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Searching for Synergy: Identifying Optimal Antiviral Combination Therapy using Hepatitis C Virus (HCV) Agents in a Replicon System

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Abstract

Direct acting antiviral agents (DAAs) are potent inhibitors of Hepatitis C virus (HCV) that have revolutionized the treatment landscape for this important viral disease. There are currently four classes of DAAs that inhibit HCV replication via distinct mechanisms of action: nonstructural protein (NS) 3/4a protease inhibitors, NS5A inhibitors, NS5B nucleoside polymerase inhibitors, and NS5B non-nucleoside polymerase inhibitors. Combination therapy with two or more DAAs has great potential to further enhance antiviral potency. The purpose of this study was to identify optimal combinations of DAAs against genotype 1 HCV replicons that maximized the inhibition of replicon replication. All possible two-drug combinations were evaluated against genotype 1a and 1b HCV replicons using a 96-well plate luciferase-based assay in triplicate. The Greco Universal Response Surface Area mathematical model was fit to the luciferase data to identify drug-drug interactions (i.e.: synergy, additivity, and antagonism) for antiviral effect against both genotypes. This information was used to rank-order combinations of DAAs based on their ability to inhibit replicon replication against genotype 1a and 1b HCV. These preclinical findings can provide information as to which antiviral regimens should move on in the development process.

Keywords

Combination Treatment; Drug Interaction Mathematical Modeling; Antiviral Agents; Hepatitis C Virus

1. Short Communication

Hepatitis C virus (HCV) is estimated to affect nearly 2%–3% of the world's population and is the primary cause of cirrhosis and hepatocellular carcinoma. Liver-related morbidities associated with chronic infections are responsible for nearly 400,000 deaths each year

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(World Health Organization, 2017), highlighting the substantial global burden attributed to HCV disease. Although a protective vaccine does not currently exist, ongoing HCV infections can be successfully managed with antiviral therapy.

Pegylated interferon alpha plus ribavirin has historically been the standard of care for HCV. This regimen was largely ineffective in the majority of patients with genotype 1 infections (the predominant genotype in the United States) and was associated with intolerable side effects that often resulted in poor adherence and treatment discontinuation (Fried et al., 2002; Fried, 2002). Limitations related to the traditional standard of care have been overcome with the advent of direct-acting antiviral agents (DAAs), which have since revolutionized antiviral therapy for HCV. DAAs target and inhibit the function of viral proteins that are essential for HCV replication. There are presently four different classes of DAAs including nonstructural protein (NS) 3/4a protease inhibitors, NS5A inhibitors, NS5B nucleoside polymerase inhibitors, and NS5B non-nucleoside polymerase inhibitors. Each class has shown potent activity against genotype 1 HCV in preclinical and clinical studies (Lawitz et al., 2013; Liu et al., 2014; Wyles et al., 2014).

Combination regimens with two or more DAAs have demonstrated great clinical success based upon impressive cure rates (>90%) coupled with fewer side effects and abbreviated care regimens when compared to interferon-based treatments (Afdhal et al., 2014; Andreone et al., 2014; Lawitz et al., 2014). There are currently several therapeutic regimens available for the treatment of chronic HCV, each containing various combinations of DAAs from different classes. However, it is important to understand that not all combinations are equally effective and the ability of each regimen to suppress HCV is greatly influenced by a variety of factors including: i) the potency of each agent as monotherapy; ii) the drug-drug interaction for antiviral effect (i.e.: synergy, additivity, or antagonism) between the DAAs in combination; and iii) the HCV genotype under evaluation. Here, we evaluated these factors for four DAAs (each representing a different drug class) as single agents and two-drug combinations against genotype 1a (GT1a) and genotype 1b (GT1b) HCV in an attempt to identify an optimal combination of DAAs that maximizes viral inhibition for both genotypes.

