with NS3-S122G (P-20, no fold-change to either DCV/ASV), or NS5A-Q54H (P-13, no fold-change to DCV). P-13 was the only patient with HCV-RNA <15 IU/mL (target detectable) at Week 6 and was, therefore, considered a treatment failure. Treatment-emergent resistance at Week 1 in the five patients could not be determined because of PCR failure. A comparison of initial virologic response versus dose and polymorphisms associated with resistance revealed no differences. Among null-responders who received ASV 600mg, mean HCV-RNA declines at Week 1 for those with versus without RAVs were –4.6 versus –4.3 $\log_{10}$ IU/mL, which were similar to the Week 1 declines among those who received ASV 200mg (–4.5 $\log_{10}$ IU/mL with RAVs (one patient) versus –4.3 $\log_{10}$).

Baseline HCV-RNA levels did not impact response to treatment; patients with high baseline viral load still experienced rapid and robust responses to therapy (Fig. 1; Table 1).

Ineligible/intolerant patients

Virologic response

Virologic response at Week 4 was greater in alfa/RBV ineligible patients than null-responders. Undetectable HCV-RNA at Week 4 was observed in 86% of the ineligible group versus 52% of null-responders. However, by Week 12, undetectable HCV-RNA was similar in both groups. Early HCV-RNA declines appeared unaffected by *IL28B* genotype, the presence of baseline polymorphisms associated with resistance, or virologic outcome (Fig. 3). Adherence to therapy, assessed through pill counts, was found to be high in six of the seven patients experiencing virologic failure. However, DCV/ASV exposures were high in the one non-compliant patient (P-31) who subsequently experienced relapse.

Baseline analysis

Baseline *IL28B* genotype, polymorphisms associated with resistance, and virologic outcome are shown in Table 2 and Fig. 2B. Three patients presented with DCV resistance at baseline: one (P-25) with an NS5A-L31M-Y93H combination (7,105-fold DCV resistance [13]), and two with an NS5A-Q54Y-Y93H (58-fold resistance). All three subsequently experienced viral breakthrough at Week 10 or 16.

Other patients had baseline polymorphisms conferring minimal or low-level resistance to DCV and/or protease inhibitors; NS5A-Y93H (n=4), NS5A-L28M-R30L (n=1), NS3-T54S (n=1), and NS3-Q80L (n=5). Variable responses were observed among these patients (Fig. 2B); the majority responded, but two patients with baseline NS5A-Y93H experienced post-treatment relapse. One patient (P-24) with baseline NS5A-L28M-R30L-Q54H-A92T and NS3-Q80L-S122G had a slower response to treatment at Week 1 when compared with mean HCV-RNA reductions (SD) for ineligible/intolerant patients on the study (–3.4 versus –4.74 [0.58] $\log_{10}$ IU/mL) but subsequently achieved SVR with only 16 weeks' treatment. Neither NS3-Q80L-S122G nor NS5A-L28M-R30L-Q54H-A92T conferred resistance to ASV or DCV, respectively.

Baseline viral load did not appear to affect response; mean HCV-RNA levels (SD) were 6.4 (0.7) $\log_{10}$ IU/mL among patients achieving SVR compared with 6.8 (0.3) $\log_{10}$ IU/mL among patients experiencing virologic failure. However, four of six patients with the *IL28B* CT allele subsequently failed treatment (three breakthroughs, one relapse) versus only three of 16 patients with *IL28B* CC (all relapsed).

Genotypic analysis of patients with viral breakthrough

Treatment-emergent RAVs were assessed through post-treatment Week 48 in the three patients with virologic breakthrough (Table 3).

Patient P-25: This patient was *IL28B* CT genotype with baseline HCV-RNA 6.8 $\log_{10}$ IU/mL and a linked baseline NS5A-L31M-Y93H/Y polymorphism. Despite undetectable HCV-RNA by Week 4 (Fig. 4A), viral breakthrough occurred at Week 16, associated with high-level resistance to both DCV (NS5A-L31M-P58A-Y93H; 65,000-fold) and ASV (D168A; ~120-fold in GT1b). Other minor variants detected at baseline by clonal analysis (NS5A-Q62R, -A92T) were not present at breakthrough. NS5A variants present at end of therapy persisted through follow-up Week 48, and although P58A had largely changed to P58G (73% of 33 clones, Fig. 5A) by Week 36, a similar ratio of P58G to A was detected at follow-up Week 48. By contrast, NS3-D168A had mostly been replaced by wild-type at Week 48 (83% of 64 clones).

