasma HIV viral load level (< 50 copies/mL) in response to a stable antiretroviral treatment, both at baseline and at the end of treatment.

Amplification and direct sequencing of HCV-NS5A ISDR/PKRBD

Viral RNA was extracted from plasma using a QIAamp Viral RNA Mini Kit (QIAGEN, Hamburg, Germany) following the manufacturer's instructions. The NS5A region was amplified using reverse transcription (RT) nested PCR with primers specific for subtypes 1a and 1b. The outer primers used for the amplification of NS5A were NS5Af (5′-CTCACYGTRACCCAGCTCCTGAG-3′) and NS5 AIA (5′-CCCCCTCMAGGGGGGGCATG-3′).

The inner primers used for subtype 1a were ISDRs (5′-TCCATGCTCACTGATCCCTC-3′) and ISDRa (5′-GT GAGGACCACCGTCC-3′), and for subtype 1b, they were ISDR1bF (5′-GCAGTGCTCACTTCCATGCTCA C-3′) and ISDR1bR (5′-GGACTCTAGCAGTGGAGG GTTGTA-3′).

Table 1 Demographic data of all study patients

Mean (± SD)	Nitazoxanide (n = 11)	Peg-IFN + RBV (n = 11)	p-value
Gender (M/F)	9/2	10/1	NS
Age (yrs)	41.9 (6.2)	41.3 (4.5)	NS
Weight (kg)	72.3 (11.8)	72.7 (12.3)	NS
CD4 + T (cell/mm3)	466 (148)	507.4 (257.2)	NS
ALT (U/l)	73.4 (31.3)	84.1 (41.2)	NS
Baseline HCV RNA (log copies/ml)	5.98 (0.79)	5.5 (0.5)	NS

The nested PCR products were sequenced directly and bidirectionally using an ABI PRISM 3100 Genetic Analyzer and a BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, USA).

Nucleotide and amino acid sequences and phylogenetic analysis

The nucleotide and deduced amino acid sequences of HCV NS5A ISDR/PKRBD obtained from both groups of patients were aligned using MAFFT [26] and compared at three different levels: first, at the intra-patient level, by comparing paired sequences obtained at baseline and at the end of therapy with NTZ or peg-IFN + RBV; second, at the inter-host level, by comparing the 44 NS5A sequences obtained from the 22 patients undergoing these antiviral therapies; and last, by comparing the NS5A sequences obtained prior to each therapy to NS5A sequences downloaded from a database (Los Alamos National Laboratory, http://www.lanl.gov). For this purpose, a dataset was constructed including all currently available NS5A sequences classified according to HCV genotype, such as 1a (n = 544) and 1b (n = 1055), together with available information about the outcome of IFN therapy, with the patients classified as "non-responder" or "sustained virological responder".

Considering both the NS5A -genotype-related differences in IFN antagonism [27] and the modest effectiveness of NTZ monotherapy in achieving a sustained virological response in patients who are chronically infected with hepatitis C virus genotype 4 [28–30], 43 NS5A genotype 4 sequences from patients with known peg-IFN + RBV therapy outcome were included in the analysis. Currently, there are no NS5A nucleotide sequences available from NTZ-treated patients in any database.

Different HCV genotype 1a, 1b, and 4 consensus sequences were constructed for each IFN therapy outcome in order to avoid concerns related to using an arbitrary isolate as a reference (Fig. 1).

To establish the phylogenetic relatedness of the sequences, reference sequences for HCV genotypes 2, 3, 5 and 6 were also retrieved, but without any information regarding therapy outcome. A pairwise identity matrix was generated using BioEdit 7.0.5 [31]. A maximum-likelihood phylogenetic tree was performed for the whole NS5A sequence database. The most appropriate substitution model for investigating our dataset was GTR + I + G, which was selected using jModelTest 3.7 software [32]. Phylogenetic relationships among HCV strains were estimated with PhyML version 3.0 [33]. The phylogenetic consistency of individual nodes in the most likely tree topology was estimated using a non-parametric bootstrap test (1000 replicates). The tree topologies were midpoint-rooted for illustration purposes and drawn using the Dendroscope v2.7.4 program [34].

