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Evaluation of antiviral drug synergy in an infectious HCV system

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Abstract

Background—Direct-acting antivirals (DAAs) have greatly improved the treatment of HCV infection. To improve response and prevent resistance, combination regimens have been the focus of clinical development. Regimens are often first assessed *in vitro,* with most combination studies to date using subgenomic replicon systems, which do not replicate the complete HCV life cycle and preclude study of entry and assembly inhibitors. Infectious full-length HCV systems have been developed and are being used to test drug efficacy.

Methods—Using cell-based HCV Con1b replicon and an infectious full-length HCV (HCVcc-Luc) infection system, we systematically tested the synergy, additivity or antagonism of combinations of protease, NS5A and nucleotide NS5B inhibitor classes as well as the combination of these DAAs with host-targeting agent cyclosporin A or non-antibody entry inhibitor (*S*)-chlorcyclizine. Two computational software packages, MacSynergyII and CalcuSyn, were used for data analysis.

Results—Combinations between different classes showed good consistency across the two viral assay systems and two software platforms. Combinations between NS5A and nucleotide NS5B inhibitors were synergistic, while combinations of protease inhibitors with the other two classes were additive to slightly antagonistic. As expected, combinations of antivirals of the same class were additive. Combination studies between these DAA classes and cyclosporin A or (*S*)-chlorcyclizine demonstrated additive to synergistic effects and highly synergistic effects, respectively. Combinations of these drugs did not show any added or unexpected cytotoxicity.

Conclusions—Our results show that *in vitro* combination studies of anti-HCV DAAs in the HCVcc system may provide useful guidance for drug combination designs in clinical studies. We also demonstrate that these DAAs in combination with host-targeting agents or entry inhibitors may improve HCV treatment response.

Introduction

HCV is a positive-stranded RNA virus infecting over 200 million people worldwide. Chronic HCV infection is a leading cause of hepatocellular carcinoma and a leading indication for liver transplantation in developed countries. Effective vaccination for HCV is

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Disclosure statement

TJL, SH and ZH, are named on patent application related to this work: US Provisional Patent Application 62/011,462, ‘Heterocyclic compounds and methods of use thereof’. BL. and HJY declare no competing interests.

not yet available. For many years, combination antiviral therapies utilizing pegylated interferon-α (PEG-IFN) and ribavirin (RBV) were the first line of care [1–3]. Recently, the US Food and Drug Administration (FDA) has approved direct-acting antivirals (DAAs), including the protease, NS5A and polymerase inhibitors tel-aprevir, boceprevir, simeprevir, paritaprevir, ledipasvir, daclatasvir, ombitasvir, sofosbuvir and dasabuvir for use in combination or with PEG-IFN and RBV. Many regimens demonstrated improved viral clearance in patients infected with HCV genotype-1 [4–6]. In light of HCV's high rate of developing mutations that confer drug resistance, combination therapies of DAAs have been crucial for the development of effective regimens for chronic HCV infection [7].

To facilitate selective development of such regimens, it is therefore important to investigate the potential synergistic or antagonistic effects of such combinations first with a robust *in vitro* HCV model system. Most *in vitro* combination studies published to date use the subgenomic replicon system to evaluate combinations of certain novel agents with well-developed antivirals that have completed advanced clinical trials or obtained FDA approval [8–10]. Recently, more combination studies have utilized an infectious HCV system [11–14]. However, replicon systems cannot reproduce the entire infectious HCV life cycle. In addition, the replicon-containing cells are highly selected and may not reflect the native cellular environment for HCV replication *in vivo.* A cell-based infection assay using infectious HCV with full-length HCV genomes is more biologically relevant to *in vivo* infection. It also expands the testing of agents to target entry and assembly phases of the HCV life cycle. To date, no systematic combination tests between the major HCV antiviral classes of HCV protease, NS5A and NS5B polymerase inhibitors in a true infection system have been conducted. A side-by-side comparison of combinations between replicon and infection systems in drug combination tests is also lacking in the current literature.

Here we present a study of systematic combination of HCV antivirals utilizing a previously described full-length HCV infection system. The system relies on a *Renilla* luciferase reporter inserted into a full-length HCV genome (HCVcc-Luc) for detection of viral replication [15,16]. We tested this method in comparison with the HCV replicon assay. Various DAAs were tested in the models, such as the protease inhibitor, NS5A inhibitor and nucleotide NS5B inhibitor classes. Antivirals with the same mechanism of action were also evaluated in combination as controls. We subsequently applied the system to test a series of combinations of these DAAs with cyclosporin A, a host-targeting agent (HTA) and chlorcyclizine, a recently described entry inhibitor [17,18]. For data analysis, we utilized two different software packages which base their calculation on alternative definitions of drug interaction and non-interaction: MacSynergyII (Bliss independence model) and CalcuSyn (Loewe additivity model). Both concepts of drug interaction have been widely applied and well established in the evaluation of drug combinations [11,19,20].

