AAC Accepts, published online ahead of print on 30 July 2012
Antimicrob. Agents Chemother. doi:10.1128/AAC.01209-12

Effect on HCV Replication by Combinations of Direct Acting Antivirals Including NS5A Inhibitor Daclatasvir

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Running Title: Characterization of Daclatasvir in DAA Combinations

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ABSTRACT

Three Hepatitis C virus (HCV) inhibitors, asunaprevir (ASV, BMS-650032), daclatasvir (DCV, BMS-790052), and BMS-791325, each targeting a different non-structural protein of the virus (NS3, NS5A, and NS5B, respectively), have independently demonstrated encouraging pre-clinical profiles and are currently undergoing clinical evaluation. Since drug-resistant variants have rapidly developed to monotherapy with almost all direct-acting antiviral agents (DAAs) for HCV, the need for combination therapies to effectively eradicate the virus from infected patients is clear. These studies demonstrate the additive/synergistic effects on replicon inhibition and clearance of combining NS3 protease or NS5B RNA polymerase inhibitors with the first-in-class, NS5A replication complex inhibitor daclatasvir (DCV), and reveal new resistance pathways for combinations of two small molecule inhibitors that differ from those that develop during monotherapy. The results suggest that under a specific selective pressure, a balance must be reached in the fitness costs of substitutions in one target gene when substitutions are also present in another target gene. Further synergies and additional novel resistance substitutions were observed during triple combination treatment relative to dual-drug therapy indicating that in combination, HCV inhibitors can exert cross-target influences on resistance development. Enhanced synergies in replicon inhibition and a reduced frequency of resistance together lend strong support to the utility of combinations of DAAs for the treatment of HCV, and the identification of altered resistance profiles during combination treatment provides useful information for monitoring resistance in the clinic.

Abbreviations: ASV, asunaprevir; BVDV, bovine viral diarrhea virus; DAA, direct-acting antiviral agent; DCV, daclatasvir; gt, genotype; h, hour; HCV, hepatitis C virus; IFN, interferon; kb, kilobase; RBV, ribavirin; SVR, sustained viral response; wt, wild type

INTRODUCTION

Hepatitis C virus (HCV) is a positive-stranded RNA virus in the *Flaviviridae* family of enveloped virions, which affects an estimated 170 million people worldwide and is the major cause of chronic hepatitis. Currently, approximately 50% of patients infected with genotype 1, the most prevalent form of the virus, fail to achieve a sustained reduction in viral load with pegylated interferon alpha plus ribavirin (alfa/ RBV) (56, 54, 58). A substantial fraction (20%) of chronically infected patients develop serious progressive liver disease, including cirrhosis or hepatocellular carcinoma. Alfa/RBV treatment is associated with a high incidence (>30%) of adverse effects, some of which are of sufficient severity to cause patients to discontinue therapy (58). Despite the recent approval of two new direct acting antiviral agents (DAAs), boceprevir and telaprevir, for use in combination with alfa/ RBV (18,49), their use may be limited by poor efficacy in some patient populations, inconvenient 3-times daily dosing of the DAA, and association with side effects including anemia, rash, and gastro-intestinal effects, in addition to the well documented spectrum of adverse effects associated with alfa/RBV. Although addition of these DAAs to the standard of care for HCV represents a significant improvement in patient therapy, there is still an unmet medical need for new agents and more tolerable treatment regimens for newly diagnosed patients and those failing current therapies.

The 9.6 kb HCV genome encodes a polyprotein of about 3000 amino acids *via* translation of a single, uninterrupted open reading frame. The polyprotein is cleaved co- and post-translationally in infected cells by cellular and virus-encoded proteases, to produce a multi-component replication complex (8, 34). The serine protease encoded by the N-terminal region of NS3 is thought to be responsible for all downstream cis and trans proteolytic cleavages (9, 17).

NS5A possesses no known enzymatic activity, but exists in different states of phosphorylation, and influences multiple functions at various stages of the viral replication cycle (43, 61). It has been shown to interact with an extensive array of host proteins, and to play a role in IFN resistance (38, 42). NS5B is the RNA-dependent RNA polymerase responsible for replication of HCV RNA (1, 4).

The essential roles of non-structural proteins NS3-NS5 in viral replication render each an attractive target for antiviral intervention (2). Clinical proof-of-concept has been achieved for a number of DAAs targeting some of these proteins, including the serine protease activity of NS3 (11, 16, 26, 32, 33, 47, 55) and the RNA-dependent RNA polymerase activity of NS5B (60, 21, 27, 48). More recently, daclatasvir (DCV) (Table 1) was the first NS5A replication complex inhibitor to show proof-of-concept in the clinic, demonstrating in early clinical testing the potential for this class of inhibitor to become a valuable component of an all-oral treatment regimen for HCV (15).

The high turnover rate and error-prone nature of the HCV RNA polymerase contribute to the production of potentially resistant viral quasi-species. In practice, resistance has emerged to all small molecule inhibitors of HCV tested as monotherapy except some nucleoside/nucleotide NS5B inhibitors. Resistance mutations have been identified both *in vitro* and *in vivo* upon treatment with nearly all inhibitors of HCV serine protease, NS5A, or allosteric RNA polymerase inhibitors advanced to date (3, 20, 23, 24, 25, 31, 37, 39, 53, 54, 59, 63, 65), with good correlation observed between resistance emergence in the replicon system and *in vivo*. Recent literature indicates that treatment with combinations of non-cross-resistant inhibitors not only improves antiviral activity during treatment, but also suppresses the post-treatment viral rebound often associated with monotherapy (20, 22, 27). To achieve a sustained viral response (SVR), it will be essential to use combination therapies similar to those that have recently been explored in replicon (5, 10, 30),

animal models (46), and patients (14, 53) as a viable approach to improving the efficacy, tolerability, and compliance issues associated with current therapies. In this report, the effects of a combination approach to HCV therapy have been studied in the HCV replicon system using two- and three-drug combinations that include NS5A replication complex inhibitor (DCV), NS3 protease inhibitor asunaprevir (ASV), and non-nucleoside NS5B RNA polymerase inhibitor BMS-791325.