Nucleotide sequence accession numbers

The sequence data reported in this paper have been deposited in the GenBank nucleotide sequence database with the accession numbers JX296115 through JX296136, JQ065825, JQ065827, JQ065829, JQ065831, JQ065833, JQ065835, JQ065837, JQ065839, JQ065841, JQ065843, JQ065845, JQ065847, JQ065849, JQ065851, JQ065853, JQ065855, JQ065857, JQ065859, JQ065861, JQ065863, JQ065865, and JQ065867.

Statistical analysis

ANOVA with Tukey's post-test (one-way ANOVA for comparisons between groups) was used. $P < 0.05$ was considered statistically significant.

Results

HCV-NS5A ISDR/PKRBD nucleotide and inferred amino acid sequence analysis

Once the intra-individual analysis of treated patients was carried out, the comparison between 44 NS5A ISDR/PKRBD amino acid sequences obtained at baseline and end-of-therapy showed that most of the paired sequences were conserved (Fig. 1; Table 2). At the inter-host analysis, several well-defined NS5A ISDR/PKRBD amino acid substitutions were found. The A2217T/V, E2228Q and I2252 V NS5A variations were the most frequently found among HCV-1a isolates, while T2217A and R2218C were observed in all HCV-1b isolates (Fig. 1).

In addition, when comparing the NS5A amino acid sequences of NTZ- or IFN-exposed patients to those of each IFN response group reported in the database, no

