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The Combination of Alisporivir plus an NS5A Inhibitor Provides Additive to Synergistic Anti-Hepatitis C Virus Activity without Detectable Cross-Resistance

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Alisporivir (ALV), a cyclophilin inhibitor, is a host-targeting antiviral (HTA) with multigenotypic anti-hepatitis C virus (HCV) activity and a high barrier to resistance. Recent advances have supported the concept of interferon (IFN)-free regimens to treat chronic hepatitis C. As the most advanced oral HTA, ALV with direct-acting antivirals (DAAs) represents an attractive drug combination for IFN-free therapy. In this study, we investigated whether particular DAAs exhibit additive, synergistic, or antagonistic effects when combined with ALV. Drug combinations of ALV with NS3 protease, NS5B polymerase, and NS5A inhibitors were investigated in HCV replicons from genotypes 1a, 1b, 2a, 3, and 4a (GT1a to -4a). Combinations of ALV with DAAs exerted an additive effect on GT1 and -4. A significant and specific synergistic effect was observed with ALV-NS5A inhibitor combination on GT2 and -3. Furthermore, ALV was fully active against DAA-resistant variants, and ALV-resistant variants were fully susceptible to DAAs. ALV blocks the contact between cyclophilin A and domain II of NS5A, and NS5A inhibitors target domain I of NS5A; our data suggest a molecular basis for the use of these two classes of inhibitors acting on two distinct domains of NS5A. These results provide *in vitro* evidence that ALV with NS5A inhibitor combination represents an attractive strategy and a potentially effective IFN-free regimen for treatment of patients with chronic hepatitis C. Due to its high barrier and lack of cross-resistance, ALV could be a cornerstone drug partner for DAAs.

Hepatitis C virus (HCV) is the major causative agent of chronic hepatitis, cirrhosis, and hepatocellular carcinoma in the United States (1). Nearly 200 million people worldwide (3% of the population), including 4 to 5 million in the United States, are chronically infected with HCV, and 4 million new infections occur every year (2, 3). Although the addition of the recently approved protease inhibitors boceprevir and telaprevir improved the efficacy of pegylated-interferon (IFN)/ribavirin (RBV) treatment, there remains the need for the development of more effective and better-tolerated anti-HCV regimens, especially oral therapies that are effective against all HCV genotypes (1, 2). In this regard, it is noteworthy that the new direct-acting antiviral (DAA) combinations under advanced development have a relative deficiency in their ability to effectively treat genotype 3.

To date, some 30 anti-HCV agents have been investigated, representing two main classes of anti-HCV agents: direct-acting antivirals (DAAs) and host-targeting antivirals (HTAs). The current DAAs target the viral NS3 protease, the NS5B polymerase, or the NS5A protein. The function of NS5A is not clear, but it appears to play multiple key roles in viral replication, including regulating the activity of the NS5B polymerase, cell signaling pathways, and viral particle release (4). The HTAs currently being tested in clinical trials target host proteins critical for HCV replication, such as cyclophilin A and microRNA 122 (miRNA-122) (5). The cyclophilin inhibitors, which neutralize the isomerase activity of cyclophilin A, have demonstrated great efficacy for the treatment of HCV (5). ALV, a synthetic cyclophilin inhibitor derived from cyclosporine, is the most advanced cyclophilin inhibitor currently in clinical development for treatment of chronic hepatitis C (6).

Conceptually, an ideal IFN-free therapy would consist of a combination of several anti-HCV agents with different mechanisms of action in order to enhance antiviral effectiveness and avoid viral resistance. We investigated in this study whether particular DAAs exhibit additive, synergistic, or antagonistic effects when combined with the effective HTA ALV.

MATERIALS AND METHODS

Compounds. The NS5A inhibitor daclatasvir (Bristol Myers Squibb), the NS5B polymerase inhibitors sofosbuvir (Gilead) and mericitabine (Roche), and the NS3 inhibitors boceprevir (Merck) and telaprevir (Vertex) were obtained from MedChemexpress (Princeton, NJ, USA). ALV was provided by Novartis, and sanglifehrin B was provided by M. A. Gregory and B. Wilkinson.

Replicons. In the present study, we used several HCV replicons, derived from HCV G1, G2, G3, and G4 (Fig. 1). The GT1a subgenomic *Renilla* luciferase reporter replicon H77 RLucP (7) was generously provided by W. Delaney (Gilead). The GT1b subgenomic firefly luciferase reporter replicon pFK-I389/NS3–3′ (8) was generously provided by R. Bartenschlager. The GT1B subgenomic NS3, NS5A, and NS5B mutants were created via homologous recombination using the In-Fusion HD cloning kit (Clontech). The GT2a genomic luciferase reporter replicon

Received 4 January 2014 Returned for modification 3 February 2014
Accepted 24 March 2014
Published ahead of print 31 March 2014
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This is publication no. 25033 from the Department of Immunology & Microbial Science, The Scripps Research Institute, La Jolla, CA.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.00016-14.

