Research Article

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Viral Hepatitis

Post-treatment resistance analysis of hepatitis C virus from phase II and III clinical trials of ledipasvir/sofosbuvir

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Background & Aims: Ledipasvir/sofosbuvir combination treatment in phase III clinical trials resulted in sustained viral suppression in 94–99% of patients. This study characterized drug resistance in treatment failures, which may help to inform retreatment options.

Methods: We performed NS5A and NS5B deep sequencing of hepatitis C virus (HCV) from patients infected with genotype (GT) 1 who participated in ledipasvir/sofosbuvir phase II and III clinical trials.

Results: Fifty-one of 2144 (2.4%) (42 GT1a and 9 GT1b) treated patients met the criteria for resistance analysis due to virologic failure following the end of treatment. The majority of patients with virologic failure (38 of 51; 74.5%) had detectable ledipasvir-specific resistance-associated substitutions (RASs) at the time of virologic failure (1% deep sequencing cut-off). The percent of patients with NS5A RASs at virologic failure were 37.5%, 66.7%, 94.7% and 100% in patients treated for 6, 8, 12 and 24 weeks, respectively. The common substitutions detected at failure were Q30R/H, and/or Y93H/N in GT1a and Y93H in GT1b. At failure, 35.3% (18/51) of virologic failure patients' viruses had two or more NS5A RASs and the majority of patients harbored NS5A RASs conferring a 100–1000-fold (n = 10) or >1000-fold (n = 23) reduced susceptibility to ledipasvir. One patient in a phase II study with a known ledipasvir RAS at baseline (L31M) developed the S282T sofosbuvir (NS5B) RAS at failure.

Conclusions: In GT1 HCV-infected patients treated with ledipasvir/sofosbuvir ± ribavirin, virologic failure was rare. Ledipasvir resistance in NS5A was selected or enhanced in most patients with virologic failure, one of whom also developed resistance to sofosbuvir.

Lay summary: Clinical studies have shown that combination treatment with ledipasvir/sofosbuvir efficiently cures most patients with genotype 1 hepatitis C infection. For the few patients failing treatment, we show that resistance to ledipasvir was observed in most patients, whereas resistance to sofosbuvir was less common. This has important implications for the selection of optimal retreatment strategies for these patients.

Introduction

In recent years, direct-acting antiviral drugs (DAAs) developed for the treatment of hepatitis C virus (HCV) have replaced the previous standard of care of pegylated interferon and ribavirin. Inhibitors targeting the viral NS3/4A protease (telaprevir, boceprevir, simeprevir, paritaprevir and grazoprevir), NS5B polymerase (the nucleotide inhibitor sofosbuvir and the non-nucleoside inhibitor dasabuvir), and NS5A protein (ledipasvir, daclatasvir, ombitasvir and elbasvir) have been approved by the USA FDA, with more in late-phase clinical trials. Combination DAA regimens that are interferon-free have treatment success rates of well over 90% in most patients groups, and have become the preferred treatment option [1].

Sofosbuvir is a HCV NS5B-directed nucleotide inhibitor that displays potent pan-genotypic antiviral activity. Its effective concentration causing 50% maximal inhibition (EC_{50}) values range from 0.014 to 0.11 μM across HCV genotype (GT) 1–6 replicons [2].

Ledipasvir is a HCV NS5A-directed inhibitor that displays picomolar potency against HCV GT1a and GT1b *in vitro* and significant antiviral activity in HCV GT1-infected patients [3]. When tested against a panel of GT1a and 1b HCV clinical isolates, ledipasvir demonstrated EC_{50} values ranging from 0.004 to 0.085 nM [4]. In cell-based studies, replicons bearing resistance mutations to other investigational NS5A inhibitors were shown to be cross resistant to ledipasvir [5]. In addition, clinical studies have shown a selection of NS5A substitutions during monotherapy with ledipasvir [3,4].

Keywords: Hepatitis C virus; Drug resistance; NS5A; NS5B; DAA; Ledipasvir; Sofosbuvir; Ribavirin.