Methods

Antiviral agents, viruses and cell lines

Antiviral agents included two active site protease inhibitors telaprevir (Selleckchem, Houston, TX, USA) and boceprevir (ChemScene, Monmouth Junction, NJ, USA), NS5A inhibitor daclatasvir (Selleckchem), two nucleotide NS5B polymerase inhibitors, sofosbuvir

(Advanced Chemblocks, Burlingame, CA, USA) and 2′-*C*-methylcytidine (US Biological, Salem, MA, USA), host-targeting cyclophillin inhibitor cyclosporin A (Sigma-Aldrich, St Louis, MO, USA) and entry-inhibitor (*S*)-chlorcyclizine (NCATS, Bethesda, MD, USA).

The HCVcc-Luc infectious virus consisted of a full-length J6/JFH-1 HCV with insertion of a luciferase reporter gene at the 3′ end of the p7 gene [16].

Huh7.5.1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Serum Source International, Charlotte, NC, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2, at 37°C. A stably expressing Con1b replicon cell line with luciferase reporter under the direction of 5′-NTR for quantification described previously [21,22] was grown in the same condition as Huh7.5.1 cells with the addition of 500 μg/ml G-418.

Efficacy assays

Cells were seeded in 96-well plates (10^4 cells/well) and cultured overnight in the above-mentioned culture medium in absence of G-418. In the infectious efficacy assay, Huh7.5.1 cells underwent simultaneous infection at a multiplicity of infection of 0.05 and treatment with a matrix of concentrations of two compounds. No viral inoculum was used in the Con1b replicon efficacy assay.

Eight or nine concentrations of each agent were used, for a total matrix of 72 concentration combinations. Concentration ranges were chosen to include at least one point of approximately 0% inhibition and at least one point of approximately 100% inhibition for each drug alone. Concentrations were in ½ log10 increments (for example, 1 μM, 0.316 μM, 0.1 μM and so on), unless the individual dose–response curves were too steep (>70% change in inhibition between adjacent ½ log10 increments). In such cases, the assay was repeated with ¼ log10 increments to more accurately define dose–response relationships. After 48 h, a *Renilla* luciferase assay kit (Promega, Madison, WI, USA) was used to measure HCV inhibition, normalized to DMSO-control treatment. All experiments were conducted in triplicate.

Cytotoxicity assay

Cytotoxicity was assessed in parallel assays under the same conditions as in the HCVcc-Luc or Con1b replicon efficacy assay using a luminescence-based ATP sensor (ATPlitc 1-Step Kit; PerkinElmer, Waltham, MA, USA).

Data analysis

Experimental data were analysed according to two drug combination models, Bliss independence and Loewe additivity.

Bliss independence and MacSynergyII

Data were analysed using the software MacSynergyII [23]. From experimental individual drug effects, the program calculates a theoretically expected 'additive' combined-drug effect

if the drugs act independently, for each combination of agents at specific concentrations. From (1−A)(1−B)=(1−C), the theoretical additive combined-drug effect, C, is determined when A and B are the experimental individual drug effects. The program then compares this theoretically expected additive effect to the experimental combined-drug effect. If the experimental antiviral activity significantly deviates (95% confidence level) from the theoretical additive effect at any concentration combination, MacSyncrgyII quantitates that synergy or antagonism. This can be described by a surface plot of peaks and valleys of synergy and antagonism values, with the log volumes (LV) of these peaks and valleys informing a quantitative measure of synergy or antagonism over the sum of the concentrations tested. LV ≥2, −2< LV <2 and LV ≤−2 indicate synergy, additive effect and antagonism, respectively.

Loewe additivity and CalcuSyn

Data were also analysed using the software CalcuSyn (BioSoft, Ferguson, MO, USA), which draws on the methodology of Chou and Talaly [24]. The program takes the drug concentrations required to produce a given effect in an experimental combination and compares them to the drug concentrations that would be needed individually to achieve that same effect. Inputting concentrations and effect values from individual antiviral agent treatments, we identified the concentration at 50% efficacy (EC_{50}) for each agent. We then selected the concentration that was closest to the approximated EC_{50} of each drug (for example, for EC_{50}s of 0.29 μM and 0.9 μM, we select 0.316 μM and 1 μM, respectively) and took the ratio of those concentrations. We subsequently inputted the concentrations and effect values for all experimental combinations that had the same ratio of drug concentrations. Using this, CalcuSyn interpolates the drug concentrations needed in combination at that ratio to produce effects of 50%, 75% and 90% inhibition. It compares these combined drug concentrations with the concentrations from the two drugs' individual dose–effect curves needed to achieve 50%, 75% and 90% inhibition. The extent of synergy or antagonism is reported in a combination index (CI) value, calculated by $CI = a_c/a_i + b_c/b_i$, where a_c and b_c are experimental drug concentrations needed to achieve a stated effect, and a_i and b_i are the individual drug concentrations needed to achieve that effect according to individual drug dose curves. CI >1.1, 1.1≥ CI ≥0.9 and CI <0.9 indicate synergy, additive effect and antagonism, respectively.