Patient P-29: This patient was *IL28B* CT genotype, with baseline HCV-RNA 6.7 $\log_{10}$ IU/mL and a pre-existing linked NS5A-Q54Y-Y93H/Y (Fig. 5B) and NS3-Q80L. Undetectable HCV-RNA by Week 3 was followed by viral breakthrough at Week 16 (Fig. 4A) associated with NS5A-L31M-Q54Y-Y93H (6,467-fold DCV resistance) and NS3-Q80L-D168V (~280-fold ASV resistance). These RAVs remained stable through 48 weeks post-treatment.

Patient P-43: This patient was *IL28B* CT genotype with baseline HCV-RNA 7.0 $\log_{10}$ IU/mL, and a pre-existing NS5A-Q54Y-Y93H variant (Fig. 5C). HCV-RNA was undetectable at Week 2 and breakthrough occurred at Week 10 (Fig. 4A), associated with a linked NS5A-L31M-Q54Y-Y93H variant (Fig. 5C; 6,467-fold DCV resistance) and a NS3-D168V variant (~270-fold ASV

resistance). Again, NS5A variants remained stable through Week 48 post-treatment while NS3-D168V was replaced by wild-type (100% of 60 clones).

For the three patients experiencing viral breakthrough, DCV and ASV trough exposures were less than drug levels required to achieve a 90% effective concentration (EC_{90}) value against emergent RAVs (Table 3).

Genotypic analysis of patients experiencing post-treatment relapse

Four ineligible patients with undetectable HCV-RNA at end of treatment experienced relapse (Fig. 4B). Resistance polymorphisms through Week 48 off-treatment are shown in Table 3. Baseline polymorphisms associated with resistance were not detected in two patients (P-32 and P-36), but both displayed post-relapse resistance by follow-up Weeks 8 and 4, respectively. Patient P-32 relapsed with NS5A-L31M-P58L-Y93H (8,300-fold DCV resistance) and NS3-D168V (270-fold ASV resistance). Patient P-36 relapsed with a NS5A-L31V/M-Y93H genotype (L31V-Y93H: 14,789-fold DCV resistance versus L31M-Y93H: 7,105-fold) [13] and NS3-D168V. The remaining two patients had detectable NS5A-Y93H at baseline (24-fold DCV resistance) and additional substitutions at NS5A-L31 and NS3-D168 were detected after relapse. Patient P-31 displayed NS5A-L31M-Y93H (7,105-fold DCV resistance) [13] and NS3-D168A (~120-fold ASV resistance); patient P-37 relapsed with the same NS5A-L31V/M-Y93H and NS3-D168V, as described for patient P-36.

Baseline HCV-RNA and *IL28B* genotype did not appear to influence relapse; three of four relapse patients were *IL28B* CC genotype and baseline HCV-RNA was not appreciably higher

than for those with SVR (mean HCV-RNA [SD]: 6.8 [0.4] vs. 6.4 [0.7] $\log_{10}$ IU/mL, respectively).

Changes in the DCV resistance pattern present at relapse through follow-up Week 48 were seen in three of four relapsers; comprising Y93H changing to wild-type (100% of 68 clones) in patient P-32. Clonal analysis of the baseline sequence revealed the presence of Y93H as a minor species (~2%; 1/61 clones). Genotypic changes resulting in a lower level of phenotypic resistance (L31V-Y93H to L31M-Y93H) were detected in patients P-36 and P-37. NS3 substitutions observed at relapse were not detectable by population sequencing by follow-up Week 36. The D168V substitution detected in patient P-37 was replaced by D168E (78-fold ASV resistance [19]) at follow-up Weeks 36 and 48. As with the patients who experienced virologic breakthrough, ASV and DCV trough values in the three drug-compliant patients who relapsed were less than the observed EC_{90} values for the respective RAVs.

Discussion

This study assessed resistance and virologic failure in a difficult-to-treat population of null-responders and alfa/RBV ineligible/intolerant patients treated with the dual oral combination of DCV and ASV. Overall, 77% achieved an SVR [11], with all viral breakthroughs and post-treatment relapses occurring in the ineligible/intolerant subpopulation. It is possible that pharmacokinetics may have played a role in these failures, since patients experiencing failure had DCV and/or ASV trough values below median or documented non-compliance [11]. However, since most patients with troughs below the median achieved SVR, the influence of drug exposure is hard to assess.

