Virology 443 (2013) 278–284

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Emergence of resistance-associated variants after failed triple therapy with vaniprevir in treatment-experienced non-cirrhotic patients with hepatitis C-genotype 1 infection: A population and clonal analysis ☆

Richard J.O. Barnard [a,*], Carolyn M. McHale [a], William Newhard [a], Carol A. Cheney [a], Donald J. Graham [a], Amy L. Himmelberger [a], Julie Strizki [a], Peggy M.T. Hwang [a], Amber A. Rivera [b], Jacqueline D. Reeves [b], David Nickle [a], Mark J. DiNubile [a,**], Daria J. Hazuda [a], Niloufar Mobashery [a]

[a] Merck Sharp & Dohme, Whitehouse Station, NJ, USA
[b] Monogram Biosciences Inc., South San Francisco, CA, USA

ARTICLE INFO

Article history:
Received 26 March 2013
Returned to author for revisions
18 April 2013
Accepted 10 May 2013
Available online 10 June 2013

Keywords:
Vaniprevir
Hepatitis C-genotype 1
Resistance-associated variants

ABSTRACT

Background: Vaniprevir with P/R improved SVR rates over P/R alone in treatment-experienced patients with chronic HCV-genotype 1 infection, but treatment failure presents therapeutic challenges. We identified RAVs from non-cirrhotic patients failing to achieve SVR on vaniprevir-containing regimens from a dose/duration-ranging trial of triple-combination therapy.
Methods: Using population analysis, resistance sequencing was performed on all baseline samples and on samples at virologic failure in the vaniprevir arms. Longitudinal clonal analyses were performed on viral isolates from six vaniprevir recipients experiencing breakthrough viremia.
Results: Baseline RAVs were detected in two patients subsequently experiencing virologic failure. At virologic failure, the majority of RAVs had substitutions at R155, A156, or D168. Clonal analyses identified novel double/triple variants emerging with continuing vaniprevir dosing.
Conclusions: RAVs were predominantly observed at R155, A156, and/or D168 during virologic failure on vaniprevir/P/R. Double/triple RAVs were identified in patients remaining viremic on triple therapy, suggesting evolution of resistance under selective pressure.

Introduction

Peginterferon-alfa and ribavirin (P/R) had been the standard of care for chronic hepatitis C virus (HCV) infection until the recent introduction of direct-acting antiviral agents (Manns et al., 2001; Fried et al., 2002; Ghany et al., 2011). The two licensed NS3/4A serine protease inhibitors [boceprevir (VICTRELIS™; Merck, Whitehouse Station, NJ, USA)] and [telaprevir (INCIVEK™; Vertex Pharmaceuticals, Cambridge, MA, USA)] when added to P/R therapy significantly improved sustained virologic response (SVR) rates in previously untreated patients as well as in patients who had partially responded to or relapsed after a standard course of P/R alone (Poordad et al., 2011; Jacobson et al., 2011; Sherman et al., 2011; Bacon et al., 2011; Zeuzem et al., 2011). Accumulating evidence indicates that even previous null responders have an appreciable chance at SVR with the newer regimens. However, virologic failure ultimately occurs in >20% of patients with genotype 1 HCV infection given triple therapy (Poordad et al., 2011; Jacobson et al., 2011; Sherman et al., 2011; Bacon et al., 2011; Zeuzem et al., 2011).

The low fidelity of the HCV RNA-dependent RNA polymerase coupled with the high rate of virion production gives rise to a population of genetically related viruses in an infected patient that harbor minor differences in their RNA genome. As a consequence, it is likely that the majority of single and double-resistant resistance-associated variants (RAVs) are present prior to treatment with direct acting antiviral (DAA) agents (Ribeiro et al., 2012). HCV protease inhibitors are routinely given as part of combination regimens with P/R in clinical practice (Ghany et al., 2011) or with other direct-acting antiviral agents in experimental trials.

Abbreviations: DAA, directly acting antiviral agent; HCV, hepatitis C virus; IC50, inhibitory concentration 50%; IU/mL, international units per milliliter; LLD, lower limit of detection; LLQ, lower limit of quantification; P/R, peginterferon alfa/ribavirin; SVR, sustained virologic response

☆The parent study and the current analysis were sponsored and funded by Merck.

* Correspondence to: Merck Research Laboratories, West Point, PA, USA.
** Corresponding author.
E-mail addresses: richard_barnard@merck.com (R.J.O. Barnard), mark_dinubile@merck.com (M.J. DiNubile).

0042-6822/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2013.05.013

Virologic failure in patients treated with DAAs is often accompanied by the emergence of viral variants with resistance to the inhibitor class, and detectable RAVs can persist up to three years after cessation of therapy (Sarrazin and Zeuzem 2010; Gambarin-Gelwan and Jacobson 2012; Welsch and Zeuzem 2012). Accordingly, the enduring clinical implications of RAVs are not yet fully understood.