doi:10.1128/AAC.00016-14

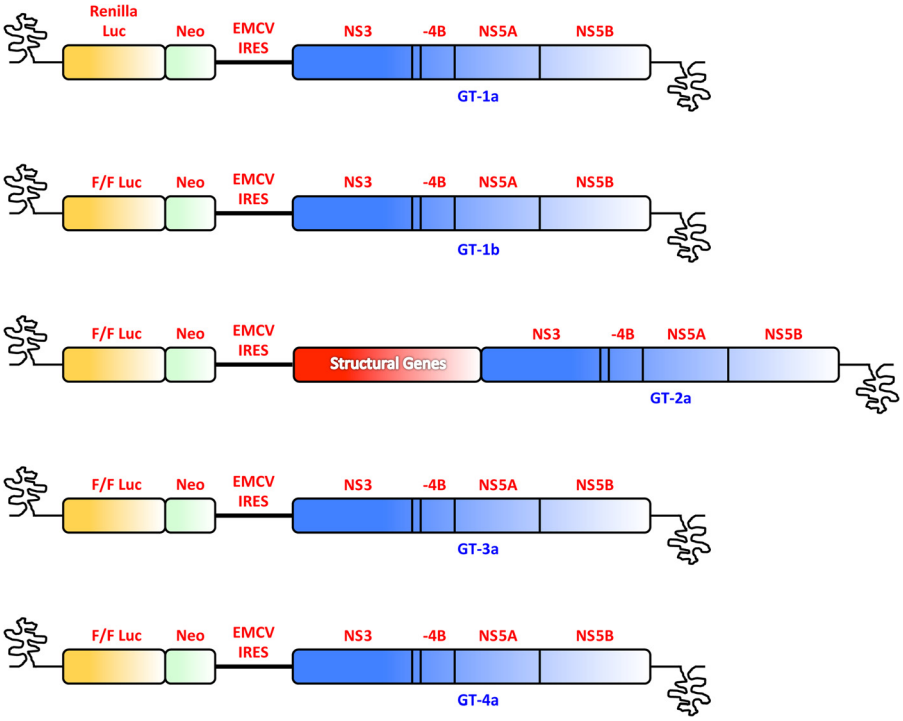

FIG 1 Schematic representation of the luciferase reporter HCV replicons used in the study.

Luc-Neo-JFH-1 was created as follows. The plasmid pFK-Luc-JFH1 was generously provided by T. Wakita and T. Pietschmann (9, 10), and the XbaI site in the firefly luciferase gene and the NotI site in the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) were utilized to clone the luciferase/ubiquitin-NPT II (the neomycin phosphotransferase II gene) fusion cassette out of pFK389ILuc-Neo (wild-type replicon from GT1b) (8, 10) and placed into the pFK-Luc-JFH1 plasmid, creating the full-length Luc-Neo-JFH-1 construct. The GT3a subgenomic firefly luciferase reporter replicon S52/SG-Feo (AI) and the GT4a subgenomic firefly luciferase reporter replicon ED43/SG-Feo (11) were generously provided by C. Rice and J. Bukh. Replicons were stably expressed in Huh7.5 or Huh7.5.1 cells under G418 selection.

Half-maximal effective concentration (EC_{50}) measurements. On day 1, 5,000 cells were plated in 96-well plates in complete Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). On day 2, increasing concentrations of each drug (2-fold steps spanning from picomolar to millimolar concentrations) were added to each well. On day 5, 72 h after drug addition, medium was removed and replaced with 100 μl of fresh complete medium. To each well, 100 μl of the fluorogenic, cell-permeant peptide substrate glycyl-phenylalanyl-amino fluorocoumarin was added in complete medium as per the manufacturer's recommendation (Promega Corp., Madison, WI). The CellTiter-Fluor cell viability assay is a nonlytic, single-reagent-addition fluorescence assay that measures the relative number of live cells. Cells were incubated at 37°C for 1 h and assayed on a fluorescence plate reader (Synergy H4; Biotek) at an excitation of 390 nm and emission of 505 nm. Medium was removed, and cells were washed and lysed in 20 μl of cell culture lysis reagent. Luciferase activity was determined using the luciferase assay system (Promega) in a Berthold luminometer.

Drug combination analyses. Drugs were tested in pairs in seven dosing combinations for each compound, centered around the calculated EC_{50} in replicon cell lines specific to each genotype. Cell viability and reporter assays were carried out as described above in five replicates for each drug combination, and the normalized data were used to analyze the synergy between different combinations using the MacSynergyII program. This program is based on the Bliss independence model that is defined by the following equation: $E_{xy} = E_x + E_y - (E_x \times E_y)$, where E_{xy} is the additive effect of drugs x and y as predicted by their individual effects, E_x and E_y (12).