MATERIALS AND METHODS

Cell lines. Bovine viral diarrhea virus (BVDV) and HCV replicon cell lines were previously described (28, 45) and were propagated in DMEM containing 2 mM L-glutamine, 10% FBS, and penicillin-streptomycin, with or without 0.3 – 0.5 mg/ml geneticin (G418).

Efficiency of replicon clearance from cultured cells. HCV replicon cells (6×10^4 per well in 6-well plates) and BVDV replicon cells (4×10^4 per well) were treated with various EC_{50} multiple concentrations of inhibitors in cell growth media for one week. After 7 days, media containing inhibitor was removed and cells were maintained in growth media containing 0.5 mg/ml G418. Media was changed twice weekly for a period of ~ four weeks. Plates were washed and colonies were counted after staining cells with 0.2% crystal violet. All conditions were tested in duplicate and repeated in separate experiments.

Selection of populations with reduced susceptibility to HCV inhibitors. HCV replicon cells were plated at a density of 6×10^4 per 60 mm plate, and maintained in growth media with 0.3 mg/ml G418 and various concentrations of inhibitor(s). BVDV replicon cells, plated at 2×10^4 per 60 mm plate, were maintained in growth media containing 0.5 mg/ml G418 and treated in parallel

with the same inhibitors. Fresh media containing compound was added every 3 to 5 days for a total of four weeks, after which plates were washed and cells were stained with 0.2% crystal violet or further selected for testing. All concentrations were tested in duplicate and selections were repeated in separate experiments. Genotype (gt) 1b cultures undergoing triple combination treatment required gradual (1.5 to 2-fold) ramping of drug concentration, starting with the 5x-selected population, allowing 4 weeks incubation for each escalation in concentration in order to select high level resistance.

HCV replicon luciferase and FRET assays. To evaluate compound efficacy, HCV replicon cells were incubated in 96-well plates in the presence of compound for 3 days. For replicons containing a luciferase reporter gene, Renilla luciferase activity was then assayed using a Renilla Luciferase Assay System or DualGlo Luciferase Assay System (Promega Corporation, Madison, WI), according to the manufacturer's directions. Plates were read on a TopCount NXT Microplate Scintillation and Luminescence Counter (Packard Instrument Company, Meriden CT). For replicons lacking a reporter gene, a FRET assay monitored NS3 protease activity as a measure of HCV replicon (45). The 50% effective concentration (EC_{50}) was calculated using the four-parameter logistic formula y = A+((B-A)/(1+((C/x)^D))), where A and B denote minimal and maximal % inhibition, repsectively, C is the EC50, D is hill slope and x represent compound concencentration.

Cell-based inhibitor combination assays. For combination studies, inhibitors were each tested at eleven concentrations. The compounds were tested as monotherapies and in combinations at various concentration ratios. Cells were exposed to compounds for 3 days and the amount of HCV inhibition was then determined using the Dual-Glo luciferase assay as described above. The

potential cytotoxicities of these combined agents were also analyzed in parallel by alamar blue staining. The CC_{50} values were calculated using the four-parameter logistic formula described above.

The degree of antagonism, additivity, or synergy was determined from combination dose response curves which were fit to assess the antiviral effects of the drug treatment combinations. The concentration ratios were analyzed using the method of Chou (6). All estimates were computed using biostatistical software SAS Proc NLIN, and a four parameter logistic. Combination indices were tested for departure from additivity using isobologram methods. Asymptotic confidence intervals were also calculated for each of the combination indices. These intervals are used to test for departure from additivity by comparing the bounds to one - a lower bound of the interval greater than 1 indicates antagonism, an upper bound of less than 1 indicates synergism, and a value of 1 contained in the interval indicates additivity.

Identification of mutations selected in resistant populations. RNA was isolated from populations of resistant cells using either Trizol™ or RNeasy 96 Kit (Qiagen Inc., Valencia, CA) in accordance with the manufacturer's directions. First strand cDNA synthesis was performed on 1-3 µg of total RNA using Superscript III™ Reverse Transcriptase (Invitrogen, Carlsbad, CA) primed with gene specific oligonucleotide primers. PCR was performed on the cDNA using pairs of primers flanking the genes of interest (NS3 - NS5B). PCR products were sequenced and mutations were identified relative to vehicle-treated populations. PCR products were purified and cloned using TOPO®PCR Cloning methods (Invitrogen, Carlsbad CA). DNA was sequenced from ~100 clones to establish the frequency and co-incidence of mutations in a population.

To generate mutant replicons, point mutations were generated with the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, and mutations were confirmed by sequencing.

Transient replication assays. Replicon clones were linearized with Sca1 and transcribed *in vitro* using the Ambion T7 MegaScript kit (Ambion, Austin, TX), or the T7 RiboMAX^{TM} Express Large Scale RNA Production System (Promega, Madison, WI) according to manufacturer's directions. Transcribed RNA (3-5 μg) was transfected into cured Huh-7 cells (~2 x 10^6 in 60 mm dishes) with DMRIE-C reagent (Invitrogen Corporation, Carslbad, CA) following the manufacturer's protocol. After 4 to 6 h, transfected cells were transferred to 96 well assay plates (10^4 cells/well) and incubated in the presence of inhibitors for 72 h. Renilla luciferase assays were performed as described above.