NS5A-Y93H was identified as the predominant polymorphism at baseline in all three patients with viral breakthrough and in two of the four patients with relapse. However, three null-responders and two ineligible/intolerant patients also had a pre-existing NS5A-Y93H polymorphism and all achieved SVR, making the significance of Y93H alone for response in the broader patient population difficult to assess. Furthermore, where Y93H polymorphisms existed at baseline, their effects on DCV inhibition were minimal (Y93H EC_{50} = 49 pM [6] compared with C_{trough} values that ranged from 75 to 620 nM. The global prevalence of NS5A-Y93H is approximately 4%, based on data from the Los Alamos database [20] and unpublished data from nine DCV studies, and approximately 11% in other recent Japanese DCV studies [21], which is considerably lower than the 23% (10/43) prevalence observed in this study. Further analysis of DCV study data indicates that Y93H pre-exists at higher levels in patients infected with GT1b (10%) than GT1a (1%); however, the link with *IL28B* is not so clear given that most failures to date with DCV have been observed in GT1a patients with no baseline Y93H. Other polymorphisms observed at a higher frequency among this GT1b population included NS3-Q80L (~19%, 8/43), versus Q80K which has been observed more frequently in GT1a populations [18, 19].

Baseline HCV-RNA did not appear to influence virologic response in either population, and response was too rapid to allow successful genomic sequencing after 1 week of treatment. ASV dose (600mg or 200mg twice-daily) did not impact the initial decline in HCV-RNA in null-responders, and the *IL28B* CT allele, present in 86% (18/21) of null-responders, did not prevent patients achieving a very high (90%) SVR. By contrast, although only 27% (6/22) of ineligible/intolerant patients were *IL28B* CT, this genotype was present in all three viral breakthroughs and one of four relapses. While *IL28B* genotype is known to influence

response to alfa/RBV, its apparent impact on virologic suppression in alfa-sparing regimens is unexpected. However, given the small number of patients, any such correlation will require evaluation in a larger dataset.

The emergent RAVs at viral breakthrough or relapse (signature NS5A-L31 and -Y93 substitutions for DCV and NS3-D168 substitutions for ASV) were similar to observations from other clinical studies of DCV, and also with *in vitro* GT1b replicon resistance studies with ASV [19], although this study represents the first demonstration of emergent clinical ASV resistance. It is possible that signature resistance variants to both DCV and ASV pre-existed as minor species and subsequently enriched by selective pressure, as predicted by viral kinetic modeling [22]. Although a combination of these NS3 and NS5A variants were not detected by clonal sequencing at baseline, their low-level pre-existence cannot be ruled out. However, assessment of minor NS3 plus NS5A variants from the same RNA sequence is currently not feasible using available deep-sequencing technologies. Nevertheless, additional studies to assess the presence and dynamics of minority baseline variants under drug selection are indicated.

Interestingly, ASV-resistant NS3-D168 substitutions generally decayed during the off-drug follow-up period, implying a lack of replicative fitness relative to wild-type in the absence of selective drug pressure. Indeed, a reduction in replicative fitness has been observed for D168 variants in replicons [19]. Neither of the secondary variants associated with D168V in this study (Q80L or S122G) had an impact on fitness *in vitro* (replication capacity similar or higher than that observed for parental GT1b [Con1] replicon), with both double variants possessing replicative capacities similar to D168V alone [19]. However, clonal analysis indicated that ASV-resistant variants were still detectable in some post-treatment samples

as minority species, although not detectable by population sequencing. Deeper sequencing techniques will be required to fully establish the dynamics of decay and whether ASV-resistant strains remain enriched for long periods relative to baseline. Since the re-treatment of patients with prior NS3 protease inhibitor failure have only been assessed in small studies [23], it is not clear whether these NS3 RAVs will form a stable minority capable of rapid overgrowth on re-treatment. By contrast, NS5A variants associated with DCV resistance were observed to be linked and relatively stable through at least 48 weeks post-treatment, although change of DCV-resistance substitutions was noted in four of seven patient samples. As described above, the prevalence of the NS5A variant Y93H, that confers low level resistance to daclatasvir, is approximately 10% in the general HCV genotype 1b population. Linked NS5A RAVs conferring high level resistance to daclatasvir are less prevalent (<1%). While NS3 RAVs (substitutions at positions V36, T54, R155, or D168) associated with first-generation protease inhibitors have been reported to be present at ≤2.7% by population sequencing [5, 24], emergent NS3 RAVs have been shown to persist for up to 4 years in long-term follow-up studies [25]. Therefore, longer-term studies are indicated to assess what, if any, replicative impairment is conferred by these linked NS5A changes and how long these potentially transmissible drug-resistant strains persist without DCV selection pressure.