The two software packages CalcuSyn and MacSynergyII diagnose synergy and antagonism based on two different definitions of non-interaction on which the alternative concepts of interaction are based. It is possible that the results generated from the two analysis methods sometimes may differ depending on the mechanisms of drug action. One should take this into consideration in interpreting the results.

Results

Comparison of HCVcc-Luc infection assay and replicon assay in evaluating combinations of DAAs

We systematically tested the effects on HCV inhibition of combinations of antivirals of different DAA classes, including protease inhibitors telaprevir and boceprevir, NS5A

inhibitor daclatasvir, and nucleotide NS5B polymerase inhibitor sofosbuvir in both HCVcc-Luc infection and Con1b replicon systems. Results in CalcuSyn will be discussed first, followed by results in MacSyncrgyII, in order to facilitate the comparison of the two viral assay platforms. Table 1 shows EC_{50} and concentration at 50% cytotoxicity (CC_{50}) values of the antivirals from individual dose–response curves in both systems.

Combinations of daclatasvir with telaprevir in HCVcc-Luc and Con1b replicon yielded CIs in CalcuSyn representing slight-to-moderate antagonism (1.23–1.47) and moderate antagonism (1.48–1.50), respectively, at EC_{50}, EC_{75}, and EC_{90} (Figure 1A and Table 2). The combinations resulted in the same level of synergy or antagonism (additive, slight, moderate, major) or differing only by 1 level. The combination of daclatasvir and boceprevir demonstrated additivity (1.02–1.08) in HCVcc-Luc and slight antagonism (1.26–1.29) in Con1b replicon, when analysed in CalcuSyn (Figure 1B and Table 2). For combinations of sofosbuvir and telaprevir or boceprevir in the HCVcc-Luc and Con1b replicon systems, CalcuSyn interaction assessments generated results varying widely from 0 to 2 levels. CI values for sofosbuvir with telaprevir were additive (1.00) and additive to moderately antagonistic (1.07–1.49) for the HCVcc-Luc and replicon systems, respectively (Figure 1A, 1B and Table 2). Sofosbuvir and boceprevir were slightly synergistic to slightly antagonistic (0.85–1.26) and slightly antagonistic (1.12–1.17) in HCVcc-Luc and Con1b systems, respectively (Figure 1A, 1B and Table 2). The combination of daclatasvir and sofosbuvir was moderate to highly synergistic in the HCVcc-Luc synergy assay with CI values 0.44–0.75 and highly synergistic in replicons with CI 0.45–0.54 (Figure 1A, 1B and Table 2).

In MacSynergyII, combinations between daclatasvir and telaprevir or boceprevir yielded additive-level log volumes for both the HCVcc-Luc and replicon systems. Sofosbuvir with telaprevir or boceprevir straddled additivity, resulting in additivity, minor synergism or minor antagonism across the two HCV systems in MacSynergyII. Daclatasvir and sofosbuvir combinations were slightly synergistic in HCVcc-Luc and highly synergistic in replicon when analysed with MacSynergyII. These results show general similarity with small differences in determination of the level of synergism or antagonism between analyses of data using either CalcuSyn or MacSynergyII (Table 2).

To test whether combinations of these drugs have any cytotoxicity at the concentrations used, parallel ATPlite assays were performed. No toxicity was observed except at the highest concentration of a single drug in a single test, which was excluded from analysis in CalcuSyn and MacSynergyII.

Combination studies of DAAs of the same class in HCVcc-Luc assay

Additivity represents a situation in which the combination of two drugs results in effects no greater or less than the addition of their individual effects. Although the definition of additivity and the expected result varies depending on which concept of drug non-interaction is used, additivity would be expected in combinations of drugs of the same mechanism of action or in combinations of a drug with itself [25]. To validate this hypothesis in the HCVcc-Luc system, we tested such combinations. As expected, combinations between daclatasvir with itself, protease inhibitors telaprevir and boceprevir, and nucleotide polymerase inhibitors sofosbuvir and 2′-*C*-methylcytidine yielded additive or nearly

additive (minor synergy or minor antagonism) CIs of 0.95–1.30, 1.17–1.18 and 0.83–0.95, respectively (Figure 1C and Table 2). MacSynergyII yielded log volumes indicating additivity for the latter two combinations (Table 2). As MacSynergyII calculates additivity assuming the two drugs bind to dissimilar sites, it becomes nonsensical to test a drug combination with itself [23,26,27], For completeness, the result of minor synergism for the combination of daclatasvir with itself is still reported in Table 2. No toxicity was observed in the parallel ATPlite cytotoxicity assay at the concentrations tested.