RESULTS

Inhibition of replicon with combinations of HCV inhibitors. The HCV replicon system is widely acknowledged as a predictive tool for the development of DAAs that target HCV RNA replication (2). In the genotype 1 HCV replicon system, DCV, ASV, and BMS-791325 exhibit pM to low nM potency (Table 1). The virology profile and clinical effects of DCV (15, 44, 47), ASV (37,38), and BMS-791325, a non-nucleoside allosteric inhibitor of NS5B that binds a site on the surface of the thumb domain of the RNA-dependent RNA polymerase (60, 19, and Gentles, et al; manuscript in preparation) have been reported. These inhibitors were used to investigate the spectrum of events resulting from simultaneously targeting multiple essential proteins of HCV *in vitro*. Of particular interest was determining the effect of NS5A replication complex inhibitor, DCV, in combination with other DAAs.

We used the method of Chou (6) to evaluate the additive, antagonistic, or synergistic effects of combination therapies on HCV inhibition in a 3 day replicon assay. Table 2 shows the results of testing DCV and other inhibitors as monotherapies or in combination in HCV replicon. The CC_{50}s of these combined agents, analyzed in parallel by alamar blue staining, were greater than the highest tested inhibitor concentration. The effects of DCV in combination with ASV or BMS-791325 indicate mixed additivity/synergy over a range of molar ratios of inhibitors. When the NS5A, NS5B, and NS3 inhibitors were tested in a 3-drug combination, additive effects were observed at all effective doses. Taken as a whole, the results from multiple experiments demonstrate that combinations of these inhibitors display mixed additivity and/or synergy at the 50%, 75% and 90% effective dose levels of drug. Importantly, no antagonistic effects were observed with any of these combinations, nor was any increase in cytotoxicity observed.

Resistance selection with combinations of HCV inhibitors. Upon prolonged exposure of replicon cells to HCV inhibitors, colonies develop as resistant variants are selected. Figure 1 shows the relative densities of resistant colonies when DCV was combined with either ASV (panel a) or BMS-791325 (panel b) in cultures examined after continuous four week DAA exposure in the presence of G418. In multiple experiments, resistant colonies were markedly reduced when cells were treated with the DCV/ASV (Fig. 1a) or the DCV/BMS-791325 (Fig. 1b) combination compared with similar EC_{50} multiples of the DAAs alone. BVDV replicon cells treated in parallel showed no reduction in colony formation, suggesting a specific anti-HCV effect (data not shown). Interestingly, fewer resistant colonies were observed in combinations composed of 5x EC_{50} of DCV with any concentration of NS5B or NS3 inhibitor than were seen with alternate combinations pairing 5x EC_{50} of either NS5B or NS3 inhibitor with any concentration of DCV (Fig. 1a and 1b). For example, although both plates were exposed to a total of 35x EC_{50} of DAA, the 5x DCV/30x BMS-791325 plate developed far fewer resistant colonies than the 30x DCV/5x BMS-791325 plate (Fig. 1b). A similar phenomenon was observed for DCV in combinations with ASV (Fig. 1a).

Triple DAA treatment of HCV replicon cells with inhibitors at 10x and 30x their respective EC_{50}s resulted in complete clearance of HCV replicon (Fig.1c), while having no effect on BVDV replicon cells (not shown). In the presence of 5x EC_{50} of the three DAAs (total 15 EC_{50} multiples) fewer colonies were observed than after treatment with 30x of any single, or many pairs of DAAs. Similarly, in gt 1a replicon, combinations of two or three DAAs more effectively reduced resistant colony formation than any single DAA at similar EC_{50} multiples. The overall suppressive effect in gt 1a was slightly reduced compared to gt 1b (not shown).

Replicon clearance studies were also performed with the DAAs in the absence of G418 selection to evaluate the ability of various inhibitor combinations to eradicate HCV replicon RNA

from the cells. Similar to the resistance studies, replicon clearance occurred more efficiently in gt 1b than gt 1a. In both gt 1a and 1b, low EC_{50} multiples of DCV effected a much greater reduction in resistant colony formation compared with the same multiple of the other DAAs and a greater than additive effect on replicon clearance was observed using combinations of DAAs. Overall clearance with the DAA dual combinations in gt 1a was reduced 2 to 4-fold compared to gt 1b at 3x and 9x EC_{50}; the difference was greater (6 to 17-fold) at 27x EC_{50}.

Genotypic and phenotypic analysis of resistant variants. Resistant variants identified from selection with each DAA inhibitor class used in these studies have been previously described (12, 13, 28, 30, 31, 39, 47, 54, 64). The major gt 1b resistant substitutions are at residues 168 for NS3, 31 and 93 for NS5A and 495 for NS5B; while the major gt 1a resistant substitutions are at residues 155 for NS3, 28, 30, 31 and 93 for NS5A and 495 for NS5B. To study the impact of different DAA combinations on the emergence of resistance, and in an attempt to use these HCV specific inhibitors as a tool to study potential interactions of viral proteins, this report only emphasizes the genotypic and phenotypic analysis of unique or less frequently observed substitutions identified from dual and triple DAA selections. Resistant variants selected with one, two and three DAAs in HCV 1a and 1b replicons were analyzed by both population and clonal sequencing to determine the percentage and linkage of mutations within and between genes in the population. Substitutions observed with a frequency of $\geq$10% were engineered into wild type replicon to evaluate the contribution of the altered residue to resistance and impact on replication fitness in transient replication assays.