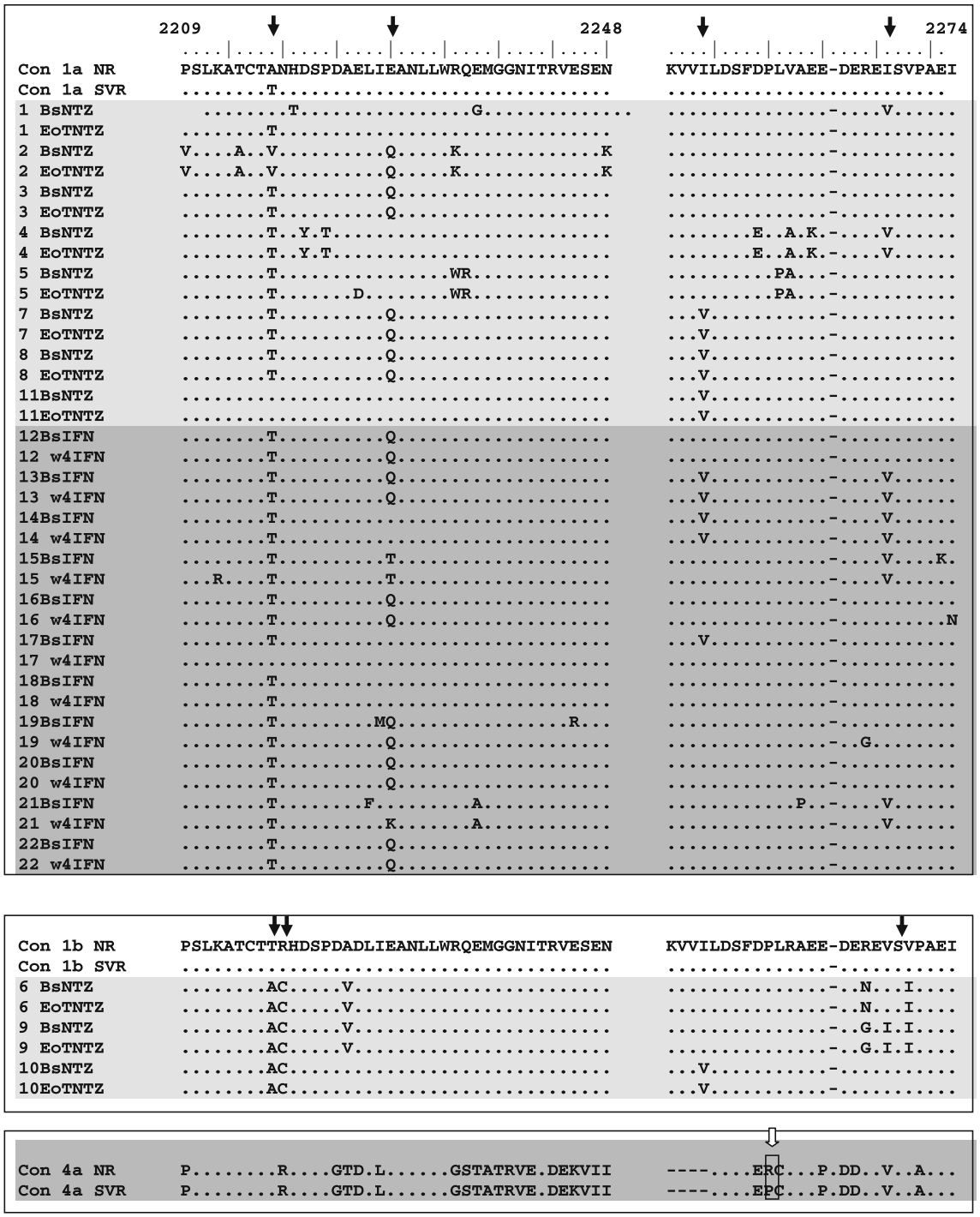


Fig. 1 Alignment of paired amino acid sequences of the HCV-1a (*top*), HCV-1b (*middle*), and HCV-4 (*bottom*) NS5A PKR-binding domain (amino acids 2209–2274), including the interferon sensitivity-determining region (ISDR), 2209–2248 and the region downstream from ISDR, 2249–2274. For each HCV genotype, consensus sequences ("Con") were created from database sequences (http://www.lanl.gov) and references [29] following the interferon response reported. The HCV amino acid sequences obtained from patients treated with NTZ appear in a *light gray block*, while those from peg-IFN + RBV-treated patients appear in a *dark gray block*. *Black arrows* indicate the most frequently mutated amino acid positions. *White arrows* indicate the discrepant amino acids between the 4a consensus sequences. Bs, baseline; EoT, end of treatment; w4, 4 weeks of IFN therapy; NTZ, nitazoxanide

significant difference was found, irrespective of the IFN therapy outcome ($p > 0.05$). As expected, after comparing HCV NS5A genotype 4 to genotype 1a or 1b sequences, significantly lower amino acid identity was found ($p < 0.0001$), regardless of the IFN or NTZ therapy outcome.

Phylogenetic analysis of NS5A-ISDR/PKRBD nucleotide sequences and its relationship to the IFN response

Phylogenetic analysis of NS5A ISDR/PKRBD nucleotide sequences revealed a clustering of pairs of sequences in the trees that was statistically supported by bootstrap analysis (≥95 %, Fig. 2). The analysis also showed that there were no specific clusters of baseline NS5A nucleotide sequences that appear to be related to the different observed responses to peg-IFN plus RBV therapy among HCV isolates belonging to genotypes 1a, 1b, and 4 (Fig. 2). Sequences from later time points were clustered according to the individual patients; that is, each patient's sequences more closely resembled other sequences from the same patient than they did those of other patients, irrespective of whether they were treated with peg-IFN + RBV or NTZ.

Discussion

The cost of the treatment of HCV infection with peg-IFN + RBV, especially with the addition of direct-acting antivirals (boceprevir and telaprevir) is very high for the health system in developing countries such as Argentina. In this scenario, it is of substantial interest to explore and understand the resistance phenotype to new and lower-cost drugs in order to minimize the development of resistance and expand the treatment options, mainly in special populations such as HIV/HCV-coinfected patients. The antiviral activity of both nitazoxanide and interferon-based therapies against HCV appears to be related to an immunomodulator stimulus of the host cell defense. Considering their common target, it is plausible that both therapeutic alternatives also share predictive factors for the response to therapy. In this regard, selected domains of the HCV nonstructural 5A region (NS5A) may repress IFN-induced RNA-dependent protein kinase (PKR) activity and thus have the potential to influence the response of HCV to both therapies.

We have recently reported the genetic heterogeneity of the NS5A and E2-PePHD regions of the HCV genome in HCV/HIV-coinfected individuals as a predictor of treatment outcome with combination therapy in Argentinean patients with chronic HCV genotype 1a infection [35, 36]. However this issue remains controversial for interferon-based therapy, and its relationship to the outcome of nitazoxanide treatment is still unexplored.