Combination studies of DAAs and an HTA in HCVcc-Luc assay

Combinations involving host-targeting agents (HTAs) provide another possibility aside from DAAs for IFN-free regimens, especially as host-targets provide a higher barrier to resistance and pan-genotypic activity [3]. Cyclosporin A, an immunosuppressive agent and cyclophillin inhibitor, has been demonstrated to have antiviral activity against HCV replication [28–30]. Therefore, cyclosporin A was tested as an HTA in this study. In HCVcc-Luc, *in vitro* combinations of cyclosporin A with daclatasvir or sofosbuvir were slightly to moderately synergistic, with CI values 0.49–0.93 and 0.63–0.74, respectively, and MacSynergyII synergy log volumes were 2.27 and 7.22, respectively (Figure 1D and Table 3). Combinations between cyclosporin A and telaprevir or boceprevir were additive in CalcuSyn, with CIS of 0.97–0.99 and 1.03–1.06, respectively. This near additivity resulted again in MacSynergyII with minor synergism for the telaprevir combination with log volume 2.17 and additivity for the boceprevir combination with log volume 1.83 (Table 3). No toxicity was observed at the concentrations tested.

Combination studies of DAAs and an entry inhibitor in HCVcc-Luc assay

A major advantage of HCVcc-Luc infection over the replicon system is its ability to assess the combinatorial effect of HCV inhibitors targeting all stages of the HCV replication cycle. We and others previously reported the entry-related antiviral activity of FDA-approved antihistamine chlorcyclizine (CCZ) [17,18]. In the current study, we performed an extensive analysis of the combination of DAAs and CCZ in accordance with the commonly used fixed ratio and fitting method of CalcuSyn to calculate the projected CI values at 50%, 75% and 90% effective concentrations. Combinations between (*S*)-CCZ and daclatasvir, telaprevir, boceprevir, sofosbuvir, 2′-C-methylcytidine or cyclosporin A were highly synergistic with CI values ranging from 0.16 to 0.67 (Figure 2 and Table 3). In Table 3, we also report the moderate to major synergy in MacSynergyll. No toxicity was observed at the concentrations tested.

Discussion

Because of the importance of combination drug regimens against HCV to prevent resistance and the high cost to clinically evaluate all combinations, there is a need for robust *in vitro* systems for analysis of antiviral combinations. The commonly used method of subgenomic replicons does not represent the complete HCV life cycle and precludes investigation of entry and assembly inhibitors [21,22]. A full-length infectious system would capture all stages of the HCV life cycle, and therefore could be used to test HCV inhibitor combinations involving every aspect of HCV life cycle. A systematic combination of three

classes of DAAs in a full-length HCV cell culture system compared to a replicon system has not been conducted to date. A comprehensive study of these drug combinations *in vitro* using the HCVcc infection system may provide useful information for clinical drug combination designs and future anti-HCV drug development.

In the current study, we analysed the results of drug combination studies using the HCVcc-Luc system in five aspects. First was a systematic study of various combinations of three different classes of DAAs (protease inhibitors, NS5A inhibitors and nucleotide NS5B inhibitors). Second was comparison of combinations of these three different classes of DAAs in our infectious system to a subgenomic replicon system. Third was to test if combinations of compounds of the same class or a compound with itself were additive. Fourth was to analyse synergy, additivity or antagonism using two different software packages, CalcuSyn and MacSynergyII that are based on two different concepts of drug non-interaction. Last, we verified no added or unexpected cytotoxicity in the combinations used in parallel ATPlite assays.

In our study of telaprevir, boceprevir, daclatasvir and sofosbuvir, only the combination of NS5A inhibitor (daclatasvir) and nucleotide NS5B inhibitor (sofosbuvir) was synergistic. Combinations between protease inhibitors and NS5A inhibitors or nucleotide NS5B inhibitors exhibited additivity in general and even some antagonistic effects despite the differences in the drug target. Consistency over two protease inhibitors verified that the effect is indeed real for this class of DAA. Previous combination studies of protease inhibitors with NS5A inhibitors or nucleotide NS5B inhibitors using the Chou-Talaly method [24] in subgenomic replicons have shown mixed results from moderately antagonistic to moderately synergistic CI values [10,31–33].

The differences between studies may be methodological. We had observed especially steep changes in the dose-response curves for telaprevir and boceprevir in experiments, such as a >70% difference between the two concentrations of 1 μM and 0.316 μM in telaprevir. Due to these steep changes in inhibition, we suspected that more intermediate concentrations are needed to generate adequate data for the independent dose-response curves. from which Chou and Talaly's method in CalcuSyn draws upon for calculation of CI values. To prevent any errors that might result from poor curve fitting we repeated the combinations in ¼ log10 concentration increments to yield greater resolution of independent drug curves. Indeed, in the case of the theoretically additive combination of two protease inhibitors, telaprevir and boceprevir, ½ log10 dilutions resulted in synergistic CI values from 0.53–0.82 and synergy log volume 7.14, whereas ¼ log10 dilutions yielded near-additivity as expected, with CIs of 1.17–1.18 and synergy log volume 1.77. This highlights the variability of results in *in vitro* combination tests based on drug dose-responsiveness and selected dilution factors. Future studies should make note to ensure that one should select dilution factors capable of yielding good resolution of the drug's dose–response curve. Based on our results, we therefore believe that protease inhibitors act mostly in additive effect with the other two DAA classes.