Analysis of NS5A/ NS3 dually resistant populations. Similar to previous reports, the major gt 1b resistance substitutions selected by DCV or ASV were L31M and Y93H in NS5A (12, 28) and D168V in NS3 (39, 47) (Table 3). For the dual selection performed in parallel, the most striking

substitution was N77S in NS3. The N77S variant has been reported in gt 1b under dual selection conditions with boceprevir and a 2-C-methyl-adenosine NS5B inhibitor. (5) In that case N77S exhibited no phenotype as it conferred no resistance to boceprevir and did not affect replication efficiency. However in this case N77S conferred a low level of resistance to ASV by itself (3- to 9-fold), and when combined with R155Q, which was itself associated with 8- to 9-fold ASV resistance, demonstrated an amplified level of resistance of 93- to 113-fold in the dually substituted N77S-R155Q variant. In NS5A, R30Q/H linked with L31M emerged in the dual selection. R30H, which has not been reported before, conferred minimal resistance to DCV (2- to 8-fold) but displayed significant resistance in combination with L31M (150- to 330-fold). When linkage of N77S-R155Q in NS3 occurred with multiple substitutions in NS5A (R30Q-L31M-Y93H), a very high level of resistance to both DCV (31,000- to 37,000-fold) and ASV (80- to 210-fold) was observed. The observation of the N77S substitution in NS3 only during dual selection was unexpected and indicates the influence of the NS5A inhibitor on the emergence of NS3 resistance *in vitro*. In gt 1a replicon cells, R155K was selected by ASV, while a D168G substitution was also observed during dual selection with ASV and DCV although substitutions at R155 and D168 in NS3 were not linked (Table 4). Selection with DCV yielded two predominant NS5A substitutions, Q30H and K68R, while in the dual selection, an additional NS5A resistance substitution, M28T, was also observed. The novel arginine substitution at residue 68 of NS5A did not confer any resistance but appeared to increase the relative fitness of the 1a variant (1-4 times the efficiency of wt) whereas replication of the Q30H-K68R mutant was greatly impaired. A dramatic improvement was observed with the dually selected D168G / Q30H-K68R clone which replicated at 30-70% the level of wt while the NS3 and NS5A mutants alone each had extremely impaired replication efficiency (4-9% the level of wt), suggesting an NS3-NS5A interaction that restored replicase

activity. Interestingly, combination of the Q30H-K68R substitutions in NS5A with D168G in NS3 reduced the level of NS5A resistance.

Analysis of NS5A/ NS5B dually resistant populations. Gt 1b replicon cells treated with DCV and BMS-791325 alone or in combination yielded resistant populations with substitutions at known positions in NS5A (R30, L31, Y93) (12) and NS5B (P495) (Lemm, J.A. et al; manuscript in preparation) (Table 3). For dual selection with NS5A and NS5B inhibitors, L31M and L31F were linked with R30Q (Table 3) while only L31M was selected by DCV alone. Phenotypic analysis of R30Q-L31F, with and without NS5B substitution P495S, revealed much greater (46- to 85-fold) resistance to DCV compared to the single L31F (5-fold) (12) and R30Q (Table 3) substitutions. While the NS5A R30Q-L31F variant replicated as efficiently as wt, linkage with the NS5B P495S substitution greatly impaired replication (Table 3).

In gt 1a, selection with BMS-791325 yielded substitutions of A421V and P495L in NS5B while, as stated earlier, DCV selection generated the Q30H-K68R variant in NS5A (Table 4). In dual selections, an additional L392I substitution in NS5B was also observed. Clonal analysis of the dual selections revealed linkage of Q30H-K68R in NS5A with each of the single NS5B substitutions (L392I, A421V, P495L) as no two NS5B changes were found in the same clone. The A421V NS5B substitution conferred no resistance to BMS-791325; however, it did increase the replication efficiency of the Q30H-K68R NS5A variant 10- to 15-fold (Table 4). The L392I substitution in NS5B has not been reported for gt 1a although it was previously shown in gt 1b to confer low level resistance (15- to 20-fold) to an indole-N-acetamide nonnucleoside inhibitor (52). In gt 1a, L392I conferred low level resistance to BMS-791325 (5- to 16-fold) while the P495L variant gave the expected high level resistance (60- to 100-fold).

Triple selection with NS5A, NS5B and NS3 inhibitors. Gt 1b cells treated with a fixed

299 concentration greater than 5x EC_{50} of DCV, ASV, and BMS-791325 together were unable to
300 survive. However, resistant variants emerged when cells were serially passaged with gradually
301 increasing concentrations of the three inhibitors. Populations treated in this way with up to 15x EC_{50}
302 selection consisted of an interesting mix of previously seen and new variants. Compared to the dual
303 selections, a different set of NS3 substitutions, Q41R with Q80R, and Q80R with R155Q, were
304 selected during triple DAA treatment. Presumed to be adaptive or compensatory changes,
305 substitutions of glutamine residues at positions 41 and 80 in NS3 are well documented, and Q80R
306 was selected with very low frequency in gt 1b replicon upon treatment with ASV (39). ASV
307 resistance to Q41R or Q80R was low but measurable (4- to 5-fold), and roughly additive when both
308 substitutions were present (7- to 12-fold) (Table 5). The combination of Q80R with R155Q
309 conferred greater resistance (30- to 100-fold) to ASV, which was 4-10x higher than observed with
310 each NS3 mutation alone. Despite differences in the NS3 sites affected by the triple DAA regimen,
311 the sites of resistance in NS5A and NS5B during triple-drug combination treatment were the same
312 as those targeted during dual drug selection regimens: R30, L31, and Y93 in NS5A and P495 in
313 NS5B (Table 5). Replication efficiency for these re-constituted triply resistant variants was
314 significantly impaired.