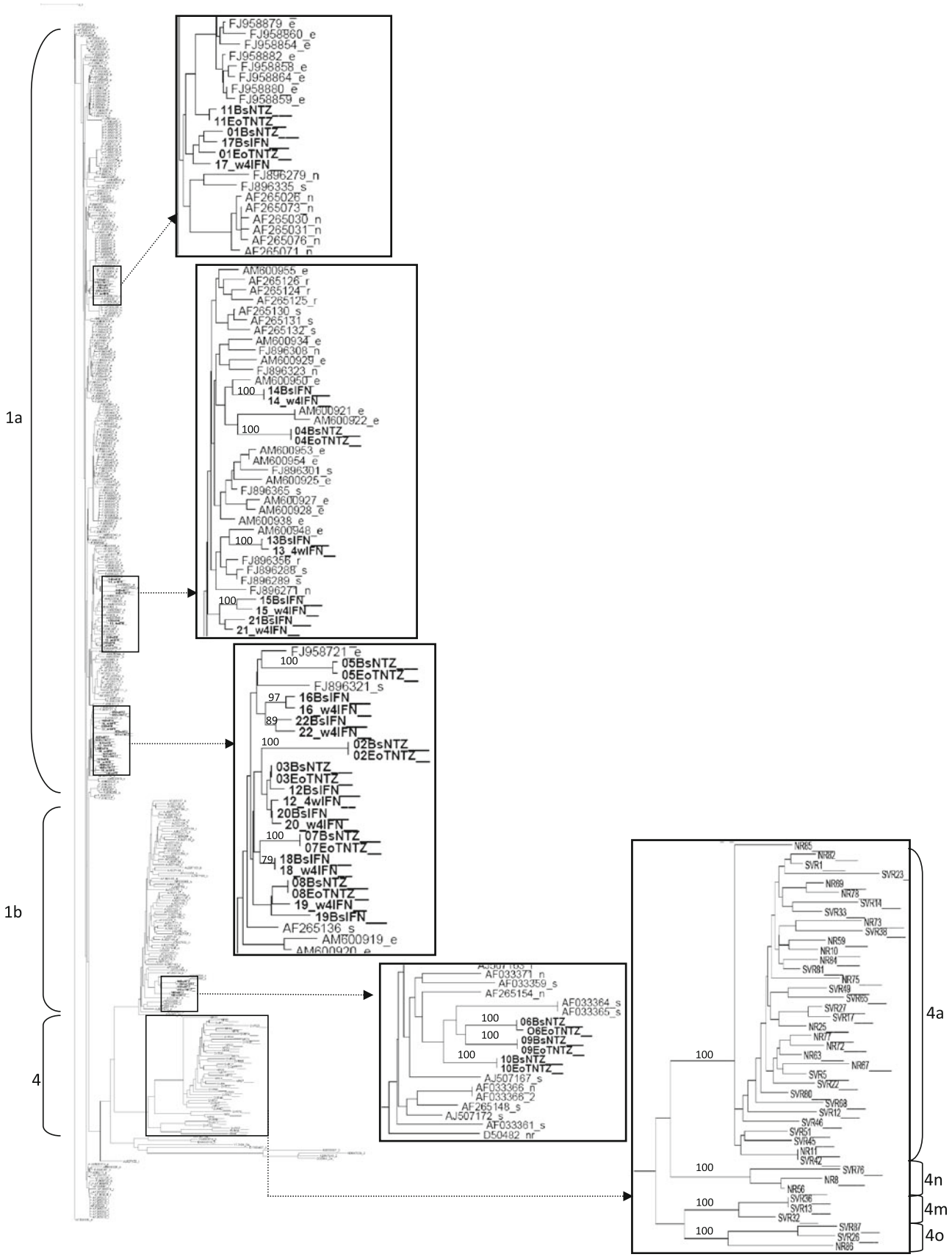
In the present work, analysis of the genetic heterogeneity of the HCV NS5A region from HIV/HCV-coinfected patients revealed a small number of amino acid substitutions and an almost unchanged HCV viral load within the first 4 weeks of therapy in both groups. Such findings could imply on one hand that the virus in non-responder patients is intrinsically highly fit and resistant to the antiviral effect of nitazoxanide or peg-IFN + RBV [37–39]. On the other hand, virus-mediated inhibition of the innate and/or adaptive immune responses may be responsible for this apparent absence of interferon/nitazoxanide pressure [40]. A limitation of this study is that the examination of consensus sequences is likely to be less sensitive than quasispecies analysis to determine changes in overall molecular patterns.