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Both sofosbuvir and ledipasvir have well-characterized *in vitro* resistance profiles. The primary *in vitro* resistance substitution for sofosbuvir is S282T in NS5B across all GTs; though it is rarely selected for *in vivo* [6,7]. Moreover, after sofosbuvir-based treatment, L159F and V321A have been observed to emerge in 15% and 5% of patients, respectively. Intensification of sofosbuvir treatment with ledipasvir reduced the emergence of L159F or V321A to 2% (1 of 50 each) at virologic failure [7]. Recently, E237G has been observed to emerge in a few patients treated with ledipasvir/sofosbuvir ± ribavirin [8,9]. The primary NS5A resistance-associated substitutions (RAS) selected by ledipasvir *in vitro* are Q30E and Y93H for GT1a and Y93H for GT1b [5]. The mutation spectrum in patients with GT1a infection after 3 days of ledipasvir monotherapy was more complex and included single or multiple mutations at NS5A amino acid positions 24, 28, 30, 31, and 93; while in GT1b infected patients, Y93H was the most prevalent RAS [4]. The following NS5A RASs confer a >100-fold reduction of *in vitro* susceptibility to ledipasvir in GT1a; Q30H/R/E, L31M, H58D and Y93C/H/N [4].

Analysis of baseline RASs in the phase II and III studies of ledipasvir/sofosbuvir and association with treatment outcome has been reported elsewhere [10]. High sustained virologic response 12 weeks after treatment (SVR12) were achieved even in the presence of baseline NS5A RASs, whether or not ribavirin was added to the regimen, in patients treated according to current guidelines [10]. Of the few patients not achieving SVR, we here present comprehensive HCV resistance analyses involving sequence-based and phenotypic analysis. Characterization of the resistance profile of these patients is of potential importance for determining optimal retreatment strategies.

Patients and methods

Ledipasvir/sofosbuvir clinical trials

Plasma samples were analysed from patients who did not achieve SVR12 enrolled in two phase II and three phase III clinical trials of ledipasvir/sofosbuvir containing regimens (Table 1) [11–15]. Before enrollment and before any study procedures were undertaken, written informed consent was obtained from all patients. The studies were conducted in accordance with the 1975 Declaration of Helsinki and Good Clinical Practice.

Resistance analyses

Resistance analyses included deep sequencing and phenotypic analysis for patients who met the criteria of the resistance analysis population. The resistance analysis population includes any patient who received at least one dose of a ledipasvir/sofosbuvir containing regimen, but did not achieve SVR12 due to virologic failure or early discontinuation, and had a HCV RNA $\geqslant$1000 IU/ml at the sequencing time point. NS5A and/or NS5B PCR amplicons at baseline and virologic failure time points, generated by DDL Diagnostic Laboratory (Rijswijk, The Netherlands) or Monogram Bioscience (South San Francisco, CA, USA), were subjected to deep sequencing primarily using the Illumina MiSeq platform (Illumina, San Diego, CA) at WuXi AppTec (Shanghai, China) or Monogram Biosciences. Internally developed software (Gilead Sciences) was used to process and align sequencing data. The consensus sequences are available at NCBI Genbank, (https://www.ncbi.nlm.nih.gov/genbank, accession numbers KY189697–KY189898). RASs from clinical trials were recently summarized by the HCV Drug Resistance Advisory Group (DRAG) group [16]. For patients with GT1a HCV infection, ledipasvir-specific NS5A RASs were defined as the following substitutions: K24G/N/R, M28A/G/T, Q30E/G/H/L/K/R/T, L31I/F/M/V, P32L, S38F, H58D, A92K/T, and Y93C/F/H/N/S. For patients with GT1b HCV infection: L31I/F/M/V, P32L, P58D, A92K, and Y93C/H/N/S [4,17–21]. NS5B nucleotide inhibitor (NI) RASs that are reported here are S96T, N142T, L159F, S282any, C289I/L, E237G, L320F/I/V, and V321A/I [6,8,9,22–27], of which S282T, L159F and V321A have been associated with resistance to sofosbuvir [6,28].