RESULTS

Determination of EC_{50} for ALV and selected DAAs. ALV is effective (nanomolar range) against all genotypes: GT1a (EC_{50} = 10.8 nM), GT1b (EC_{50} = 16.6 nM), GT2a (EC_{50} = 9.8 nM), GT3a (EC_{50} = 24.0 nM), and GT4a (EC_{50} = 28.9 nM) (Table 1). This demonstrates the multigenotypic activity that ALV exhibits when it is tested with genotype-specific replicons. The protease inhibitors boceprevir and telaprevir were less effective than ALV. EC_{50}s of boceprevir were 266.6, 258.1, 301.5, 578.5, and >16,000 nM for GT1a, -1b, -2a, -3a, and -4a, respectively. EC_{50}s of telaprevir were 354.0 and 720.6 nM for GT1b and -2a, respectively. The NS5A inhibitor daclatasvir was effective: the calculated EC_{50}s of daclatasvir were 0.018, 0.014, 0.099, 5.295, and 0.016 nM for GT1a, -1b, -2a, -3a, and -4a, respectively. In contrast to ALV, which exerts a similar efficacy among genotypes (10 to 30 nM), the NS5A inhibitor daclatasvir is more effective on particular genotypes than others: it is highly effective on GT1a, -1b, and 4a, effective on GT2a, and less effective on GT3a. The NS5B polymerase inhibitors sofosbuvir and mericitabine were effective against all genotypes at levels similar to those of boceprevir and telaprevir. We calculated EC_{50}s of sofosbuvir of 236.1, 333.8, 337.3, 1,676.4, and 725.4 nM for GT1a, -1b, -2a, -3a, and -4a, respectively. EC_{50}s of mericitabine were 946.9, 762.1, 1,111.3, 3,337, and 726.3 nM for GT1a, -1b, -2a, -3a, and -4a, respectively. Together these data demonstrate that all selected DAAs are effective against a majority of genotypes and therefore have the potential to be combined with ALV in clinical studies.

TABLE 1 EC_{50}s of ALV and selected DAAs for different HCV genotypes

Antiviral	EC_{50} (nM)[a]				
	GT-1a	GT-1b	GT-2a	GT-3a	GT-4a
ALV	10.8 ± 1.3	16.6 ± 0.8	9.8 ± 0.6	24.0 ± 2.1	28.9 ± 1.8
Boceprevir	266.6 ± 18.5	258.1 ± 8.5	301.5 ± 24.6	578.5 ± 39.8	>16,000
Telaprevir		354.0 ± 24.6	720.6 ± 39.5		
Daclatasvir	0.018 ± 0.002	0.014 ± 0.003	0.099 ± 0.002	5.295 ± 0.426	0.016 ± 0.002
Sofosbuvir	236.1 ± 13.5	333.8 ± 18.5	337.3 ± 20.3	1,676.4 ± 88.4	725.4 ± 38.9
Mericitabine	946.9 ± 47.2	762.1 ± 26.7	1,111.3 ± 78.5	3,337 ± 148.4	726.3 ± 57.2

[a] Data are means ± standard errors of the means for 5 replicates.

Combination of ALV and selected DAAs. We then conducted combination studies with the intention of identifying an optimal partner for ALV. Drugs were tested in pairs in seven dosing combinations for each drug, centered nearby calculated EC_{50}s. The combination of ALV with the NS5A polymerase (daclatasvir), an NS5B polymerase (sofosbuvir and mericitabine), or the protease inhibitor (boceprevir) was mainly additive for GT1a (Fig. 2, first column). As for GT1a, each ALV combination exerted an additive effect on GT1b, even the ALV-telaprevir combination (Fig. 2, second column), suggesting that the combination of ALV with each class of selected DAAs causes an additive antiviral effect on GT1. In contrast to GT1, where we observed a strict additive effect, we observed a moderately synergistic effect when ALV was combined with daclatasvir (Fig. 2, third column). A significant synergistic effect on GT2a was detected when ALV was combined with sofosbuvir, whereas a moderately synergistic effect was observed when ALV was combined with mericitabine. Only an additive effect was observed when ALV was combined with boceprevir. In contrast, the combination of ALV with daclatasvir was synergistic on GT3a (Fig. 2, fourth column). The combinations of ALV with sofosbuvir and mericitabine were moderately synergistic, whereas the combination of ALV with boceprevir was additive. In control experiments, the combination of ALV plus ALV exerted an additive effect on GT3a. In contrast to the combination of ALV and daclatasvir, which exerted a synergistic effect on GT3a, the combination of sofosbuvir and daclatasvir exerted only an additive antiviral effect. Thus, the synergistic effect between ALV and NS5A inhibitors appears to be specific. Note that some drug combinations at specific drug concentrations appear be antagonistic, especially for GT1 and GT4a. We then tested the possibility that a combination of two classes of anti-HCV inhibitors—cyclophilin inhibitors and NS5A inhibitors—mediates a synergistic effect on specific genotypes. Specifically, we examined the antiviral activity of the combination of the cyclophilin inhibitor sanglifehrin B and daclatasvir. Similarly to the ALV-daclatasvir combination, the sanglifehrin B-daclatasvir combination exerted an additive effect on GT1a and synergistic effects on GT2a and -3a (Fig. 3). We next examined the antiviral effect of ALV combinations on GT4a (ED43/SG-Feo). We found that all drug combinations exerted an additive antiviral effect (Fig. 2, fifth column). Altogether, these data suggest that the combination of ALV and NS5A inhibitors exerts an additive effect on GT1 and -4, a moderately synergistic effect on GT2, and a synergistic effect on GT3.