Vaniprevir (MK-7009) is an investigational macrocyclic NS3/4A protease inhibitor which exhibits potent activity against HCV-genotype 1. In vitro resistance selection experiments and sequence data from Phase 1 and 2a clinical studies with vaniprevir have identified several viral NS3/4A protease variants associated with decreased susceptibility to vaniprevir. A recently completed dose/duration-ranging study (Protocol 009) of vaniprevir combined with P/R in treatment-experienced patients demonstrated higher SVR rates than those previously reported with the approved first-generation protease inhibitors combined with P/R (Lawitz et al., 2012). Using samples from the non-cirrhotic cohort of this Phase 2b trial, the current report examined the types and frequencies of vaniprevir RAVs detected in viruses isolated from patients randomized to vaniprevir/P/R at baseline and at the time of virologic failure. Since the technology for HCV population sequencing is widely available, our results can be directly applied to contemporary clinical practice. In addition, the evolution of resistance was examined by longitudinal clonal sequence analysis in six patients that experienced virologic failure with vaniprevir RAVs.

Results

Of the 169 treated non-cirrhotic patients in the four vaniprevir groups (Fig. 1), 73 (43%) had genotype 1a virus, 94 (56%) were infected with genotype 1b virus and two (1%) patients were infected with a genotype 1 virus with an indeterminate subtype. A total of 157 vaniprevir recipients (93%) were included in the primary efficacy analysis: 67 with genotype 1a virus (43%), 88 with genotype 1b virus (56%), and two (1%) with an indeterminate genotype 1 subtype. Patient histories of prior P/R failure in the primary efficacy population encompassed 62 relapses (39%), 42 null responses (26%), 30 partial responses (19%), and 23 virologic breakthroughs (15%).

Baseline population NS3/4A-sequence data were obtained for the 155 patients with typeable HCV infections included in the primary analysis. Seven (5%) patients had viruses that harbored variants with a decreased resistance to first-generation HCV protease inhibitors, including V36M, T54S, V55A/I/T/V, and R155K (Table 1). NS3/4A genes harboring variants at positions V36 and T54 confer a <4-fold decrease in sensitivity to MK-7009 in vitro with a cell-based assay. Although variants at these positions have not been identified in patients dosed with vaniprevir or in replicon resistance selection studies *in vitro*, these variants caused a synergistic decrease in sensitivity to vaniprevir when combined with the R155K variant. Notably two patients who had been null responders on their previous P/R regimen harbored R155K variants in combination with either V36M or T54S at baseline, both of whom experienced virologic failure on vaniprevir/P/R; one patient had a null response and the other had breakthrough viremia.

Population resistance analyses revealed that the majority of emergent RAVs had amino acid substitutions at positions R155, A156, or D168 (Table 2), all of which conferred decreased vaniprevir susceptibility relative to the reference strains (Table 3). Of the 28 patients who experienced virologic failure in the vaniprevir-dosing arms of this study, resistance data were available at virologic failure for 26 patients. The NS3/4a gene could not be amplified from samples obtained at virologic failure from the remaining two patients. Of the 26 patients with resistance data at virologic failure, 17 had genotype 1a virus and 9 had genotype 1b virus. RAVs were detected in 17 of 17 (100%) genotype 1a infections and 9 of 9 (100%) genotype 1b infections. The most frequently detected protease variants associated with virologic failure among genotype 1a infected patients were at positions R155, A156, and D168, whereas the most commonly detected protease variants among patients with genotype 1b infections were at positions D168 and A156. Similar to other studies with first-generation HCV proteases, R155K was only found by population sequencing in genotype 1a.

All 26 patients included in the primary efficacy analysis whose HCV isolate was sequenced at the time of virologic failure had RAVs (Table 4). Of these 26 vaniprevir recipients experiencing virologic failure with sequence data, 12 had virologic breakthrough, 1 had a null response, 2 had partial responses, and 11 had relapses. In the 12 patients who experienced virologic breakthrough, variants were detected at positions R155K, A156, and D168. Compared to patients with relapse, partial response, or null response, patients with breakthrough more often had RAVs detected at more than one locus. RAVs were detected at multiple loci (R155/D168, R155/A156, or A156/D168) in 5/12 breakthrough patients, while the remainder had variants at only R155 or D168 detected. Of the 11 relapse patients, only one had RAVs detected at more than one locus (R155K and D168V). The remaining patients