The reason for a lack of synergy between protease inhibitors and NS5A or NS5B inhibitors is not apparent. It is known that inhibition of NS3/4a protease activity prevents cleavage of the HCV polyprotein, resulting in a reduction of NS5A and NS5B protein levels. Thus the

action of protease inhibitors may not be totally independent of NS5A and NS5B inhibitors. For example, the reduction in NS5A and NS5B protein levels from inhibition of NS3/4a and polyprotein processing may result in reduced effectiveness of NS5A and nucleotide NS5B inhibitors due to a reduction in the concentration of their targets. Recently it has also been shown that telaprevir may have additional inhibitory effects on HCV RNA synthesis independent of its effect on reducing HCV non-structural protein levels [34]. These possibilities would therefore suggest against a synergistic effect and in favour of additive or antagonistic effects.

In general, our results showed systematic near-agreement in synergistic, additive or antagonistic effects between the replicon and infectious virus system with combinations of protease inhibitors, NS5A inhibitors and NS5B inhibitors. Combinations yielded deviation of no more than one level of synergism or antagonism (additive, minor, moderate, major) between the infectious and replicon systems in CalcuSyn, and only a few deviated by two levels in MacSynergyII. It is likely that one level of difference in synergy or antagonism is insignificant for these software tools, as theoretically additive combinations of DAAs of the same class were sometimes one level off the additive effect (minor synergism and minor antagonism). Making note of this variation, results in *in vitro* combination studies should be regarded with caution, unless results indicate strong synergy, such as in the case of combinations of replication inhibitors with entry inhibitor (*S*)-CCZ in this study.

For variations greater than one level of synergy, the differences could be attributed to the fact that the HCVcc-Luc system represents more steps of the HCV life cycle than the replicon. However, it is also important to consider that the HCVcc-Luc system used in this study is based on a genotype-2a clone, whereas the replicon system used is based on a genotype-lb clone. It is well known that drugs may have different efficacy depending on genotype both *in vitro* and *in vivo* [4,31]. The differences in results between the two systems could be in part due to differences in genotype. Nonetheless, the functional relevance of testing drugs of different or same targets in combination should still remain valid regardless of the genotype of the virus. Therefore, it is still of interest from the perspective of dissecting drug mechanism of action to test combination in both a full-length infectious virus and a replicon virus.

We applied our system to test a previously described non-antibody entry inhibitor, chlorcyclizine [17,18] and demonstrated a highly synergistic effect of combinations with active site protease inhibitors, NS5A inhibitors, nucleotide NS5B inhibitors and cyclosporin A. Recently, Xiao *et al.* [35] showed synergistic effects in combinations between antibodies against HCV entry factors and different classes of DAAs with methods and software packages similar to those in our study, though their study focused on entry inhibitors in the HCVcc-Luc system. Collectively our studies support the potential promise of developing combination therapies utilizing HCV entry and replication inhibitors.

Our study compares the accuracy of an infectious HCV virus system for analysis of the combinatorial effect of HCV antivirals to a replicon HCV cell culture system. In studying the three DAA classes, we found only the combination of NS5A and NS5B inhibitors to be synergistic in HCVcc-Luc and Con lb replicons, while the combinations of protease

inhibitors with NS5A or nucleotide NS5B inhibitors were additive to antagonistic. It is interesting that our *in vitro* findings are correlated with recent *in vivo* data in clinical studies of drug combinations. Combination of NS5B polymerase inhibitor sofosbuvir and an NS5A inhibitor appears to be highly effective, whereas combinations of other classes tend to require 3–4 drugs (protease inhibitor, NS5A inhibitor, non-nucleoside analogue NS5B inhibitor and ribavirin) to achieve high clinical efficacy [5]. The combination of a replication inhibitor with an entry inhibitor, like (*S*)-CCZ, demonstrates strong synergy *in vitro*, supporting future development of such combination regimens. Finally our study supports that this HCVcc-Luc system designed for *in vitro* combination studies of new HCV antivirals, including drugs targeting other stages of the HCV life cycle, is promising in guiding the efficacy studies of drug combinations *in vivo.*