Cross-resistance studies. We then examined the sensitivity of ALV-resistant variants to DAAs. A double NS5A substitution (D320E/Y321N) renders HCV more resistant (~3- to 5-fold) than single substitutions (D320E or Y321N) (13, 14). We thus used the D320E/Y321N NS5A mutant clone as the ALV-resistant replicon and tested its sensitivity to ALV and DAAs (Table 2). Although the D320E/Y321N NS5A mutant has reduced susceptibility to ALV—the EC_{50} is 17.2 nM for the wild type but 46.7 nM for the D320E/Y321N NS5A replicon (2.72-fold)—it remained fully susceptible to DAAs, including (i) boceprevir (EC_{50} of 239.5 and 262.3 nM for wild-type and D320E/Y321N NS5A replicons, respectively [1.10-fold]), (ii) telaprevir (EC_{50} of 358.0 and 361.5 nM for wild-type and D320E/Y321N NS5A replicons, respectively [1.01-fold]), (iii) daclatasvir (EC_{50} of 0.041 and 0.036 nM for wild-type and D320E/Y321N NS5A replicons, respectively [0.88-fold]), (iv) sofosbuvir (EC_{50} of 223.1 and 231.4 nM for wild-type and D320E/Y321N NS5A replicons, respectively [1.04-fold]), and (v) mericitabine (EC_{50} of 751.1 and 732.4 nM for wild-type and D320E/Y321N NS5A replicons, respectively [0.96-fold]).

We then examined whether ALV is effective against DAA-resistant variants. To test this possibility, we generated the following DAA-resistant Con1 replicons: (i) the protease inhibitor-resistant R155Q/A156T NS3 replicon, (ii) the daclatasvir-resistant L31V NS5A replicon, and (iii) the polymerase inhibitor-resistant S282T NS5B replicon (15). We then established stable cell lines for each DAA-resistant variant. We next measured the EC_{50} of each selected DAA against all created DAA-resistant replicons. R155Q/A156T substitutions within NS3 increased the EC_{50} for both boceprevir (from 239.5 nM for the wild type to 19,887.4 nM for the R155Q/A156T NS3 replicon [83.0-fold]) and telaprevir (from 358.0 for the wild type to 5,573.8 nM for the R155Q/A156T NS3 replicon [15.6-fold]). Similarly, the S282T substitution within NS5B increased the EC_{50} for sofosbuvir (from 223.1 for the wild type to 2,317.2 nM for the S282T NS5B replicon [10.4-fold]) as well as for mericitabine (from 751.1 for the wild type to 3,278.8 nM for the S282T NS5B replicon [4.3-fold]). The L31V substitution within NS5A increased the EC_{50} for daclatasvir (from 0.041 for the wild type to 0.851 nM for the L31V NS5A replicon [20.8-fold]). Within a specific class of DAAs, the chosen substitutions increased the EC_{50} over a similar range: 15- to 83-fold for protease inhibitors, 4- to 10-fold for polymerase inhibitors, and 21-fold for the NS5A inhibitor. We observed no cross-resistance between classes of DAAs. Boceprevir and telaprevir inhibited the replication of wild-type replicon at levels similar to those of polymerase inhibitor-resistant and NS5A inhibitor-resistant replicons. Daclatasvir suppressed the replication of the wild-type replicon at levels similar to those of protease inhibitor-resistant and polymerase inhibitor-resistant replicons. Sofosbuvir and mericitabine blocked the replication of the wild-type replicon at levels similar to those of protease inhibitor-resistant and NS5A inhibitor-resistant replicons. Most importantly, ALV inhibited the rep-