315 Triple selection in the gt 1a replicon cells readily yielded a cell line that displayed
316 significant resistance to all three inhibitors. Along with known substitutions at amino acids 155
317 and 168, several additional substitutions were identified in NS3, and clonal analysis revealed
318 100% linkage between NS3 D168E and M179T, R155K and M179A, and Q80R and Y134H; as
319 well as T389I and A421V in NS5B (Table 6). The Q89R, Y134H and M179A/T NS3 mutations
320 showed no resistance to ASV nor did they significantly affect resistance levels in combination with
321 other substitutions except M179A, which slightly enhanced R155K resistance (3 to 5-fold) in the

context of the triple mutant (Table 6). Alone, the Q89R change significantly enhanced replication (2 – 6x the efficiency of wt) and may play a similar role in the fitness of triple mutants to allow replication of these highly altered replicons. Along with substitutions at amino acid 495, a new combination of NS5B mutations was identified in the triple selection, T389I-A421V, that conferred low level resistance to BMS-791325 (3 to 8-fold). It also appears that high level resistance to NS3 and NS5B inhibitors were not selected in the same clone. High level NS5B resistance due to the P495L substitution is associated with low level NS3 resistance from Q80R and conversely, high level NS3 resistance (Q89R-R155K-M179A) is linked to T389I-A421V changes in NS5B which confer low level resistance. Levels of NS5A resistance remain consistent in all three triple mutants. Table 7 provides a summary of the resistant substitutions observed during treatment with combinations of DAAs in these studies. However, as reported, additional replication enhancing substitutions were also observed, particularly during triple DAA treatment.

DISCUSSION

Having shown promising antiviral activity in early clinical trials (15), the picomolar NS5A replication complex inhibitor DCV was examined in combination with other DAAs targeting distinct steps in the replication process. Since it has yet to be established whether efficacy and resistance profiles for combinations of DAAs will reflect the sum of the profiles of the individual drugs, *in vitro* replicon studies may provide insight into clinical outcomes for combinations of non-cross-resistant DAAs in terms of impact on viral replication and emergent resistance pathways.

In 72 h replicon assays, combination of DCV with NS3 protease and/or NS5B polymerase inhibitors demonstrated additive to synergistic inhibitory effects on replicon activity. Replicon curing performed over a seven-day treatment period provided evidence of a similar, greater than

additive effect from combining these inhibitors. Clearance from gt 1b replicon was generally twice as efficient as from gt 1a, a genotype difference that appears to extend to clinical outcomes for DCV (15, 35). Moreover, a low EC_{50} multiple of DCV coupled with either the NS3 or NS5B-targeted DAA had a much more profound impact on limiting resistance development than did a low EC_{50} multiple of either NS3 or NS5B inhibitor combined with DCV. The impressive dose response curve for curing with DCV compared to the other inhibitors suggests that NS5A inhibition more readily attenuates the ability of HCV replicon to survive. Since data suggest homotypic oligomeric interactions of the NS5A protein (7, 36, 62), it is possible that binding of a single NS5A inhibitor molecule may induce conformational changes that translate to adjacent NS5A molecules, cooperatively impacting the functionality of the entire replication complex. Higher concentrations of NS5A would not have such an impact as the system may become saturated. Such a model could explain the steep dose-response curve of clearance with DCV, and suggests an advantage of including DCV in DAA combination regimens.

It is known that minor changes in selection conditions can affect the pattern of resistance observed. Here we performed selections with single and multiple inhibitors in parallel to compare resistance emergence between monotherapy and combination DAA treatment under the same selective conditions. In general, the resistant variants observed in this study were all at reported or predicted positions but were distinct in terms of frequency and emergence among different DAA combinations. In multiple experiments, the NS3 substitution N77S emerged in gt 1b replicons during two-DAA treatment. Substitution at this residue has been detected in gt1a replicons selected with a close analog of ASV (40) and in gt 1b replicon selected with a combination of protease inhibitor (boceprevir) and nucleoside analog NS5B inhibitor, without an apparent phenotype, but to our knowledge N77S has not previously been reported as the predominant resistance variant in

either gt 1b or 1a with any protease inhibitor. This substitution is located at the end of the enzyme's EF loop and may interact with the bulky P2 moiety of inhibitors like ASV. Although it is a low level resistance substitution, N77S may influence subsequent changes in the population, as it was observed first under low dual selective pressure and was 98% linked to R155Q in populations selected under higher selective pressure with the two DAAs. In contrast, populations of cells treated with any level of ASV pressure alone were homogeneous for D168V. In these studies, the opposite was observed in gt 1a where substitution at amino acid 168 only occurred during combination treatment while monotherapy elicited a R155K substitution. In NS5A, the mix of amino acid changes at position 30, 31, and 93 differed in gt 1b populations emerging under single and dual drug selection. The new R30H substitution in gt 1b was observed only in the NS5A/NS3 dual selection and while it showed minimal resistance itself, it enhanced resistance of L31M ~100-fold. In contrast, position 30 plays an important direct role in resistance in gt 1a NS5A, where the change from wild type glutamine to histidine (Q30H) conferred significant resistance to DCV (>1000-fold), highlighting the genotype context-dependent role of this residue. In general, resistance in NS5B for both genotypes arose predominantly at amino acid 495 where the different relative frequencies of S/L/A variants correlated with different levels of resistance. However, an additional NS5B substitution (L392I) occurred in the gt 1a NS5A/NS5B dual selection, generating a variant with low level resistance and suggesting that alterations to NS5A, either through inhibitor interactions or via resistance substitutions, can effect emergence of resistance in NS5B, consistent with reports of NS5A-NS5B interactions (50, 57).

From the mutations that emerged only in dual selections, we can infer that cross-target influences allowed the different substitutions to emerge. Given that the nonstructural proteins targeted by these DAAs are believed to function together as a multi-component complex, one can

envision that simultaneously targeting multiple proteins may necessitate additional changes to allow formation of an active replicase complex. Others have also reported novel mutations (5) or increased prevalence of minor variants (30) in dual-DAA-selected populations. Although the overall differences in sequence and resistance levels we observed between single- and dually-treated populations were not dramatic, they alert us to the potential for unexpected outcomes during combination treatment. The results indicate that the replicon may not tolerate more dramatic differences given that the target proteins still need to associate into an active replication complex. Whether the mutations that emerged *in vitro* as a result of dual and triple DAA challenges will also be observed in patients treated with the same combinations will be determined as clinical data are reported. The HCV replicon system will be extremely useful for analyzing samples from subjects treated with combinations of DAA.