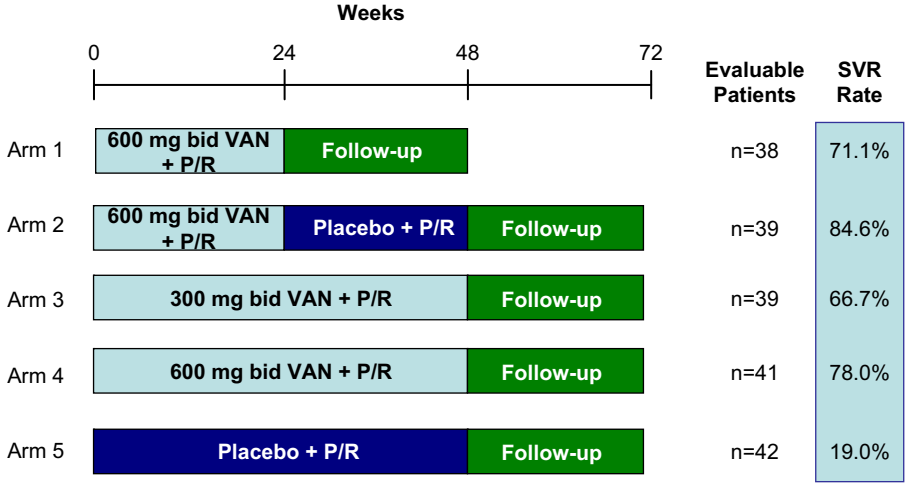

Fig. 1. Dosing arms for MK-7009 P009 Phase 2b trial in treatment-experienced non-cirrhotic patients. After stratification by type of previous failure, patients were treated with 4 regimens combining vaniprevir with P/R (arms 1–4). The control arm (arm 5) used PR alone.

had RAVs detected at position R155 or D168 alone. The patient with a null response had RAVs detected at R155K while the two patients with partial responses had D168T detected at virologic failure. Other than the presence of RAVs at more than one locus in breakthrough patients, there was no specific variant associated with a particular type of treatment failure.

By design, stopping rules for futility were not stipulated until week 12. Therefore, patients were kept on vaniprevir or placebo and P/R therapy until week 12 of the study irrespective of their virologic response during this period. In order to characterize the evolution of RAVs in patients under these circumstances, longitudinal clonal analysis was performed on six patients with breakthrough viremia (4 with genotype 1a virus and 2 with genotype 1b virus) who had variants detected at positions R155 or D168 at virologic failure by population sequencing. In this analysis, 40–80 clones were sequenced at each time point, allowing linkage of any double/triple variants to be established.

In all patients, clonal analyses revealed that the overwhelming majority of viruses in each patient sequenced at virologic failure harbored vaniprevir RAVs indicating that wild-type viruses were suppressed during vaniprevir/P/R dosing (Fig. 2). In addition, the number of RAVs detected after virologic failure increased during continued vaniprevir/P/R dosing so that double/triple RAVs were detected more frequently in most patients at later time points. In 2/6 genotype 1b patients, single RAVs were observed at position D168 at virologic failure. However, in one patient, double RAVs were observed at positions R155 and D168 20 days after virologic failure, during vaniprevir/P/R dosing. This pattern of increasing RAV frequency and the selection of double-RAV viruses was observed in all genotypes 1a and 1b infected patients during the vaniprevir/P/R dosing period after virologic failure. The number of double/triple RAVs declined after discontinuation of therapy.

Table 1
Summary of HCV-protease variants detected at baseline by population sequencing from vaniprevir recipients according to HCV genotype.

NS3/4A Protease variants[a]	Genotype 1A ($N=73$ patients)	Genotype 1B ($N=94$ patients)
V36M	2	None
T54S	1	2
V55A	3	None
V55I	2	None
T54S, V55I	1	1
V36M/L, T54T/S, V55T/I/A/V	1	None
T54S/R155K[b]	1	None
V36M/R155K[b]	1	None

[a] 167 of the 169 baseline isolates were typeable and are listed here.
[b] Only these 2 variants which included R155K exhibited phenotypic resistance to vaniprevir. Both patients with genotype 1A HCV harboring R155K variants experienced virologic failure.

Discussion

The parent Phase II study evaluated four treatment regimens combining vaniprevir with P/R compared to 48 weeks of PR alone

Table 2
Summary of emergent HCV-protease variants during or after vaniprevir regimens.

Vaniprevir dosing arm	N in primary analysis[a]	N failed	N with VF	N with sequence data	N with RAV	HCV genotype	Category of virologic failure	RAVs detected by population sequencing
Arm 1 V600/P/R × 24 wk	38	11	9	8	8	1A	Breakthrough	R155K, D168F/N/T/A/Y/S
						1A	Breakthrough	R155K, D168Y
						1A	Relapse	D168V
						1A	Relapse	D168V
						1A	Relapse	D168H
						1A	Relapse	R155K, D168V
						1A	Relapse	R155K
						1B	Relapse	D168T
Arm 2 V600/P/R × 24 wk+P/R × 24 wk	39	6	3	3	3	1A	Null response	R155K
						1B	Breakthrough[b]	A156G, D168T
						1B	Breakthrough[b]	D168T
Arm 3 V300/P/R × 48 wk	39	13	11	10	10	1A	Breakthrough	R155K
						1A	Breakthrough	R155K
						1A	Breakthrough	D168T
						1B	Breakthrough	D168 Y,V
						1B	Breakthrough	D168E
						1B	Breakthrough	D168V,Y,F
						1A	Relapse	R155K
						1A	Relapse	R155K
						1A	Relapse	R155K
						1B	Partial responder	D168T
Arm 4 V600/P/R × 48 wk	41	9	5	5	5	1A	Breakthrough	R155K, A156S
						1A	Breakthrough	R155K, D168N
						1B	Partial Response	D168T
						1A	Relapse	R155K
						1B	Relapse	D168A

N, number of non-cirrhotic patients in the designated category; V, vaniprevir; P, peginterferon alpha; R, ribavirin; VF, virologic failure.
Variants detected at baseline are not tabulated here. No RAVs were identified in patients dosed with PR alone.
[a] These counts do not include patients discontinued for administrative reasons who had undetectable viral loads at the last visit on record.
[b] The two viral breakthroughs in Arm 2 occurred within the first 24 weeks of treatment while the patients were still receiving vaniprevir.