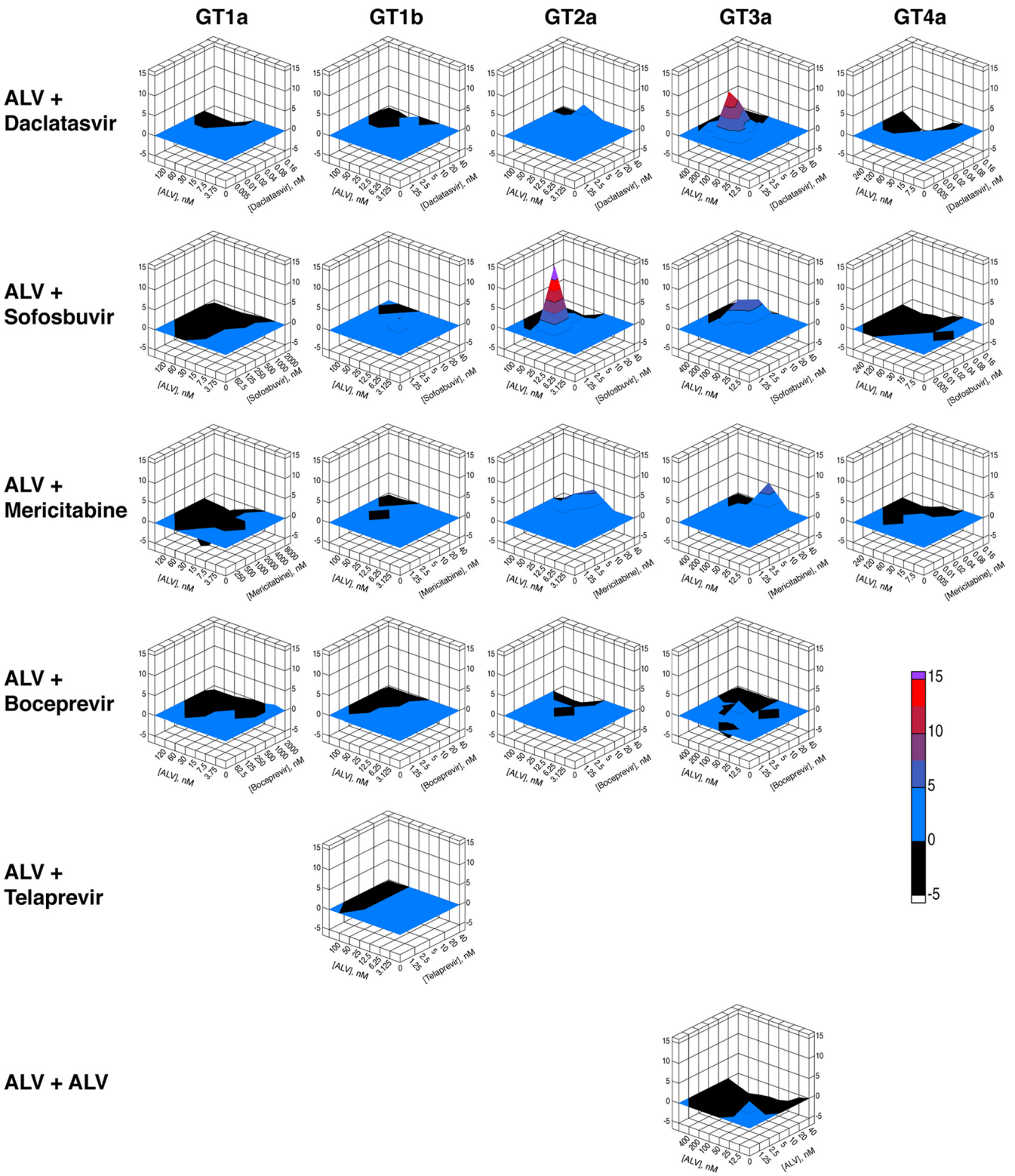

FIG 2 Synergy analyses for the combination of ALV with selected DAAs. The results of combinations were analyzed in a mathematical model, MacSynergy II. The three-dimensional response surface plot represents the differences between actual experimental effects and theoretical additive effects at various concentrations of the two compounds. The results are representative of 3 to 5 independent experiments.

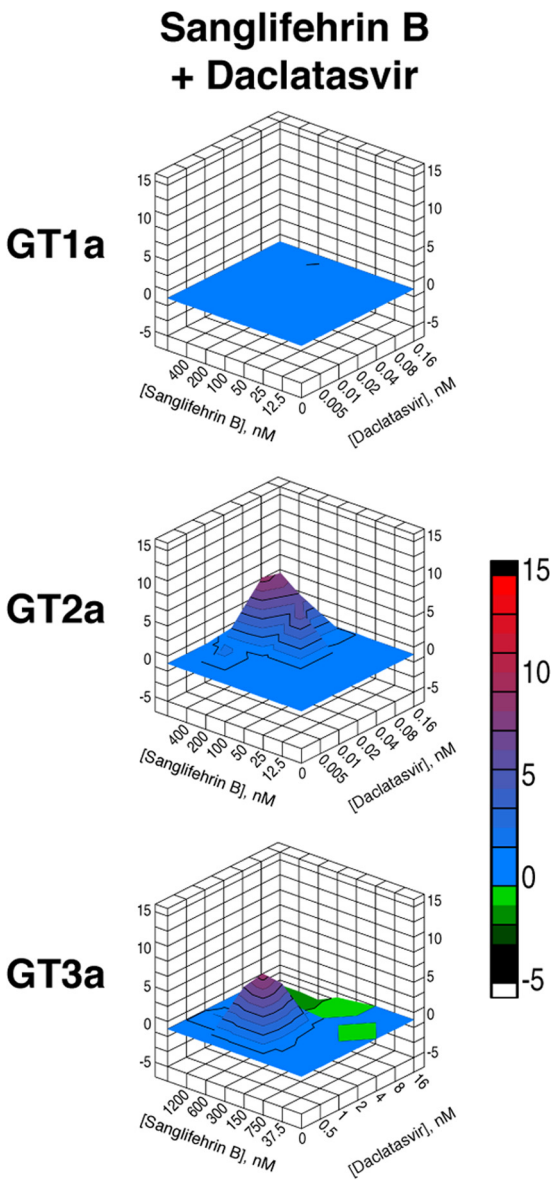

FIG 3 Synergy analyses for the combination of another cyclophilin inhibitor (sanglifehrin B) with selected DAAs. The data are as described for Fig. 2, except that MacSynergy II analyses were conducted on GT1a, -2a, and -3a. The results are representative of two independent experiments.

lication of all DAA-resistant replicons. We obtained EC_{50}s of 17.2, 16.3, 17.4, and 15.8 nM for wild-type, NS5A inhibitor-resistant L31V NS5A, protease inhibitor-resistant R155Q/A156T NS3, and polymerase inhibitor-resistant S282T NS5B replicons, respectively. Altogether, these data demonstrate that ALV is active against DAA-resistant replicons.