In conclusion, high response rates were achieved in this small Japanese study comprising GT1b null-responders and alfa/RBV ineligible/intolerant patients with limited treatment options. Among patients experiencing virologic failure, ASV- and DCV-resistant substitutions emerged together at the time of failure, which were similar to those reported previously. An analysis of persistence demonstrated that DCV-resistant substitutions appeared to have

greater fitness over the duration of the study. A loose association with a baseline NS5A polymorphism on virologic outcome was observed; however, further data from larger studies are required. Consequently, a greater understanding of the role and dynamics of pre-existing, emergent and persistent resistance variants to DCV and ASV will be sought from the planned Phase 3 global studies of this combination.

Acknowledgments

The authors wish to thank Arlene Carifa, Bernadette Kienzle, and Xin Huang for assistance with sequencing, and Nannan Zhou, Aaron Monikowski, Paul Falk, and Chaoqun Chen for assistance with drug-susceptibility analyses. Editorial assistance with the preparation of this manuscript was provided by Andrew Street, Ph.D. and Nick Fitch, Ph.D., of Articulate Science Ltd and was funded by Bristol Myers Squibb.

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Table 1: Baseline viral and host characteristics among genotype-1b null-responders and their virologic outcome

Patient	*IL28B* GT	HCV-RNA, log_{10} IU/mL	NS5A polymorphism(s)[a]	NS3 polymorphism(s)[a]	Virologic outcome
P-1	CT	7.2	Q54H/Q-Q62Q/E-Y93H/Y	T54S-Q80L	SVR
P-2	CT	7.0		Q80L-V170I/M	SVR
P-3	CT	7.4	Q54H		SVR
P-4	CT	6.7	R30Q		SVR
P-5	CT	7.0	L31L/M-P58P/S		SVR
P-6	CC	5.3	P58P/T-Q62E		D/C at WK2 due to SAE[b]
P-7	CC	7.2		S122S/G	SVR
P-8	CT	7.0	Q54H	Q80L	SVR
P-9	CT	7.1	Q54H-Y93H/Y	S122N	SVR
P-10	CT	6.4	L28M-R30Q		SVR
P-11	CT	6.8			D/C at WK12 due to AE; SVR
P-12	CT	6.4	Q54H-P58S-Q62E		SVR

P-13	CT	7.4	Q54H		D/C at WK6; PDR not achieved[c]
P-14	CT	6.5			SVR
P-15	CT	6.3	R30Q/R-Q62Q/R		SVR
P-16	CT	6.6	Q54H		SVR
P-17	CT	6.6	Q54H-Q62E		SVR
P-18	CT	6.9	Q54Y	Q80L	SVR
P-19	CT	6.6	Q54H-Y93H	N77A	SVR
P-20	CT	7.0	R30Q	S122G	SVR
P-21	CC	6.6	Q54L		SVR

[a]All NS3 and NS5A amino acids were examined with focus on polymorphisms at positions known to be associated with resistance to NS3 protease inhibitors (36,43,54,55,77,78,79,80,122,123,138,155,156,158,168,170,175) and NS5A inhibitors (21,23,24,28,30,31,32,54,58,62,92,93). When a mixture of substitutions is indicated, the most predominant is identified first.

[b]HCV-RNA undetectable at post-treatment week 24

[c]Alfa/RBV added; HCV-RNA undetectable at post-treatment week 24 following 52 weeks' therapy

D/C, discontinued; GT, genotype; HCV, hepatitis C virus; PDR, protocol-defined response; SAE, serious adverse event; SVR, sustained virologic response; WK, Week.

Table 2: Baseline viral and host characteristics among genotype-1b ineligible/intolerant patients and their virologic outcome

Patient	*IL28B* GT	HCV-RNA, log_{10} IU/mL	NS5A polymorphism(s) [a]	NS3 polymorphism(s) [a]	Virologic outcome
P-22	CC	7.1			SVR
P-23	CC	6.9	A92T	Q80L-S122G/S	SVR
P-24	CC	6.6	L28M-R30L-Q54H-A92T	Q80L-S122S/G	D/C at WK12 due to AE; SVR
P-25	CT	6.8	L31M/L-Y93H/Y		VBT (WK16)
P-26	CC	5.3			SVR
P-27	CC	6.9	Q54H-Y93H/Y	T54S	SVR
P-28	CC	6.8	Y93H/Y	Q80L	SVR
P-29	CT	6.7	Q54Y-Y93H/Y	Q80L	VBT (WK16)
P-30	CT	6.7	Q54H		SVR
P-31	CC	6.6	P58S/P-Y93Y/H	S122G	Relapse (FUWK12)
P-32	CT	6.7	P58L	S122G	Relapse (FUWK4)
P-33	CT	5.2	Q54H-Q62P/S		D/C at WK12 due to patient request; SVR
P-34	CC	6.6		Q80L	SVR
P-35	CC	6.4	Q54H-Q62E/A-A92T		SVR