Table 3
In vitro phenotypic data for RAVs using a secreted alkaline phosphatase assay.

Genotype	Variant	IC50 (nM)	Fold-change in IC50
1a	Wild type (H77)	7.97	N/A
	V36A	15.33	1.9
	V36M	28.87	3.6
	V36M/R155K	4589	575.8
	T54A	19.96	2.5
	T54S	13.17	1.7
	T54S/R155K	2341	293.7
	R155K	342	42.9
	R155K/A156S	6589	826.7
	A156S	52.03	6.5
	A156T	210.4	26.4
	A156V	1151	144.4
	D168A	746.2	93.6
	D168E	98.88	12.4
	D168F	6753	847.3
	D168H	1053	132.1
	D168N	159.5	20.0
	D168T	4397	551.7
	D168V	1485	186.3
	D168Y	1244	156.1
1b	Wild type (Con1)	6.803	N/A
	V36A	4.213	0.6
	V36M	14.92	2.2
	T54A	4.69	0.7
	T54S	1.463	0.2
	T54S/R155K	2698	396.6
	R155K	445.5	65.5
	R155Q	575.2	84.6
	R155Q/D168N	4447	653.7
	A156T	403.6	59.3
	A156V	550.9	81.0
	A156S	19.51	2.9
	D168N	200.5	29.5
	D168A	1353	198.9
	D168E	141.7	20.8
	D168F	7621	1120.2
	D168H	529.9	77.9
	D168T	4911	721.9
	D168V	451.4	66.4
	D168Y	1899	279.1

Table 4
Association of the type of prior virologic failure with the development of vaniprevir resistance.

Virologic failure on vaniprevir regimen	**Prior treatment failure on P/R therapy**				
	Null response	Partial response	Breakthrough	Relapse	**Total**
Null response	1	0	0	0	1
Partial response	2	0	0	0	2
Breakthrough	6	3	3	0	12
Relapse	6	2	2	1	11
Total	15	5	5	1	26

The table shows data for all 26 patients with sequence data at the time of virologic failure, all of whom harbored RAVs. For technical reasons, sequencing could not be completed on viral isolates from two additional patients (one prior null responder and one prior partial responder).

in patients who had failed previous P/R therapy (Lawitz et al., 2012). Patients retreated with vaniprevir in addition to P/R achieved significantly higher SVR rates than patients receiving another course of P/R without a third drug. Importantly, no patients had received a direct-acting antiviral agent for HCV prior to entry.

Resistance-associated amino acid variants were predominantly observed at positions R155, A156, and/or D168 in patients failing to achieve SVR by population sequencing. More genotype 1a infected patients experienced virologic failure (17/67, 25%) compared to genotype 1b infected patients (9/88, 10%). As has been observed with other HCV protease inhibitors, R155K was seen exclusively in genotype 1a by population sequencing, likely due to the higher number of nucleotide changes required to generate the R155K RAV in genotype 1b viruses (Cento et al., 2012). However, R155Q and R155N viruses were observed by clonal sequencing in one genotype 1b patient in combination with D168N or T, respectively. In genotype 1b, R155Q and N require only a single change from the consensus sequence. As double-variants only were observed after virologic failure and resulted in a synergistic decrease in susceptibility to vaniprevir in vitro, it is possible that these double-variants have a selective advantage over the single variants during vaniprevir dosing.

Overall, 5/12 patients that experienced virologic breakthrough while on vaniprevir had RAVs detected at more than one locus in the NS3 region. As these patients all experienced virologic failure during the dosing period, it is likely that the detection of RAVs at more than one locus represents the selection and expansion of multiple variants that have a selective advantage in the presence of vaniprevir. By comparison, all but one of the 11 patients relapsing after cessation of vaniprevir dosing had RAVs detected at a single vaniprevir RAV locus. As viruses isolated at relapse are not under ongoing vaniprevir selective pressure, it is likely that the RAVs identified in these patients represent variants selected during vaniprevir dosing that were not cleared by vaniprevir/P/R therapy and are able to cause virologic failure after dosing.

In a small sample of six patients with virologic breakthrough, clonal analyses were performed to assess the evolution of resistance during continued vaniprevir/P/R dosing to Week 12 of the study. In the majority of patients, additional novel double and triple variants were identified prior to cessation of vaniprevir dosing at Week 12 of the study, indicating continued evolution of resistant viruses during failing therapy. Double-RAVs exhibited larger decreases in susceptibility to vaniprevir compared to single RAVs. Selection of double-and triple RAVs during therapy may be indicative of increased viral fitness in the face of vaniprevir selective pressure (Kieffer et al., 2007; Sarrazin et al., 2007). In all cases, the frequencies of RAVs declined following the cessation of vaniprevir/P/R therapy. However, some double-variants did persist for over 100 days.