Interesting differences in replication fitness were also observed between mono and dual treatment. For example, in both gt 1a and 1b, selection with ASV alone elicited the less 'costly' NS3 resistance substitution in terms of fitness. However, in the presence of both DCV and ASV, the more costly substitution appears to have been compensated for by the presence of additional substitutions in the second protein. For example, in gt 1b, N77S-R155Q replicates with ≤10% the efficiency of wt replicon, but when linked with NS5A substitutions, the dually resistant variants replicated at least 3x more efficiently. Such compensatory changes could explain why cells treated with either DCV or ASV developed >100-fold resistance, while cultures treated in parallel with both inhibitors developed only 5- to 10-fold resistance, and required additional passages to develop a high level of resistance. More time may be needed to derive the correct mix of compatible substitutions whose combined fitness costs were not fatal. Likewise, the poor replication (<10%) of the Q30H-K68R variant selected in gt 1a with DCV treatment suggests an additional change outside

NS5A must have been present in the selected cell line to enhance replication. The increased replication of the dually selected D168V/Q30H-K68R variant (30-70% wt), compared to variants with the same changes in the individual NS3 and NS5A proteins (both <10% wt) was noteworthy. This finding suggests NS3-NS5A cross-target influences that enhance efficiency of the replication complex.

The high clearance efficiency of the triple combination probably reflects the reduced frequency of the multiple genetic events necessary to generate a viable triply-resistant HCV replicon. However, variants resistant to all three inhibitors were eventually selected, and these had some of the same mutations that had been selected during dual combination studies, as well as new ones. In NS3, Q80R was found to be linked to either R155Q or Q41R in 1b cultures maintained in the presence of the three DAAs, but not in parallel cultures selected with monotherapy or 2-DAA combinations. These mutations, both previously identified as NS3 resistance sites, conferred low level resistance on their own, and Q80R has been shown to have an auxiliary role in enhancing the resistance of R155 or D168 mutations (29, 41, 51). In gt 1a, D168E and M179T substitutions were 100% linked, as were R155K and M179A in a more highly resistant variant. In addition, both clones contained a Q89R substitution. This substitution on its own greatly enhanced replicon replication and may be essential to permit replication of these triple mutants during the increased pressure from selection with three inhibitors. A new combination of low level NS5B resistance variants was also detected specifically in the gt 1a triple selection (T389I-A421V). The fact that the T389I-A421V substitutions enhanced replication of the Q30H-K68R NS5A variant (5 to 10-fold) suggests that together, changes in these two proteins can form a more active replicase than one with only NS5A substitutions. It is of interest that in gt 1a clones demonstrating resistance to all three inhibitors, high level resistance to both NS3 and NS5B were not detected in the same clone. This

may indicate that the fitness cost associated with highly resistant substitutions in three key proteins of the HCV replication machinery is too great to yield a genome capable of high replicative fitness. Collectively, these results indicate that challenging HCV with multiple DAAs had a compelling suppressive effect on replication and elicited novel mutations and combinations of mutations. Since interferon-α and unrelated HCV inhibitor chemotypes retained activity against the multi-DAA resistant populations generated in these experiments (data not shown), further complementation or follow-up to a combination DAA regimen should be feasible if required to achieve SVR. If the synergies demonstrated here for combinations of DAA inhibitors are predictive of reduced resistance and enhanced viral clearance in patients, a triple combination regimen targeting three different proteins of HCV replication, and particularly including an NS5A inhibitor, promises to markedly improve the rates of SVR over alfa/RBV regimens, regimens including a single DAA ± RBV, and many dual-DAA combinations. Such combinations have the potential to quickly reduce viral load and limit opportunities for the emergence of multi-drug resistance, helping to achieve SVR. Given the altered patterns of resistance observed during these combination studies, these results provide guidance for resistance monitoring during ongoing combination trials.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the technical contributions of Lourdes Valera and Bernadette Kienzle.

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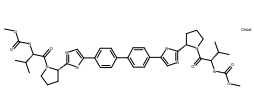
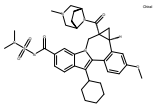
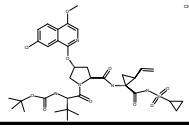
687

688 **Table 1. *In vitro* antiviral profile of BMS HCV inhibitors**

689

HCV Replicon Genotype	Daclatasvir 	BMS-791325 	Asunaprevir 
1a (H77c) EC_{50}	50 ± 13 pM	3 ±1 nM	4 ± 0.8 nM
1b (Con1) EC_{50}	9 ± 4 pM	7 ± 2 nM	3 ± 2 nM
1b (Con1) CC_{50}	17 ± 1 µM	20 ± 6 µM	26 ± 5 µM
Therapeutic Index (CC_{50} / EC_{50})	1,900,000	2900	8700

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691 Data shown represent ≥3 independent tests ± standard deviation.