Since the combination of ALV and an NS5A inhibitor gave the most promising results in terms of synergistic effect, we examined the effect of the ALV-daclatasvir combination on DAA-resistant Con1 replicons established above. This combination exerts an additive effect on wild-type, D320E/Y321N, and L31V NS5A replicons as well as R155Q/A156T NS3 and S282T NS5B replicons (Fig. 4). The ALV-sofosbuvir combination exerted a synergistic effect on D320E/Y321N NS5A and S282T NS5B replicons and an additive effect on the other DAA-resistant replicons. Similar additive and synergist effects were observed for the ALV-mericitabine combination. The ALV-telaprevir combination exerted a synergistic effect on D320E/Y321N NS5A and R155Q/A156T NS3 replicons and an additive effect on the other DAA-resistant replicons. In general, we observed a moderately to highly synergistic effect of the drug combination on the mutant replicon which is resistant to the drug used in combination with ALV.

DISCUSSION

The present study demonstrates that combinations of ALV with DAAs mediate additive effects on GT1 and -4, while a significant synergistic effect was observed for the combination of ALV with an NS5A inhibitor on GT2 and -3. ALV-resistant variants were susceptible to DAAs, and ALV was active against DAA-resistant variants, suggesting no cross-resistance between ALV and DAAs. The combination of ALV with selected DAAs mediated additive to synergistic effects on any drug-resistant variants. Thus, our data suggest that the combination of ALV with NS5A inhibitors represents a very promising multigenotypic regimen for HCV-infected patients, especially those infected with GT2 and -3.

ALV is the most advanced cyclophilin inhibitor in clinical development. The experience so far, involving more than 2,000 patients exposed to the compound, shows good tolerability and a safety profile which is markedly better in IFN-free than IFN-containing treatment regimens (16). The most common clinical and laboratory adverse events associated with ALV in combination with pegylated IFN-α/RBV were similar to those associated with pegylated IFN-α/RBV alone; however, hyperbilirubinemia and hypertension were more frequent with ALV treatment. Reports of serious adverse events of pancreatitis led to a partial clinical hold of ALV trials; however, extensive preclinical and clinical analyses demonstrated that ALV does not cause pancreatic damage, and its

TABLE 2 EC_{50} of ALV and selected DAAs for ALV-resistant and DAA-resistant HCV genotype 1b variants

Antiviral	EC_{50} (nM)[a]				
	GT1b WT	D320E/Y321N NS5A	L31V NS5A	R155Q/A156T NS3	S282T NS5B
ALV	17.2 ± 0.4	46.7 ± 6.3	16.3 ± 3.3	17.4 ± 2.1	15.8 ± 3.9
Boceprevir	239.5 ± 14.7	262.3 ± 17.6	255.6 ± 8.6	19,887.4 ± 599.6	258.3 ± 16.3
Telaprevir	358.0 ± 19.4	361.5 ± 23.6	352.1 ± 41.0	5573.8 ± 278.9	359.6 ± 27.5
Daclatasvir	0.041 ± 0.008	0.036 ± 0.004	0.851 ± 0.063	0.044 ± 0.003	0.039 ± 0.005
Sofosbuvir	223.1 ± 15.8	231.4 ± 17.8	229.7 ± 31.2	236.2 ± 19.8	2,317.2 ± 173.6
Mericitabine	751.1 ± 51.2	732.4 ± 63.4	754.6 ± 22.8	758.3 ± 42.6	3,278.8 ± 287.9

[a] Data are means ± the standard error of the mean from 5 replicates.