We found several NS5A ISDR/PKRBD amino acid substitutions at well-defined positions. Such changes could alter the protein tertiary structure, affecting its function and affinity for host or other viral regulatory sequences (i.e., the 5′-UTR) or generating proteins that further impact HCV RNA replication [17, 41]. The role of A2217T and I2252 V on IFN resistance was previously explored both *in vitro* [19, 21, 42, 43] and *in vivo* [44, 45]. We recently reported that the A2224 V and I2270 V mutations are associated with peg-IFN resistance in HIV-coinfected patients [46], yet their precise role remains to be elucidated. Nevertheless, given that these mutations were also detected in database NS5A sequences from patients with dissimilar IFN therapy outcome, even among sustained virological responders, its role remains controversial.

Previous reports have shown that NTZ seems to be modestly effective for patients monoinfected with HCV genotype 4. NTZ monotherapy in Egyptian patients infected with HCV-4, showed that HCV RNA became undetectable in 7 of 23 patients [28], but in a second study, a significantly higher rate of sustained response was observed in patients that received NTZ + peg-IFN + RBV triple therapy [47]. The marked amino acid differences in HCV ISDR/PKRBD between HCV-1 from HIV-coinfected patients and those with HCV-4 were expected. Such differences might impair the interaction

Table 2 Mean (± SD) of HCV NS5A ISDR/PKRBD amino acid mutations before and after nitazoxanide (A) or peg-interferon + ribavirin (B) treatment. p-values obtained using a correlated two-sample t-test are also shown

	Before (mean ± SD)	After (mean ± SD)	p-value
A			
PKRBD (2209–2274)	4.6 ± 1.6	4.5 ± 1.7	0.67
ISDR (2209–2248)	2.5 ± 1.0	2.5 ± 1.1	1.00
Downstream region from ISDR (2249–2274)	2.1 ± 1.2	2.0 ± 1.3	0.34
B			
PKRBD (2209–2274)	2.8 ± 1.2	2.5 ± 1.3	0.58
ISDR (2209–2248)	2.0 ± 0.9	1.8 ± 0.9	0.61
Downstream region from ISDR (2249–2274)	0.8 ± 0.9	0.8 ± 0.7	1.00


1a
1b
4
4a
4n
4m
4o
FJ958879_e
FJ958860_e
FJ958854_e
FJ958882_e
FJ958858_e
FJ958864_e
FJ958880_e
FJ958859_e
11BsNTZ
11EoTNTZ
01BsNTZ
17BsIFN
01EoTNTZ
17_w4IFN
FJ896279_n
FJ896335_s
AF265026_n
AF265073_n
AF265030_n
AF265031_n
AF265076_n
AF265071_n
AM600955_e
AF265126_r
AF265124_r
AF265125_r
AF265130_s
AF265131_s
AF265132_s
AM600934_e
FJ896308_n
AM600929_e
FJ896323_n
AM600950_e
100
14BsIFN
14_w4IFN
AM600921_e
AM600922_e
100
04BsNTZ
04EoTNTZ
AM600953_e
AM600954_e
FJ896301_s
AM600925_e
FJ896365_s
AM600927_e
AM600928_e
AM600938_e
AM600948_e
100
13BsIFN
13_4wIFN
FJ896356_r
FJ896288_s
FJ896289_s
FJ896271_n
100
15BsIFN
15_w4IFN
21BsIFN
21_w4IFN
FJ958721_e
100
05BsNTZ
05EoTNTZ
FJ896321_s
97
16BsIFN
16_w4IFN
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22BsIFN
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02EoTNTZ
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08EoTNTZ
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19BsIFN
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AM600920_e
AF033371_n
AF033359_s
AF265154_n
AF033364_s
AF033365_s
100
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10EoTNTZ
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AJ507172_s
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NR65
NR82
SVR1
SVR23
NR69
NR78
SVR14
SVR33
NR73
SVR38
NR59
NR10
NR84
SVR81
NR75
SVR49
SVR65
SVR27
SVR17
NR25
NR77
NR72
NR63
NR67
SVR5
SVR22
SVR80
SVR68
SVR12
SVR46
SVR51
SVR45
NR11
SVR42
100
SVR76
NR8
NR56
100
SVR36
SVR13
SVR32
100
SVR87
SVR26
NR88