P-36	CC	7.1		S122S/C	Relapse (FUWK4)
P-37	CC	6.6	Y93H		Relapse (FUWK4)
P-38	CC	7.5		S122T	SVR
P-39	CC	5.1	R30Q/R		SVR
P-40	CC	6.8	Q54H-A92A/T	Q80L	D/C at WK8[b]
P-41	CC	6.0		S122G	SVR
P-42	CC	6.5	A92T		SVR
P-43	CT	7.0	Q54Y-Y93H	S122G	VBT (WK10)

[a]All NS3 and NS5A amino acids were examined with focus on polymorphisms at positions known to be associated with resistance to NS3 protease inhibitors (36,43,54,55,77,78,79,80,122,123,138,155,156,158,168,170,175) and NS5A inhibitors (21,23,24,28,30,31,32,54,58,62,92,93). When a mixture of substitutions is indicated, the most predominant is identified first.

[b]Treatment discontinued at patient request; subsequently lost to follow-up

D/C, discontinued; FU, follow-up; GT, genotype; HCV, hepatitis C virus; SVR, sustained virologic response; VBT, viral breakthrough; WK, week.

Table 3: Emergence of resistance-associated variants among genotype-1b ineligible/intolerant patients experiencing viral breakthrough or relapse

Patient	Time point	DCV / ASV C_{trough} range, nM	NS5A RAVs				DCV EC_{90}, nM	NS3 RAVs			ASV EC_{90}, nM
			L31	Q54	P58	Y93		Q80	S122	D168	
VBT patients											
P-25	BL	190–261 / 25–41	M/L	–	–	H/Y	<137	–	–	–	
	WK16 (VBT)		M	–	A	H	>1000	–	–	A	540
	WK20		V	–	A	H		–	–	A	
	WK24		M	–	A	H		–	–	A	
	FUWK4		M	–	A	H		–	–	A	
	FUWK36		M	–	G	H	>5000	–	–	D/A	
	FUWK48		M	–	G/A	H		–	–	–	
P-29	BL	116–198 / 18–33	–	Y	–	H	0.04	L	–	–	1.6
	WK16 (VBT)		ND					ND			
	WK20		M/V	Y	–	H	750	L	–	V	55
	FUWK4		M	Y	–	H		L	–	V	
	FUWK36		M	Y	–	H		L	–	V	
	FUWK48		M	Y	–	H		L	–	V	
P-43	BL	243 / 69	–	Y	–	H	0.49	–	G	–	2.8
	WK10 (VBT)		M	Y	–	H	435	–	G	V	279
	FUWK4		M	Y	–	H		–	G	V	
	FUWK36		M	Y	–	H		–	G	–	
	FUWK48		M	Y	–	H		–	G	–	
Relapse patients											
P-31	BL	573–620 / 153–327	–	–	S/P	Y/H	0.02	–	G	–	
	FUWK16		ND					–	–	A	

	FUWK24		M	–	–	H	351	–	G	–	
	FUWK36		–	–	–	–		–	–	–	
	FUWK48		–	–	–	–		–	–	–	
P-32	BL		–	–	L	–	0.004	–	G	–	
	FUWK8	151–306 / 19–42	M	–	L	H		–	G	V/D	
	FUWK12		M	–	L	H	543	–	G	–	
	FUWK36		M	–	L	–	1.5	–	G	–	
	FUWK48		M	–	L	–		–	G	–	
P-36	BL		–	–	–	–		–	–	–	
	FUWK8		V/M	–	–	H		–	–	V	1190
	FUWK12	138 / 26	V	–	–	H	349	–	–	–	
	FUWK24		M/V	–	–	H		–	–	V/D	
	FUWK36		M	–	–	H	137	–	–	–	
	FUWK48		M	–	–	H		–	–	–	
P-37	BL		–	–	–	H	0.49	–	–	–	
	FUWK8		V	–	–	H		–	–	V	
	FUWK12	75–134 / 40–93	V/I	–	–	H		–	–	V	
	FUWK24		M	–	–	H		–	–	V	
	FUWK36		M	–	–	H		–	–	E/D	
	FUWK48		M	–	–	H		–	–	–	

ASV, asunaprevir; BL, baseline; DCV, daclatasvir; EC_{90}, 90% effective concentration; FU, follow-up; ND, not determined as multiple amplifications failed; RAV, resistance-associated variant; VBT, viral breakthrough; WK, week

When a mixture of substitutions is indicated, the most predominant is written first. ASV-resistant variants conferred no cross-resistance to DCV, and vice versa in a replicon assay. Dashes indicate consensus with control sequence GT1b (Con1)