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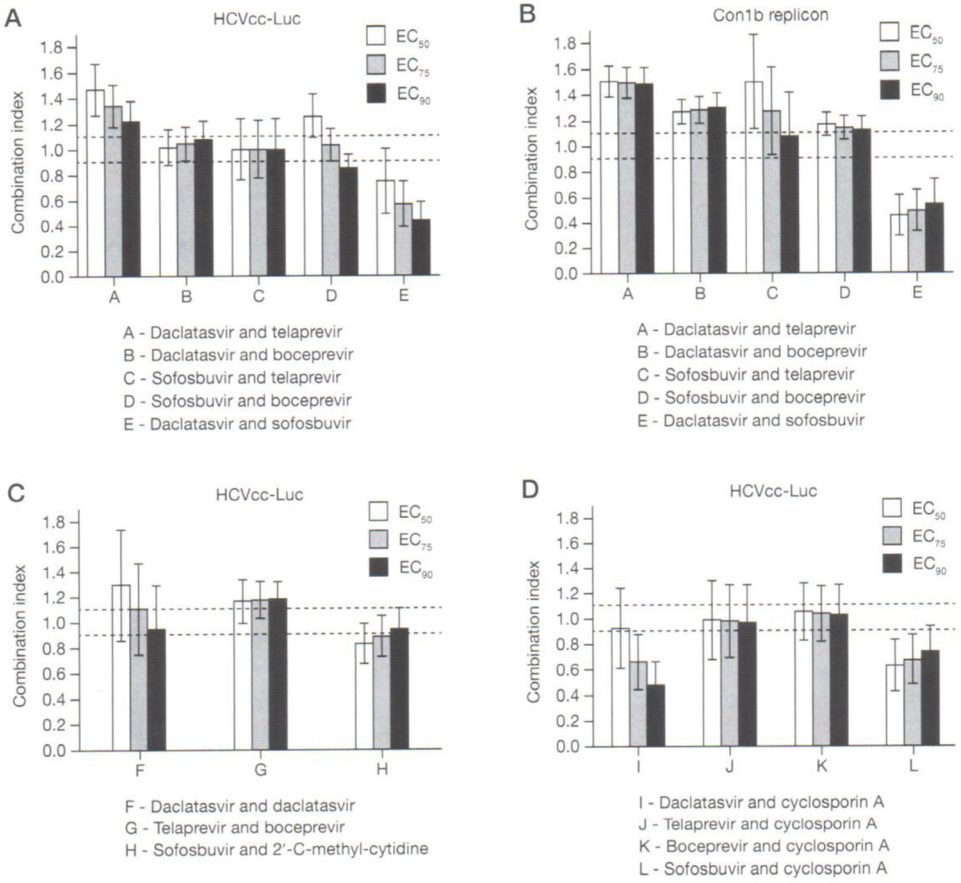

Figure 1. Combination index of combinations between different classes of DAAs or DAAs with cyclosporin A

Huh7.5.1 cells infected with HCVcc-Luc or Con1b replicon cell lines were treated with agents in serial dilutions to form a matrix of concentrations. Both were analyzed by *Renilla* luciferase activity. Combinations of different classes of direct acting antivirals (DAAs) were tested in Huh7.5.1 cells infected with (A) full-length HCVcc-Luc virus and (B) subgenomic Con1b replicons. Combinations between (C) DAAs of the same class or between (D) DAAs and host-targeting agent cyclosporin A were also tested in HCVcc-Luc-infected cells. Projected combination index (CI) SE of concentration at 50%, 75% and 90% efficacy (EC_{50}, EC_{75} and EC_{90}) from the means of experiments in triplicates are shown. Area between dotted lines represents additivity ($0.9 \leq CI \leq 1.1$). Exact CI values are shown in Tables 2 and 3.

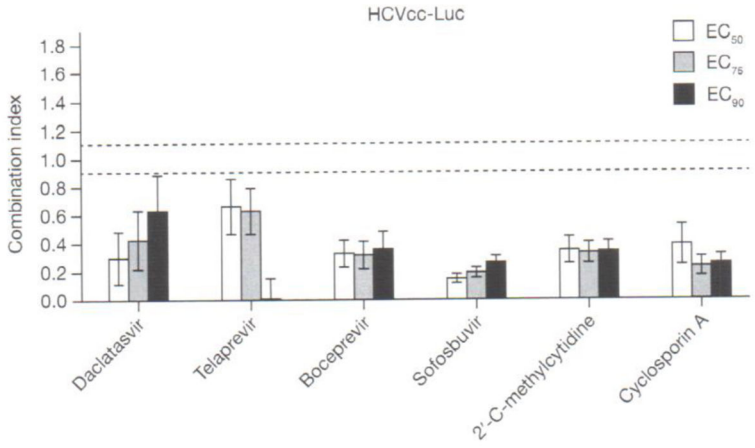

Figure 2. Combination index of combinations of entry inhibitor (*S*)-chlorcyclizine with DAAs or with cyclosporin A

Huh7.5.1 cells infected with HCVcc-Luc were simultaneously treated with (*S*)-chlorcyclizine in combination with inhibitors as indicated in the figure at effective concentrations of 50% (EC_{50}). 75% (EC_{75}) and 90% (EC_{90}), respectively. Projected combination index (CI) ± SE at EC_{50}, EC_{75} and EC_{90} from the means of experiments in triplicates are shown. Area between dotted lines represents additivity ($0.9 \leq CI \leq 1.1$). Exact CI values are shown in Table 3.