R155K variants were present in two patients prior to treatment despite never having been exposed to a HCV-protease inhibitor (Kieffer et al., 2007; Bartels et al., 2013). Both cases with baseline RAVs were followed by virologic failure on vaniprevir/P/R. Both patients were prior P/R null-responders, perhaps indicating that patients with a combination of pre-existing vaniprevir RAVs and a history of poor interferon responsiveness might be less likely to respond to a vaniprevir/P/R regimen. However, due to the small number of patients with baseline protease RAVs identified so far, Bartels et al., 2013. Vallet et al. (2011), Vicenti et al. (2012) the utility of baseline resistance testing requires further study as the use of directly-acting antiviral agents for chronic HCV infection becomes increasingly commonplace.

The presence of R155K variants may be of particular prognostic significance. Perhaps, as with initiation or change in antiretroviral regimens for HIV-infected patients, upfront resistance testing may eventually become part of the standard of care, particularly for patients exposed to protease-inhibitors during earlier treatment regimens. The lower resistance barrier of subtype 1a may make resistance sequencing more informative in infections with subtype 1a than in subtype 1b infections (Cento et al., 2012). However, unlike the HIV paradigm where resistant virus is archived indefinitely, the implications of the emergence of RAVs during treatment for HCV remain much less clear (Welsch and Zeuzem 2012).

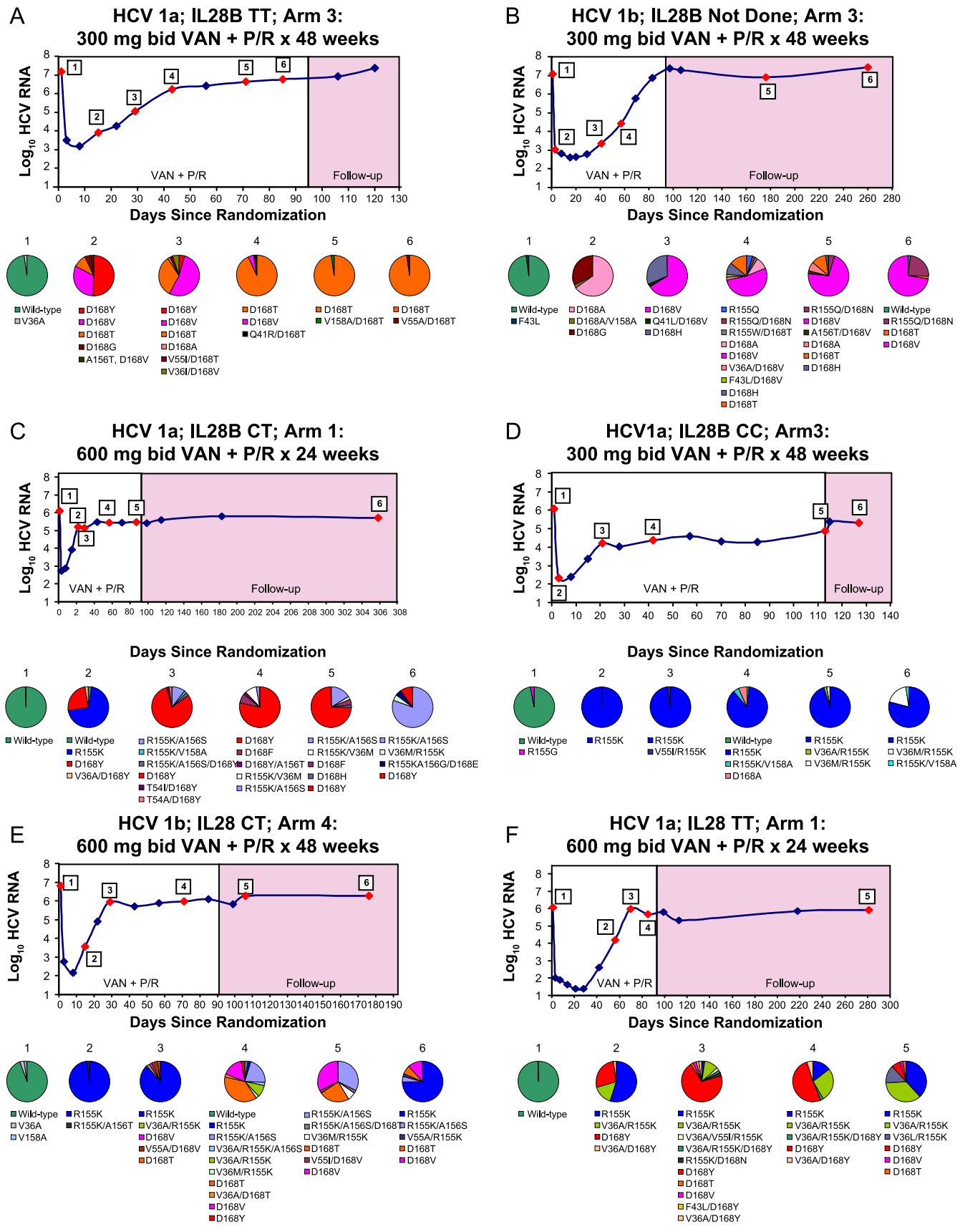

Fig. 2. Longitudinal clonal analysis for the 6 studied patients with breakthrough viremia on vaniprevir/P/R. The infecting HCV subtype, the patient *IL28B* genotype, and the assigned treatment regimen are shown for panel A–F. Note that the time on study given on the *x*-axes is not always on the same scale for each graph.