Phenotypic analyses

Phenotypic analyses were performed by Monogram Biosciences or by Gilead Sciences, Inc. [29]. Patient specimen-derived HCV NS5A or NS5B coding sequence was inserted in chimeric Con1 replicon vectors. RNA were transcribed from the vector *in vitro* and transfected by electroporation into "cured" HuH-7 cell lines. Transfected cells were cultured in the presence of different inhibitors in a range of concentrations. Luciferase activity, measured 3 days post-transfection, was used to derive EC_{50}. Inhibition data are reported as fold change relative to that of a reference vector (e.g., EC_{50} sample/EC_{50} reference) processed in the same assay batch (e.g., EC_{50} fold change from reference). In general, intra- and inter-assay precision is approximately 2–3-fold [30]. Luciferase activity in the absence of an inhibitor, expressed as a percentage of that generated by cells transfected with reference replicon RNA, is a measurement of replication capacity. Susceptibility data were analysed using Prism 6.0 (GraphPad, La Jolla, CA, USA).

Results

Of the 2144 patients initiating treatment with ledipasvir/sofosbuvir ± ribavirin within the five studies, 1603 patients had GT1a (74.8%), 529 had GT1b (24.7%) and 12 patients had other HCV genotypes or had unconfirmed genotypes (0.6%). A total of 51 out of the 2144 patients (2.4%) qualified for resistance analysis (Table 1); 42 patients had GT1a (82.4%) and nine patients had GT1b (17.6%). Forty-nine patients had HCV RNA suppressed on treatment but relapsed after completion of the treatment, while two patients treated for 24 weeks experienced breakthrough due to documented non-compliance during the dosing period (1 GT1a and 1 GT1b). Of the 51 patients not achieving SVR, 25% (13/51) had cirrhosis; 12 were treated for 12 weeks and one was treated for 24 weeks. In patients treated with ledipasvir/sofosbuvir ± ribavirin for 12 weeks (ION-1, ION-2, LONESTAR and ELECTRON), the number of patients with cirrhosis was higher in patients not achieving SVR compared with patients achieving SVR; 75.0% (12/16) *vs.* 18.0% (137/761), respectively (p value <0.001). For patients treated for 24 weeks (ION-1 and ION-2), 17.3% (113/654) of patients had cirrhosis of which only one experienced virologic failure. Of note, the recommended treatment duration for patients with cirrhosis treated with ledipasvir/sofosbuvir is 12 weeks in treatment-naïve patients and 24 weeks or 12 weeks with the addition of weight-based ribavirin in those with prior treatment experience and cirrhosis.

NS5A sequencing results

Deep sequencing data were obtained from all 51 patients in the resistance analysis population at both baseline and virologic failure time points. At baseline, NS5A RASs were observed in 43.1% (22/51) of patients; 45.2% (19/42) and 33.3% (3/9) of patients with GT1a- and GT1b-infection, respectively, using a 1% cutoff. At virologic failure, NS5A RASs were observed in 74.5% (38/51) of patients; 71.4% (30/42) and 88.9% (8/9) of patients with GT1a- and GT1b-infection, respectively (Supplementary Table 1).

In patients with GT1a-infection, 21.4% (9/42) and 38.1% (16/42) harbored $\geqslant$2 NS5A RASs at baseline and at virologic failure, respectively. Similar results were observed using a 15% cutoff (Table 2). The number of NS5A RASs per patient with GT1a-infection increased by treatment duration; for patients treated for 8 and 12 weeks, 28% (5/18) and 67% (10/15) of patients harbored $\geqslant$2 NS5A RASs at virologic failure, respectively (Supplementary Table 2). NS5A RASs were observed at amino acid positions 24, 28, 30, 31, 38 or 93, with the most predominant being Q30R and Y93H (23.8% for both) (Fig. 1A).