◀**Fig. 2** Maximum-likelihood tree of 432 HCV sequences including 44 Argentinian HCV-1 sequences. The HCV sequences from this study are in bold and are defined according to therapy (NTZ or, IFN), sampling time (baseline –Bs- or, nitazoxanide end-of-treatment –EoT-, 4-weeks of peg-IFN + RBV therapy). HCV isolates obtained from the database are identified with GenBank accession number, while HCV genotype 4 isolates are identified with the isolate number from reference 30. Each HCV isolate is followed by the peg-IFN + RBV response as follows: "s" or "SVR", sustained virological responder; "r", relapser; "e", end-of-treatment; "n" or "NR", null-responder. Bootstrap values are shown on branches. Branch lengths are proportional to the number of nucleotide substitutions per aligned site (bar = 0. 1 substitutions)

with PKR, leading to a higher rate of response to NTZ. However, when comparing HCV-4 NS5A sequences obtained from patients exhibiting the opposite peg-IFN + RBV therapy outcome [48], the amino acid at position 2258 (R or, P) appears to be the only difference (Fig. 1). Further *in vitro* assays are necessary to elucidate its role on genotype 4 peg-IFN + RBV therapy response.

Finally, NS5A amino acid changes that had been reported previously in NTZ-resistant cell lines [17] were not observed among the HCV isolates characterized from NTZ-treated patients.

From these observations, the HCV NS5A ISDR/PKRBD-mediated IFN resistance mechanism did not *per se* explain the interferon or NTZ failure, and in agreement with previous reports [16, 17, 49, 50], the data indicate that the primary HCV resistance for both therapies is associated with host factors.

An independent HCV-related factor for NTZ monotherapy or the peg-IFN + RBV response is the plasma viral load [28, 51]. All patients that were recruited for this study exhibited high HCV-RNA plasma levels, suggesting an additional factor that might explain the poor response to both therapies in patients coinfected with HIV and HCV genotype 1.

More recently, another NS5A carboxy-terminal domain [52–55] as well as additional cellular factors able to interact with NS5A [56, 57] appear to be involved in the IFN response rate, but their effect on the NTZ response rate is unknown and deserves further investigation.

The impact of HIV coinfection on the observed NTZ therapy outcome is not known; however, considering that its antiviral action is dependent on the functional competence of the immune response, HIV coinfection could have impaired drug efficacy. It is therefore pertinent to consider that both cellular (i.e., TAR RNA binding protein, TRBP) [58] and HIV-related elements (i.e., Tat protein [59, 60]) are able to suppress the PKR-mediated antiviral response. These actions could contribute to reducing the HCV-1 rate of response to NTZ among HIV-coinfected patients. NS5A inhibitors might serve as a valuable component of future therapy for HCV-infected patients [61], and there are ongoing studies in HIV/HCV-coinfected patients in which NTZ and peg–IFN + RBV are combined in triple therapy [62, 63].

In conclusion, despite both IFN and NTZ sharing the PKR as a cellular target to exert their antiviral activity, the HCV genotype 1 strategy to counteract this action mediated by NS5A ISDR/PKRBD does not explain NTZ or peg-IFN + RBV failure to control HCV replication in HIV coinfection. In this context, whether the rate of NTZ response is dependent upon other viral factors (viral load, genotype, HIV-coinfection) remains uncertain, but it appears that NTZ monotherapy cannot overcome the host′s failure to control HCV-1 replication.

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