In the pivotal boceprevir studies that used a 4-week lead-in period with P/R before addition of boceprevir, lack of interferon responsiveness ($<$ 1-log drop in HCV RNA levels by Week 4) was associated with a high rate of failure accompanied by the emergence of RAVs. In the current vaniprevir trial, no lead-in period with P/R alone was used; consequently, our data can only infer whether a poor historical response to interferon predicts vaniprevir failure and resistance. Among the 44 previous null responders in the primary efficacy analysis that received vaniprevir, there were 18 virologic failures, of whom 17 were sequenced; all 17 had RAVs by population sequencing. A null response to prior P/R therapy may represent the historical counterpart of interferon non-responsiveness during the lead-in phase in the pivotal boceprevir trials (Poordad et al., 2011; Bacon et al., 2011), predicting virologic failure with the emergence of RAVs when a single DAA is added to P/R for retreatment.

Early treatment discontinuation can help avoid unnecessarily prolonged therapy with its attendant toxicity and additional costs in patients who are very unlikely to achieve SVR. Early discontinuation is also likely to decrease the complexity and frequency of RAVs selected by a suboptimal or failing regimen. With regimens containing a protease inhibitor along with P/R, stopping rules serve to preempt the emergence of RAVs in patients destined to fail. Consequently, stopping rules for futility have been developed for the licensed protease inhibitors (Jacobson et al., 2012; Adda et al., 2013). To preempt the selection of RAVs as well as minimize unnecessary cost and toxicity, DAA-based regimens should be discontinued promptly anytime virologic failure becomes evident through frequent monitoring of HCV-RNA levels during treatment.

Methods

Objective

An analysis of RAVs present at baseline or emerging during vaniprevir combination therapy was performed by population and selective clonal sequencing of the viral NS3/4A protease to determine the resistance patterns selected in patients not achieving SVR during a Phase 2b dose/duration-ranging study in P/R-experienced patients with HCV-genotype 1 infection.

Study design

The parent study was a randomized, active-control, double-blind study performed in non-cirrhotic patients with chronic hepatitis C-genotype 1 infection who had previously failed P/R alone in order to assess the efficacy of 4 dosing regimens of vaniprevir given with standard weight-based doses of P/R relative to P/R without a protease inhibitor (Lawitz et al., 2012). The primary efficacy outcome was the SVR rate assessed by the proportion of patients achieving undetectable HCV-RNA levels 24 weeks after discontinuation of all study medications. The protocol was approved by the appropriate institutional review boards and regulatory agencies, and written informed consent was obtained before any patient was enrolled. The conduct of the trial followed the principles of Good Clinical Practice.

Patients 18 to 65 years of age with hepatitis C-genotype 1 infection who had failed prior treatment with standard P/R therapy were eligible for MK-7009 Protocol 009 if their screening HCV RNA level was $\geq 4 \times 10^5$ IU/mL. Null responders to prior P/R treatment were to be limited to ~25% of the non-cirrhotic patients enrolled in the trial. Exclusion criteria included HIV co-infection, evidence of cirrhosis by liver biopsy or noninvasive assessment, decompensated liver disease, or any condition contraindicating retreatment with P/R. Upon entry, patients were stratified on the basis of their prior response to P/R as null response, partial response, breakthrough on therapy, and relapse after completing treatment. Vaniprevir was administered in combination with P/R in 4 dosing regimens; the control group received P/R plus placebo (Fig. 1). Patients treated with ≥1 dose of vaniprevir served as the primary population for the efficacy analysis.

Specimens were obtained from all patients at baseline for population sequencing of the HCV NS3/4A protease, which was to be repeated on the first sample qualifying as virologic failure per protocol. Failure to achieve SVR was operationally defined as $<$ 2-log decline in baseline HCV RNA level by Week 12 (null response), quantifiable HCV RNA levels at Week 24 or persistently detectable but unquantifiable HCV RNA levels at Weeks 24 and 28 (partial response); a log-increase from nadir HCV-RNA level by Week 12 or a HCV RNA level $>$ 100 IU/mL during therapy after becoming undetectable (breakthrough); or detectable HCV RNA following discontinuation of all study treatment after being undetectable at the end of treatment (relapse). A convenience sample selected from patients with breakthrough viremia while on vaniprevir underwent clonal sequencing analysis.