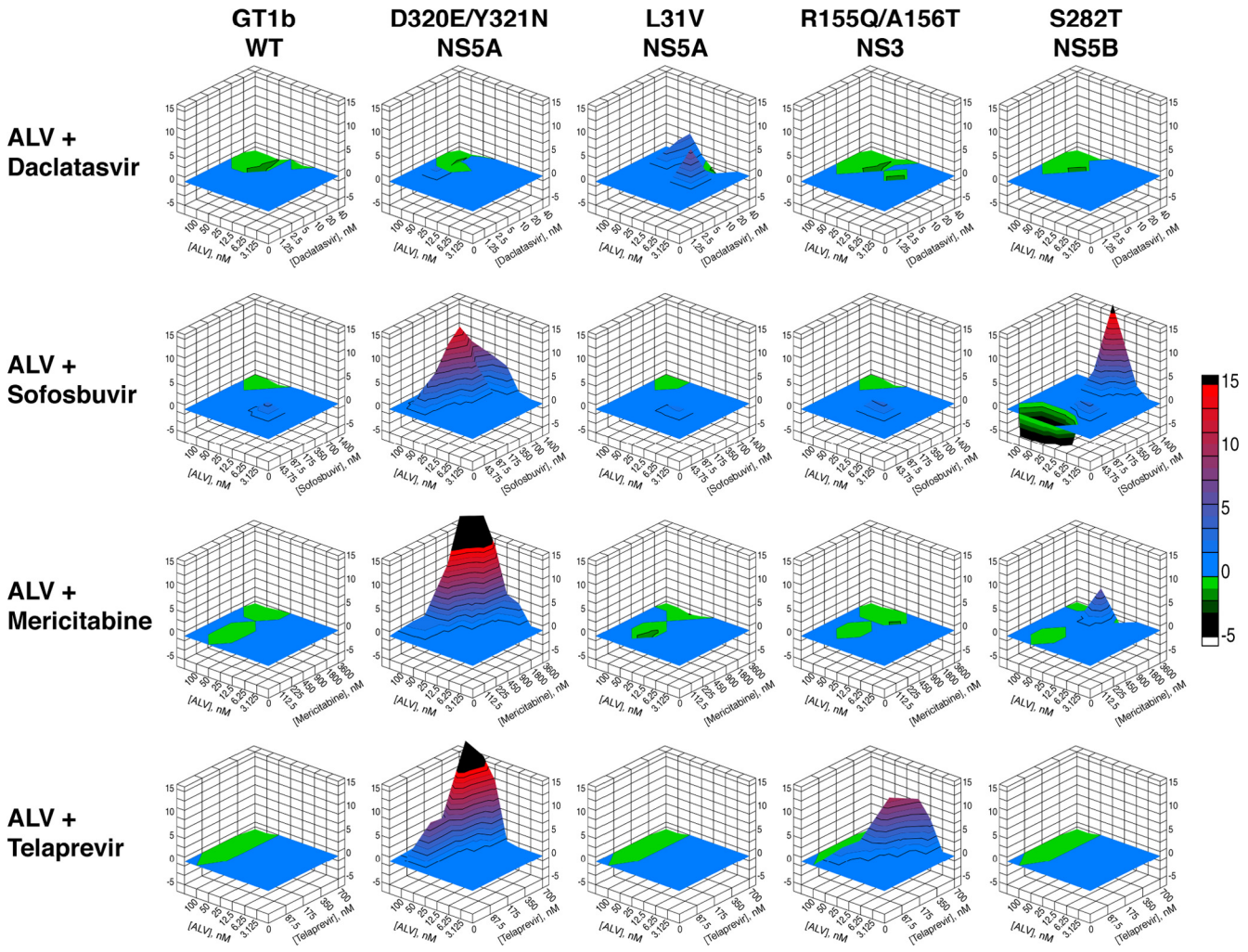

FIG 4 Synergy analyses for the combination of ALV with selected DAAs on ALV- and DAA-resistant GT1b variants. The data are as described for Fig. 2, except that MacSynergy II analyses were conducted on GT1b variants which are partially resistant to ALV (D320E/Y321N NS5A), NS5A inhibitors (L31V NS5A), protease inhibitors (R155Q/A156T NS3), and polymerase inhibitors (S282T NS5B). The results are representative of two independent experiments.

clinical development has resumed. No pancreatitis was seen in patients treated with ALV in interferon-free regimens.

Like antiretroviral drugs, HCV drugs have been shown to select drug-resistant viral variants, the outgrowth of which is responsible for virological breakthrough and disease progression. HCV drugs can be split into two groups, i.e., drugs with a low and high barrier to resistance. Drugs with a low barrier to resistance include the direct-acting antivirals first-initial NS3 and nonnucleoside NS5B and NS5A inhibitors. HCV drugs with a high barrier to resistance include nucleoside/nucleotide analogues and cyclophilin inhibitors. Because their target is a host protein, cyclophilin inhibitors such as ALV have broad genotypic coverage and a favorable resistance profile. Amino acid substitutions in NS5A can be selected by ALV, but individual mutations confer only a low-level (<5-fold) reduction in susceptibility to ALV *in vitro*, while combinations of two or more mutations are required to further reduce ALV susceptibility, usually with compromised replicative fitness (17). Due to its high barrier to resistance, very low viral breakthrough rates were observed, even in IFN-free regimens. Since ALV possesses a unique mechanism of antiviral action, that is, the prevention of cyclophilin A-NS5A interactions (6, 18), it represents an ideal component for an IFN-free drug combination. In order to identify the best DAA partner(s) for ALV, we investigated whether particular DAAs exert additive, synergistic, or antagonistic effects when combined with ALV. We first calculated the EC_{50} for ALV and for selected DAAs on GT1 to -4. ALV is effective against all genotypes tested—GT1a, -1b, -2a, -3a, and -4a (EC_{50} from 9.9 to 29 nM)—demonstrating the multigenotypic activity of ALV. Two protease inhibitors as well as two polymerase inhibitors were less active than ALV. The NS5A inhibitor daclatasvir was the most effective agent against all genotypes. The combination of ALV with daclatasvir exerts a potent inhibitory effect on GT2a, indicating that the synergistic effect of ALV with NS5A inhibitors on GT2a is independent of the degree of antiviral efficacy of the NS5A inhibitor. It is important to emphasize that the NS5A inhibitor together with ALV was the most effective *in vitro* antiviral combination evaluated in this study.