Table 1

In vitro activity of HCV antivirals

	EC_{50}, μM		CC_{50}, μM	
Antiviral name	**HCVcc-Luc (2a)**	**Con1b replicon**	**HCVcc-Luc (2a)**	**Con1b replicon**
Daclatasvir	0.00004 ±0.00002	0.00004 ±0.00002	45 ±1	22 ±1
Telaprevir	0.20 ±0.04	0.55 ±0.05	>100	97.8 ±0.9
Boceprevir	0.22 ±0.01	0.62 ±0.01	>100	>100
Sofosbuvir	0.060 ±0.002	0.4 ±0.1	>100	>100
2′-*C*-methylcytidine	1.4±0.4	1.61 ±0.07	>100	>100
Cyclosporin A	0.14 ±0.02	0.62 ±0.01	>100	>100
(*S*)-CCZ	0.024 ±0.009	N/A	33 ±2	N/A

Data shown represents means of results of ≥3 independent tests ±SEM. CCZ, chlorcyclizine; CC_{50}, concentration at 50% cytotoxicity; EC_{50}, concentration at 50% efficacy; N/A, not applicable.

Table 2

Comparison of combinations of DAAs of different classes in HCVcc-Luc and Con1b replicon assays and combination study of DAAs of same class in HCVcc-Luc assay

			MacSyncrgy II			CalcuSyn			
Virus type	**ID**	**Antiviral name**	**Synergism LV**	**Antagonism LV**	**Conclusion[a]**	**EC_{50} CI**	**EC_{75} CI**	**EC_{90} CI**	**Conclusion[b]**
HCVcc-Luc[c]	A	Daclatasvir and telaprevir	0.13	−1.95	() Additive	1.47 ±0.20	1.34 ±0.16	1.23 ±0.15	(−) Minor antagonism
HCVcc-Luc[c]	B	Daclatasvir and boceprevir	3.61	0	(+) Minor synergy	1.01 ±0.14	1.05 ±0.13	1.08 ±0.14	() Additive
HCVcc-Luc[c]	C	Sofosbuvir and telaprevir	4.1	−1.27	(+) Minor synergy	1.00 ±0.24	1.00 ±0.23	1.01 ±0.23	() Additive
HCVcc-Luc[c]	D	Sofosbuvir and boceprevir	0.42	0	() Additive	1.26 ±0.17	1.03 ±0.12	0.85 ±0.10	() Additive
HCVcc-Luc[c]	E	Daclatasvir and sofosbuvir	2.11	0	(+) Minor synergy	0.75 ±0.26	0.57 ±0.18	0.44 ±0.14	(+++) Major synergy
Con1b replicon[c]	A	Daclatasvir and telaprevir	0.4	−0.94	() Additive	1.50 ±0.12	1.49 ±0.12	1.48 ±0.13	(−) Minor antagonism
Con1b replicon[c]	B	Daclatasvir and boceprevir	1.39	−1	() Additive	1.26 ±0.09	1.28 ±0.10	1.29 ±0.12	(−) Minor antagonism
Con1b replicon[c]	C	Sofosbuvir and telaprevir	0.02	−4.83	(−) Minor antagonism	1.49 ±0.36	1.26 ±0.34	1.07 ±0.34	(−) Minor antagonism
Con1b replicon[c]	D	Sofosbuvir and boceprevir	0	−1.36	() Additive	1.17 ±0.09	1.14 ±0.09	1.12 ±0.11	(−) Minor antagonism
Con1b replicon[c]	E	Daclatasvir and sofosbuvir	19.08	−0.03	(+++) Major synergy	0.45 ±0.16	0.49 ±0.16	0.54 ±0.19	(++) Moderate synergy
HCVcc-Luc[d]	F	Daclatasvir and daclatasvir	3.06	−1.77	(+) Minor synergy	1.30 ±0.44	1.11 ±0.36	0.95 ±0.35	() Additive
HCVcc-Luc[d]	G	Telaprevir and boceprevir	1.77	0	() Additive	1.17 ±0.17	1.18 ±0.15	1.18 ±0.14	(−) Minor antagonism
HCVcc-Luc[d]	H	Sofosbuvir and 2′-*C*-methylcytidine	0.16	−0.47	() Additive	0.83 ±0.16	0.89±.0.15	0.95 ±0.16	(+) Minor synergy

[a]Extent of synergy or antagonism in MacSynergyII is defined according to absolute value of log volume (LV) as follows: () additive, LV <2; (+/−) minor, 2< LV <5; (++/−−) moderate, 5≤ LV <9; (+++/−−−) major, LV ≥9.