Viral and resistance assays

HCV RNA levels were measured by COBAS TaqMan assay (Roche Diagnostics, Branchburg, NJ, USA), which has a lower limit of detection (LLD) of 10 IU/mL and a lower limit of quantification (LLQ) of 25 IU/mL. Potential viral resistance was identified by population sequencing of NS3/4A gene. DNA amplicons obtained at baseline and at the time of virologic failure were purified and subjected to DNA-sequence analysis. Because of technical assay limitations (such as amplification sensitivity and the potential for founder effects at lower viral loads), population sequencing was only performed on samples with $>$ 1000 HCV RNA IU/mL. For the population analysis, HCV RNA was purified from patient plasma samples using the QIAamp® viral RNA purification kit (Qiagen, USA) according to the manufacturer's instructions. NS3/4a HCV cDNA was generated using SuperScript III reverse transcriptase (Invitrogen, USA) and eight independent PCR amplifications generated by nested PCR. Between 1 and 4 PCR products were sequenced at each time point using standard methods and the resultant amino acid sequences aligned to HCV genotype 1a (H77) or 1b (Con1) reference sequences. Clonal analyses were done longitudinally on a convenience subset of 6 vaniprevir recipients experiencing virologic breakthrough as defined per protocol by Monogram Biosciences (Monogram Biosciences, South San Francisco, CA, USA) or at the Merck Research Laboratories (West Point, PA, USA). For clonal sequencing, PCR amplification was performed on the NS3 region only and the resultant amplicons cloned into a TOPO TA vector (Invitrogen). Up to 80 clones were sequenced for each time point. IC_{50} of vaniprevir was measured for viruses harboring protease variants in a cell-based HCV protease assay using a secreted alkaline phosphatase assay to determine whether the genetic variant conferred phenotypic resistance (Ludmerer et al., 2008).

Disclosures

All co-authors had full access to all pertinent data upon request. A penultimate version of the paper was reviewed by the sponsor. Each co-author has approved an essentially final version of the manuscript. The opinions expressed in this report represent the consensus of the authors and do not necessarily reflect the formal position of Merck or Monogram Biosciences.

Present and former employees of Merck (as indicated on the title page) may own stock or stock options in the company.

Acknowledgments

We are indebted to all the patients, healthcare providers, and investigators involved with the parent study. We are grateful to Sanhita Bhanja and Havilland Campbell from Merck as well as Sunny Choe and Jeannette Whitcomb from Monogram Biosciences for their help and support. The expert assistance of Karyn Davis from Merck in the preparation of this manuscript is much appreciated.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.05.013.

References

Adda, N., Bartels, D.J., Gritz, L., Kieffer, T.L., Tomaka, F., Bengtsson, L., Luo, D., Jacobson, I.M., Kauffman, R.S., Picchio, G., 2013. Futility rules for telaprevir combination treatment for patients with hepatitis C virus infection. Clin. Gastroenterol. Hepatol 11, 193–195.

Bacon, B.R., Gordon, S.C., Lawitz, E., Marcellin, P., Vierling, J.M., Zeuzem, S., Poordad, F., Goodman, Z.D., Sings, H.L., Boparai, N., Burroughs, M., Brass, C.A., Albrecht, J.K., Esteban, R., 2011. Boceprevir for previously treated chronic HCV genotype 1 infection. N. Engl. J. Med. 364, 1207–1217.

Bartels, D.J., Sullivan, J.C., Zhang, E.Z., Tigges, A.M., Dorrian, J.L., De Meyer, S., Takemoto, D., Dondero, E., Kwong, A.D., Picchio, G., Kieffer, T.L., 2013. Hepatitis C virus variants with decreased sensitivity to direct-acting antivirals (DAAs) were rarely observed in DAA-naive patients prior to treatment. J. Virol. 87, 1544–1553.

Cento, V., Mirabelli, C., Salpini, R., Dimonte, S., Artese, A., Costa, G., Mercurio, F., Svicher, V., Parrotta, L., Bertoli, A., Ciotti, M., Di Paolo, D., Sarrecchia, C., Andreoni, M., Alcaro, S., Angelico, M., Perno, C.F., Ceccherini-Silberstein, F., 2012. HCV genotypes are differently prone to the development of resistance to linear and macrocyclic protease inhibitors. PLoS One 7, e39652, http://dx.doi.org/10.1371/journal.pone.0039652.

Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Gonçales Jr., F.L., Häussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J., Yu, J., 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N. Engl. J. Med. 347, 975–982.

Gambarin-Gelwan, M., Jacobson, I.M., 2012. Resistance-associated variants in chronic hepatitis C patients treated with protease inhibitors. Curr. Gastroenterol. Rep. 14, 47–54.

Ghany, M.G., Nelson, D.R., Strader, D.B., Thomas, D.L., Seeff, L.B., 2011. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the study of liver diseases. Hepatology 54, 1433–1444.

Jacobson, I.M., McHutchison, J.G., Dusheiko, G., Di Bisceglie, A.M., Reddy, K.R., Bzowej, N.H., Marcellin, P., Muir, A.J., Ferenci, P., Flisiak, R., George, J., Rizzetto, M., Shouval, T.L., Sola, R., Terg, R.A., Yoshida, E.M., Adda, N., Bengtsson, L., Sankoh, A.J., Kieffer, T.L., George, S., Kauffman, R.S., Zeuzem, S., 2011. Telaprevir for previously untreated chronic hepatitis C virus infection. N. Engl. J. Med. 364, 2405–2416.