Table 2. Combination of daclatasvir with asunaprevir or BMS-791325

Experiment	Ratio, Inhibitor X to Daclatasvir	Overall Result Asunaprevir + Daclatasvir	Overall Result BMS-791325 + Daclatasvir	Overall Result Asunaprevir + BMS-791325 + Daclatasvir 1:1:1
1	1:1	Synergy / Additivity	Synergy	
	2.5:1	Synergy	Additivity	Additivity
	1:2.5	Synergy	Synergy	
2	1:1	Additivity	Synergy / Additivity	Additivity
	2.5:1	Synergy / Additivity	Additivity	
	1:2.5	Synergy / Additivity	Synergy	
3	1:1	Synergy	Synergy / Additivity	Additivity
	2.5:1	Additivity	Synergy / Additivity	
	1:2.5	Synergy	Synergy	

Table 3. Characterization of gt 1b variants identified in dual selections using transient replication assays

Selection Condition	Variant[a]			Fold-Resistance[b]			Replication Efficiency Relative to WT
	NS3	NS5A	NS5B	Asunaprevir (ASV)	Daclatasvir (DCV)	BMS-791325	
5x or 30x ASV	**D168V**	–	–	**>100**	**0.9**	**2**	**0.3-0.5**
5x DCV	–	**L31M**	–	**1**	**2 - 5**	**1**	**0.5-1.5**
30x DCV	– –	**Y93H** **L31M-Y93H**	– –	**1** **nd**	**10-33** **4200[d]**	**1** **nd**	**0.1-0.3** **0.4**
5x or 20x 325[c]	–	–	**P495A**	**1**	**1**	**9 - 16**	**0.3-0.8**
20x 325	–	–	**P495S**	**1**	**1**	**40-86**	**0.01-0.1**
5x ASV/DCV	**N77S**	**R30Q-L31M**	–	**5 - 11**	**6 - 16**	**1**	**0.5-1.2**
30x ASV/DCV	**N77S-R155Q** **N77S-R155Q**	**R30Q-L31M-Y93H** **R30H-L31M**	– –	**80-210** **62-170**	**31000-37000** **150-330**	**1** **1**	**0.2-0.5** **0.3-0.7**
5x DCV/325	–	**R30Q-L31M**	**P495A**	**1**	**12 - 22**	**9 - 20**	**0.4-0.8**
20x DCV/325	–	**R30Q-L31F**	**P495S**	**1**	**46-62**	**111-128**	**0.1**
site-directed mutant	N77S	–	–	3 - 9	1	1	0.2-0.5
site-directed mutant	R155Q	–	–	8 - 9	1	1	0.01-0.02
site-directed mutant	N77S-R155Q	–	–	93-113	1	1	0.02-0.1
site-directed mutant	–	R30Q	–	1	2	1	0.7-1.1
site-directed mutant	–	R30H	–	1	2 - 8	1	0.1-0.2
site-directed mutant	–	R30Q-L31M	–	1	4 - 16	1	0.9-1.2
site-directed mutant	–	R30Q-L31F	–	2	57-85	1	1.0-1.2
site-directed mutant	–	R30Q-L31M-Y93H	–	1	25000-51000	1	0.7-1.4

[a] Replicons containing specific substitution(s) identified from selections were tested for resistance and replication fitness. Major selected variant constructs are in bold font. Site-directed mutants were not selected but were generated as controls in the WT backbone to assess impact on phenotype. For each selection condition, each row represents a unique variant including any linked mutations.

[b] Fold of resistance was determined by dividing the mutant HCV replicon EC_{50} by the WT replicon EC_{50}. WT EC_{50}s were 2.0 ± 0.6, 0.002 ± 0.001, and 8.3 ± 2.2 nM for ASV, DCV, and BMS-791325, respectively. Results represent the range of values from two or three independent experiments.

[c] Value was taken from the work of Fridell et al (12)

[d] 325 = BMS-791325

36

706 - indicates wt sequence

707

Table 4. Characterization of gt 1a variants identified in the dual selections using transient replication assays

Selection Condition	Variant[a]			Fold- Resistance[b]			Replication Efficiency Relative to WT
	NS3	NS5A	NS5B	Asunaprevir (ASV)	Daclatasvir (DCV)	BMS-791325	
10x or 30x ASV	**R155K**	**–**	**–**	**17 - 48**	**1**	**1**	**0.3 - 0.5**
10x or 30x DCV	**–**	**Q30H-K68R**	**–**	**1**	**1280 - 2000**	**3**	**0.04 - 0.07**
10x or 30x 325[c]	**–** **–**	**–** **–**	**A421V** **P495L**	**1** **2**	**1** **1**	**1 - 3** **88 - 100**	**0.3 - 0.9** **0.07 - 0.1**
30x DCV/ASV	**R155K** **R155K** **D168G**	**Q30H-K68R** **M28T-K68R** **Q30H-K68R**	**–** **–** **–**	**46 - 68** **34 - 61** **18 - 30**	**667 - 700** **1125-13333** **111 - 333**	**1** **1** **1**	**0.5 - 0.8** **0.4 - 1** **0.3 - 0.7**
30x DCV/325	**–** **–** **–**	**Q30H-K68R** **Q30H-K68R** **Q30H-K68R**	**A421V** **P495L** **L392I**	**1** **2** **2**	**1000** **1600** **2200**	**2** **61 - 84** **6 - 16**	**0.8 - 1** **0.03 - 0.07** **0.06 - 0.19**
site-directed mutant	D168G	–	–	8 - 16	1	1	0.07 - 0.09
site-directed mutant	–	M28T	–	1	750 - 820	1	0.2 - 0.4
site-directed mutant	–	Q30H	–	1	1111- 1967	1	0.4 - 0.6
site-directed mutant	–	K68R	–	1	1	1	1 - 4.6
site-directed mutant	–	–	L392I	1	1	5 - 7	0.1 - 0.4

[a] Replicons containing specific substitution(s) identified from selections were tested for resistance and replication fitness. Major selected variant constructs are in bold font. Site-directed mutants were not selected but were generated as controls in the WT backbone to assess impact on phenotype. For each selection condition, each row represents a unique variant including any linked mutations.

[b] Fold of resistance was determined by dividing the mutant HCV replicon EC_{50} by the WT replicon EC_{50}. WT EC_{50}s were 0.7 ± 0.3, 0.006 ± 0.002 and 2.2 ± 0.9 nM for ASV, DCV, and BMS-791325, respectively. Results represent the range of values from two or three independent experiments.