[b]Extent of synergy and antagonism in CalcuSyn is defined as follows: (+++) major synergy, combination index (CI) ≤0.7; (++) moderate synergy, 0.7≤ CI <0.8; (+) minor synergy, 0.8≤ CI <0.9; () additive, 0.9≤ CI <1.1; (−) minor antagonism, 1.1≤ CI <1.3; (−−) moderate antagonism, 1.3≤ CI <3.0; (−−−) major antagonism, CI ≥3.0.

[c]LV (95% confidence level) and CI (±SE)of combinations of different classes of direct-acting antivirals (DAAs) and with HCVcc-Luc and Con1b rcplicons.

[d]LV (95% confidence level) and CIs (±SE) of combinations of DAAs of the same class in HCVcc-Luc. Additivity is defined as a combinatorial effect equal to the sum of each drug's effect alone. Raw luciferase values of all combinations in triplicate were inputted into MacSynergyII and % inhibition values of combinations at a fixed ratio of concentration of two drugs inputted into CalcuSyn, except those exhibiting >25% toxicity.

Table 3

Combination studies of DAAs, cyclosporin A and (*S*)-CCZ in HCVcc-Luc assay

			MacSynergyII			CalcuSyn			
Virus type	**ID**	**Antiviral name**	**Synergism LV**	**Antagonism LV**	**Conclusion[a]**	**EC_{50} CI**	**EC_{75} CI**	**EC_{90} CI**	**Conclusion[b]**
HCVcc-Luc[c]	I	Daclatasvir and cyclosporin A	2.27	−0.33	(+) Minor synergy	0.93 ±0.31	0.67 ±0.22	0.49 ±0.18	(++) Moderate synergy
HCVcc-Luc[c]	J	Telaprevir and cyclosporin A	2.17	−0.47	(+) Minor synergy	0.99 ±0.31	0.98 ±0.28	0.97 ±0.29	() Additive
HCVcc-Luc[c]	K	Boceprevir and cyclosporin A	1.83	−0.09	() Additive	1.06 ±0.23	1.04 ±0.22	1.03 ±0.24	() Additive
HCVcc-Luc[c]	L	Sofosbuvir and cyclosporin A	7.22	−0.11	(++) Moderate synergy	0.63 ±0.20	0.68 ±0.19	0.74 ±0.20	(++) Moderate synergy
HCVcc-Luc[d]	N/A	Daclatasvir and (*S*)-CCZ	11.76	0	(+++) Major synergy	0.31 ±0.18	0.43 ±0.21	0.63 ±0.26	(+++) Major synergy
HCVcc-Luc[d]	N/A	Telaprevir and (*S*)-CCZ	11.90	−0.59	(+++) Major synergy	0.67 ±0.19	0.64 ±0.16	0.01 ±0.16	(+++) Major synergy
HCVcc-Luc[d]	N/A	Boceprevir and (*S*)-CCZ	17.00	−1.21	(+++) Major synergy	0.33 ±0.09	0.32 ±0.09	0.36 ±0.12	(+++) Major synergy
HCVcc-Luc[d]	N/A	Sofosbuvir and (*S*)-CCZ	30.56	0	(+++) Major synergy	0.16 ±0.03	0.20 ±0.04	0.27 ±0.05	(+++) Major synergy
HCVcc-Luc[d]	N/A	2′-*C*-methylcytidine and (*S*)-CCZ	5.66	−0.05	(++) Moderate synergy	0.36 ±0.09	0.34 ±0.07	0.35 ±0.07	(+++) Major synergy
HCVcc-Luc[d]	N/A	Cyclosporin A and (*S*)-CCZ	40.80	−2.16	(+++) Major synergy	0.39 ±0.14	0.24 ±0.07	0.26 ±0.06	(+++) Major synergy

[a]Extent of synergy or antagonism in MacSynergyII is defined according to absolute value of log volume (LV) as follows: () additive, LV <2; (+/−) minor, 2≤ LV <5; (++/−−) moderate, 5≤ LV <9; (+++/−−−) major, LV ≥9.

[b]Extent of synergy and antagonism in CalcuSyn is defined as follows: (+++) major synergy, combination index (CI) ≤0.7; (++) moderate synergy, 0.7≤ CI <0.8; (+) minor synergy, 0.8≤ CI <0.9; () additive, 0.9≤ CI <1.1; (−) minor antagonism, 1.1≤ CI <1.3;(−−) moderate antagonism, 1.3≤ CI <3.0; (−−−) major antagonism, CI ≥3.0.

[c]LV (95% confidence level) and CI (±SE) of combinations of direct-acting antivirals (DAAs) with host-targeting agent (HTA) cyclosporin A in HCVcc-Luc.

[d]LV (95% confidence level) and CIs (±SE)of combinations of previously described entry inhibitor (*S*)-chlorcyclizinc (CCZ) with DAAs and HTA cyclosporin A, in HCVcc-Luc. Raw luciferase values of combinations in triplicate were inputted into MacSynergyII and % inhibition values of combinations at a fixed ratio of concentration of two drugs inputted into CalcuSyn, except those exhibiting >25% toxicity. N/A, not applicable.