Jacobson, I.M., Marcellin, P., Zeuzem, S., Sulkowski, M.S., Esteban, R., Poordad, F., Bruno, S., Burroughs, M.H., Pedicone, L.D., Boparai, N., Deng, W., DiNubile, M.J., Gottesdiener, K.M., Brass, C.A., Albrecht, J.K., Bronowicki, J.P., 2012. Refinement of stopping rules during treatment of hepatitis C genotype 1 infection with boceprevir and peginterferon/ribavirin. Hepatology 56, 567–575.

Kieffer, T.L., Sarrazin, C., Miller, J.S., Welker, M.W., Forestier, N., Reesink, H.W., Kwong, AD, Zeuzem, S., 2007. Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. Hepatology 46, 631–639.

Lawitz E., Rodriguez-Torres M., Stoehr A., Gane E., Serfaty L., Bhanja S., Barnard R.J., Gress J.O., Hwang P.M.T, Mobashery N. 2012. A phase 2b study of MK-7009 (vaniprevir) in patients with genotype 1 HCV infection who have failed previous pegylated interferon and ribavirin treatment. J. Hepatol. Feb 21. pii: S0168-8278(13)00130-X. http://dx.doi.org/%2010.1016/j.jhep.2013.02.008.

Ludmerer, S.W., Graham, D.J., Patel, M., Gilbert, K., Stahlhut, M., Olsen, D.B., 2008. J. Virological Methods 151, 301–307.

Manns, M.P., McHutchison, J.G., Gordon, S.C., Rustgi, V.K., Shiffman, M., Reindollar, R., Goodman, Z.D., Koury, K., Ling, M., Albrecht, J.K., 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 358, 958–965.

Poordad, F., McCone Jr., J., Bacon, B.R., Bruno, S., Manns, M.P., Sulkowski, M.S., Jacobson, I.M., Reddy, K.R., Goodman, Z.D., Boparai, N., DiNubile, M.J., Sniukiene, V., Brass, C.A., Albrecht, J.K., Bronowicki, J.P., 2011. Boceprevir for untreated chronic HCV genotype 1 infection. N. Engl. J. Med. 364, 1195–1206.

Ribeiro, R.M., Li, H., Wang, S., Stoddard, M.B., Learn, G.H., Korber, B.T., Bhattacharya, T., Guedj, J., Parrish, E.H., Hahn, B.H., Shaw, G.M., Perelson, A.S., 2012. Quantifying the diversification of hepatitis C Virus (HCV) during primary infection: estimates of the in vivo mutation rate. PLoS Pathog 8, e1002881, http://dx.doi.org/10.1371/journal.ppat.1002881, accessed 11/5/12.

Sarrazin, C., Zeuzem, S., 2010. Resistance to direct antiviral agents in patients with hepatitis C virus infection. Gastroenterology 138, 447–462.

Sarrazin, C., Kieffer, T.L., Bartels, D., Hanzelka, B., Müh, U., Welker, M., Wincheringer, D., Zhou, Y., Chu, H.M., Lin, C., Weegink, C., Reesink, H., Zeuzem, S., Kwong, A.D., 2007. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. Gastroenterology 132, 1767–1777.

Sherman, K.E., Flamm, S.L., Afdhal, N.H., Nelson, D.R., Sulkowski, M.S., Everson, G.T., Fried, M.W., Adler, M., Reesink, H.W., Martin, M., Sankoh, A.J., Adda, N., Kauffman, R.S., George, S., Wright, C.I., Poordad, F., 2011. Response-guided telaprevir combination treatment for hepatitis C virus infection. N. Engl. J. Med. 365, 1014–1024.

Vallet, S., Viron, F., Henquell, C., Le Guillou-Guillemette, H., Lagathu, G., Abravanel, F., Trimoulet, P., Soussan, P., Schvoerer, E., Rosenberg, A., Gouriou, S., Colson, P., Izopet, J., Payan, C., 2011. NS3 protease polymorphism and natural resistance to protease inhibitors in French patients infected with HCV genotypes 1-5. Antivir. Ther. 16, 1093–1102.

Vicenti, I., Rosi, A., Saladini, F., Meini, G., Pippi, F., Rossetti, B., Sidella, L., Di Giambenedetto, S., Almi, P., De Luca, A., Caudai, C., Zazzi, M., 2012. Naturally occurring hepatitis C virus (HCV) NS3/4A protease inhibitor resistance-related mutations in HCV genotype 1-infected subjects in Italy. J. Antimicrob. Chemother. 67, 984–987.

Welsch, C., Zeuzem, S., 2012. Clinical relevance of HCV antiviral drug resistance. Curr. Opin. Virol. 2, 651–655.

Zeuzem, S., Andreone, P., Pol, S., Lawitz, E., Diago, M., Roberts, S., Focaccia, R., Younossi, Z., Foster, G.R., Horban, A., Ferenci, P., Nevens, F., Müllhaupt, B., Pockros, P., Terg, R., Shouval, D., van Hoek, B., Weiland, O., Van Heeswijk, R., De Meyer, S., Luo, D., Boogaerts, G., Polo, R., Picchio, G., Beumont, M., 2011. Telaprevir for retreatment of HCV infection. N. Engl. J. Med. 364, 2417–2428.