[c] 325 = BMS-791325

- indicates wt sequence

Table 5. Characterization of gt 1b variants identified in the triple selection using transient replication assays

Selection Condition	Variant[a]			Fold- Resistance[b]			Replication Efficiency Relative to WT
	NS3	NS5A	NS5B	Asunaprevir (ASV)	Daclatasvir (DCV)	BMS-791325	
10x or 15x triple[c]	**Q80R-R155Q Q80R-R155Q**	**R30Q-L31M R30Q-L31M-Y93H**	**P495A P495A**	**30-93 86-99**	**5-15 >500**	**17-22 8-22**	**0.02-0.1 0.01**
Site-directed mutant	Q41R	–	–	4	1	1	1.0-1.4
Site-directed mutant	Q80R	–	–	4-5	1	1	0.5-1.0
Site-directed mutant	Q41R-Q80R	–	–	7-12	1	1	1.0-1.2
Site-directed mutant	Q80R-R155Q	–	–	42-73	1	1	0.5-1.0

[a] Replicons containing specific substitution(s) identified from selections were tested for resistance and replication fitness. Major selected variant constructs are in bold font. Site-directed mutants were not selected but were generated as controls in the WT backbone to assess impact on phenotype. For each selection condition, each row represents a unique variant including any linked mutations.

[b] Fold of resistance was determined by dividing the mutant HCV replicon EC_{50} by the WT replicon EC_{50}. WT EC_{50}s were 2.0 ± 0.6, 0.002 ± 0.001, and 8.3 ± 2.2 nM for ASV, DCV, and BMS-791325, respectively. Results represent the range of values from two or three independent experiments

[c] Selection of viable triple mutants in gt 1b required sequential passage in gradually increasing concentrations of inhibitor as detailed in Materials and Methods.

- indicates wt sequence

Table 6. Characterization of gt 1a variants identified in the triple selection using transient replication assays

Selection Condition	Variant[a]			Fold- Resistance[b]			Replication Efficiency Relative to WT
	NS3	NS5A	NS5B	Asunaprevir (ASV)	Daclatasvir (DCV)	BMS-791325	
30x triple	**Q80R-Y134H Q89R-D168E-M179T Q89R-R155K-M179A**	**Q30H-K68R Q30H-K68R Q30H-K68R**	**P495L A421V T389I-A421V**	**10-12 15-35 67-170**	**1000 1500-2200 800-1375**	**25-70 2 5-8**	**0.03 - 0.07 0.05 - 0.12 0.72 - 1.9**
Site-directed mutant	–	Q30H-K68R	T389I-A421V	2	1400	4 - 5	0.2 - 0.5
Site-directed mutant	Q80R	–	–	6 - 11	2	2	0.4 - 0.6
Site-directed mutant	Q89R	–	–	2	1	1	2.0 - 6
Site-directed mutant	Y134H	–	–	2	2	2 - 3	0.2 - 0.3
Site-directed mutant	D168E	–	–	47 - 74	1	1	0.6 - 1.2
Site-directed mutant	M179A	–	–	1	1	1	0.6 - 1.2
Site-directed mutant	M179T	–	–	1	1	1	0.3 - 0.7
Site-directed mutant	Q89R-R155K	–	–	26 - 67	2	1	0.4 - 1.1
Site-directed mutant	–	–	T389I-A421V	1	1	3 - 5	0.5 - 1.2
Site-directed mutant	–	–	T389I	1	1	1	1.1 - 2.6

[a] Replicons containing specific substitution(s) identified from selections were tested for resistance and replication fitness. Major selected variants are in bold font. Site-directed mutants were not selected but were generated as controls in the WT backbone to assess impact on phenotype. For each selection condition, each row represents a unique variant including any linked mutations.

[b] Fold of resistance was determined by dividing the mutant HCV replicon EC_{50} by the WT replicon EC_{50}. WT EC_{50}s were 0.7 ± 0.3, 0.006 ± 0.002 and 2.2 ± 0.9 nM for ASV, DCV, and BMS-791325, respectively. Results represent the range of values from two or three independent

742 experiments

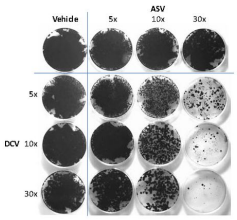
743 - indicates wt sequence

Table 7. Summary of predominant resistance substitutions selected during inhibitor treatment

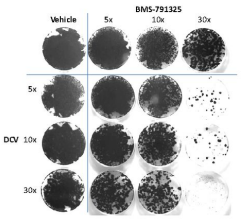
Target of Inhibitors used for Selection	Genotype 1b			Genotype 1a		
	NS3	NS5A	NS5B	NS3	NS5A	NS5B
NS3	D168V			R155K		
NS5A		L31M, Y93H			Q30H	
NS5B			P495A/S/L			P495L
NS3 - NS5A	N77S, R155Q	R30Q/H, L31M, Y93H		R155K, D168N/G	Q30H, M28T	
NS5A - NS5B		R30Q/H, L31M/F, Y93H	P495A/S/L		Q30H	L392I, P495S/L
NS3 - NS5A - NS5B	Q41R, Q80R, R155Q	R30Q/H, L31M, Y93H	P495A	Q80R, R155K, D168E	Q30H	P495L, T389I-A421V

Figure 1. Combination treatment reduces the emergence of resistant colonies. gt1b HCV replicon cells were incubated for four weeks with BMS-790052 (DCV)BMS-791325, or BMS-650032 (ASV) as mono, dual-, and triple-therapy (1a ,1b and 1c) at 5, 10 and 30x EC_{50}. Colonies were visualized by crystal violet staining. Data shown are representative of three independent experiments.

a.



b.



c.