GT1a (Robinson et al., 2010) and GT1b (Klump et al., 2006) replicon cell lines were employed as a high-throughput method to quantify the capacity of DAAs to inhibit HCV replication. The following DAAs were assessed in this investigation: sofosbuvir (SOF; NS5B nucleoside polymerase inhibitor), ledipasvir (LDV; NS5A inhibitor), vedroprevir (VDV; NS3/4A protease inhibitor), and GS-9669 (NS5B non-nucleoside polymerase inhibitor). All compounds were obtained from Gilead Sciences (Foster City, CA). DAAs were first evaluated as single agents against GT1a and GT1b replicon cell lines using a 96-well plate *Renilla* luciferase assay, as previously described (Brown et al., 2012). Briefly, 5,000 cells were inoculated into white opaque 96-well plates and incubated for 24h. Varying concentrations of each compound or 1% DMSO (a total of 10-assay points per drug) were added in triplicate to the 96-well plate and further incubated for 72h. Replicon replication kinetics were monitored after three days of treatment using the *Renilla* luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions and effective concentration 50 (EC₅₀) values were calculated using Prism 6.0 software (GraphPad,

LaJolla, CA). All DAAs evaluated were potent inhibitors of GT1a and GT1b HCV replication (Table 1). The NS5A inhibitor LDV was the most potent DAA, exhibiting EC₅₀ values in the pg/ml range for both genotypes, whereas SOF was the least potent with EC₅₀ values in excess of 200 ng/ml. GT1a replicons were overall less susceptible to DAA treatment and had higher EC₅₀ values relative to GT1b replicons. LDV effectiveness was the most influenced by HCV genotype, as GT1a replicons yielded EC₅₀ values that were 27-fold higher than those reported for GT1b replicons. In contrast, SOF exhibited better pan-genotype 1 activity, with EC₅₀ estimates that were only 1.5-fold higher in GT1a replicon cell lines compared to GT1b cells. GS-9669 and VDV were marginally influenced by genotype, with EC₅₀ differences of 5.6- and 3.4-fold, respectively between GT1a and GT1b replicons. Cytotoxicity was not observed with treatment on either cell line (data not shown), indicating that any decrease in luciferase activity was directly related to replicon inhibition and not due to treatment-related toxicities.

We next evaluated the antiviral activity of all two-drug DAA combinations against GT1a and GT1b replicons using a six concentration-by-six concentration (including a 1% DMSO control) checkerboard format in a white opaque 96-well plate. Each agent was evaluated at concentrations equivalent to the EC₁₀, EC₃₀, EC₅₀, EC₈₀, and EC₉₅. All assays were performed in triplicate. After 72h of treatment, HCV replicon levels were quantified by measuring luciferase activity. A mathematical model was fit to the luciferase output to identify drug-drug interactions for antiviral effect between the two DAAs in combination. For these evaluations, we employed the approach of Greco et al. (Greco et al., 1995) which is based on the definition of Loewe additivity. The Greco Universal Response Surface Area (URSA) model is described below:

$$1 = \frac{D_1}{EC_{50\ D1} \left(\frac{E-B}{E_{con}-E} \right)^{1/H_1}} + \frac{D_2}{EC_{50\ D2} \left(\frac{E-B}{E_{con}-E} \right)^{1/H_2}} + \frac{\alpha D_1 D_2}{EC_{50\ D1} EC_{50\ D2} \left(\frac{E-B}{E_{con}-E} \right)^{1/2_{H_1} + 1/2_{H_2}}}$$

D₁ and D₂ correspond to the concentrations of drugs 1 and 2, respectively. E signifies the measured viral effect, which in this case is luciferase activity and is reported as Log₁₀ relative light units (RLU). B is the Baseline. The EC₅₀ values of drug 1 and drug 2 are represented by EC_{50\ D1} and EC_{50\ D2}, respectively. H₁ and H₂ describe the Hill's constant, or slope parameter, for drug 1 and drug 2. Finally, α is the drug interaction term. If α is positive and its attendant 95% confidence interval (C.I.) is also positive (i.e.: the lower bound of the 95% C.I. does not cross zero), the drug-drug interaction is deemed synergistic. Conversely, if α is negative and the 95% C.I. is also negative (i.e.: the upper bound of the 95% C.I. does not cross zero), the interaction is antagonistic. If the 95% C.I. surrounding α crosses 0, the interaction is not statistically different from additivity and thus, the interaction is termed additive. Data were analyzed in the ID module of the ADAPT software package (version 5) (D'Argenio et al., 2009) using the maximum-likelihood estimation option.