Our drug combination studies revealed that the combination of ALV with protease inhibitors, polymerase inhibitors, and NS5A inhibitors exhibit additive effects on GT1a, -1b, and -4a. In con-

trast, the combination of ALV with the NS5A inhibitor daclatasvir exerted constant synergistic effects on GT2a and -3a. This synergistic effect on GT2 and -3a was genotype specific, since the combination of ALV and the NS5A inhibitor exerted only an additive effect on GT1 and -4. The synergistic effect of the combination of ALV and NS5A inhibitor on GT3a was superior to that on GT2a. This synergistic effect on GT3a seemed specific, since the combination of the NS5A inhibitors with drugs other than ALV, such as sofosbuvir, exerted only additive effects on GT3a.

The combination of ALV and an NS5A inhibitor represents a very attractive IFN-free regimen for several reasons. First, no cross-resistance exists between ALV and selected DAAs, especially NS5A inhibitors. Specifically, ALV suppresses replication of daclatasvir-resistant replicons, and daclatasvir suppresses the replication of the ALV-resistant replicon. The lack of cross-resistance of a combination of ALV with an NS5A inhibitor is important, given that daclatasvir monotherapy results in rapid viral breakthrough (19). In theory, a combination of ALV with an NS5A inhibitor should reduce the rate of emergence of NS5A inhibitor-mediated viral breakthroughs. While the D320E substitution in NS5A arose *in vitro* under ALV selection and was detected in a few ALV-treated patients, this change alone only slightly affects susceptibility to ALV (<5-fold change in EC_{50}) and does not appear to be sufficient to cause resistance or viral breakthrough (18, 20). Another reason why the combination of ALV and an NS5A inhibitor is attractive is that ALV is multigenotypic and provides a high barrier for viral resistance development (13, 18). By binding to the isomerase pocket of cyclophilin A, ALV prevents the contact between the host protein and NS5A. A recent study provided the first direct genetic evidence that cyclophilin A is a bona fide HCV replication factor *in vivo* in genetically humanized mice (20). Another reason why a combination of ALV with an NS5A inhibitor represents an appealing IFN-free regimen is because they act on two distinct domains of NS5A. ALV blocks the contact between cyclophilin A and the domain II of NS5A (17), whereas NS5A inhibitors target the domain I of NS5A (21). HCV may face difficulties developing resistance to these two classes of inhibitors when used in combination. Indeed, it is likely that the virus will have to develop substitutions in both domains I and II of NS5A to escape the drug pressure of the combination of ALV and NS5A inhibitors. Our observation that the combination of ALV and daclatasvir exerts a synergistic effect on GT2a and -3a may be due to the fact that these two classes of inhibitors act on two separate domains of NS5A. One can envision that ALV and NS5A inhibitors act in concert to diminish the affinity of NS5A to the viral RNA (see Fig. S1 in the supplemental material). Cyclophilin A was shown to enhance NS5A binding to RNA, and the cyclophilin inhibitor cyclosporine was shown to block this enhancement (22). The domain I of NS5A contains the viral RNA binding site (23, 24). Daclatasvir, by interacting with domain I, may influence NS5A binding to the viral RNA. In this scenario, the combination of ALV and an NS5A inhibitor may exert either an additive (i.e., GT1 and -4) or synergistic (GT2 and -3) inhibitory effect on NS5A binding to viral RNA, hampering viral RNA replication or the composition and activity of HCV replication complexes.

In conclusion, to our knowledge, this is the first study with a comprehensive analysis, using a full range of HCV strains (GT1a, -1b, -2a, -3a, and -4a), of the combinations of a host-targeting antiviral, such as ALV, with a panel of DAAs. Our *in vitro* data demonstrate that the combination of ALV with an NS5A inhibitor represents a scientifically supported therapeutic combination based on their mechanisms of action. NS5A inhibitors, which have a low barrier to resistance and rapid viral load decline in patients, represent ideal drug partners for ALV, which is multigenotypic, has a high barrier to HCV resistance and targets a host protein. The combination of ALV with DAAs, such as NS5A inhibitors, thus presents an attractive opportunity for future HCV therapy as an oral IFN-free regimen.

ACKNOWLEDGMENTS

We thank F. Chisari for the Huh-7.5.1 cells, C. Rice and J. Bukh for the GT3a and GT4a Huh7.5 cell lines, T. Pietschmann, T. Wakita, and R. Bartenschlager for the Luc-JFH-1 plasmid and the Huh Luc-Neo Con1 cell line, and W. Delaney for the GT1a cell line.

This work was supported by U.S. Public Health Service grant AI087746 from the National Institute of Allergy and Infectious Diseases (NIAID).

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