The mean parameter estimates from the Greco URSA model are shown in Table 2. Original attempts to fit a model to the data identified values for H₁ and H₂ that were very small and resulted in non-physiologic values for the EC₅₀'s. Consequently, we fixed both the values

for H to 1.0. This resulted in improved fits and more physiological values for the EC₅₀ values. The model fit all data sets well, yielding r^2 values ranging from 0.915 to 0.974. The estimates for alpha (the drug-drug interaction term) were positive for all two-drug regimens against GT1a and GT1b replicons, demonstrating that antagonism does not occur with any of the DAAs in combination. Combinations that were synergistic for inhibition of HCV replication are illustrated with bolded alpha values. For GT1a replicons, nearly all of the combination regimens resulted in synergy, with the one exception of LDV + VDV which was additive for replicon suppression. In contrast, additivity was achieved for all combinations against GT1b replicons with the single exception of SOF + LDV which was synergistic (Table 2). These findings are somewhat surprising because clinically, GT1b infections usually exhibit a better virological response to antiviral therapy with DAAs than GT1a infections (Forns et al., 2015; Zeuzem et al., 2016). Therefore, we expected to see more positive drug interactions (i.e.: synergy) for GT1b replicons compared to GT1a replicons. There are several possible explanations for this difference in outcome with the first being that GT1a replicons are overall less susceptible to DAA treatment compared to GT1b (Table 1). Consequently, GT1a replicons likely require higher levels of drug pressure to effectively inhibit replication, despite the synergistic interactions between DAAs. Additionally, DAAs (particularly the NS3/4a protease inhibitors and NS5A inhibitors) often have a lower genetic barrier to resistance for GT1a HCV, resulting in higher frequencies of HCV harboring resistance-associated variants (RAVs) (Sarrazin et al., 2016; Zeuzem et al., 2017). These RAVs have been shown to significantly reduce the susceptibility of GT1a HCV to DAA treatment; however, the impact of RAVs on the susceptibility of GT1b HCV is substantially lower (Liu et al., 2015; Sarrazin et al., 2016; Zeuzem et al., 2017). Our evaluation only focuses on the ability of DAAs in combination to inhibit HCV replication and does not consider the emergence of resistance. Thus, it is possible that combination therapy is synergistic for replicon suppression against wild-type replicons and that synergy is lost with the emergence of resistance.

The SOF + LDV combination was the only regimen to achieve statistically significant synergy for both GT1a and GT1b HCV replicons with alpha values of 3.21 and 2.54, respectively. These findings suggest that the NS5B nucleoside polymerase inhibitor + NS5A inhibitor is the most effective DAA regimen at inhibiting genotype 1 HCV replication and thus, is identified in our evaluations as the best combination. Interestingly, SOF + LDV (Harvoni®) is the only combination assessed here that is licensed for the treatment of HCV and has demonstrated great clinical success. These results highlight the potential utility of preclinical assays for the selection and rank-ordering of DAA combinations for further preclinical development.

Drug-drug interactions for antiviral effect were identified here using the Greco URSA model which employs Loewe additivity as the null reference model. Cheng et al. (Cheng et al., 2016) conducted similar evaluations with LDV + GS-9669, LDV + VDV, and LDV + SOF on GT1a replicon cells, but instead utilized the MacSynergy II program (Prichard et al., 1993) that defines additivity based on Bliss Independence. The results reported by Cheng et al. were similar to ours with slight differences in two circumstances: 1) LDV + GS-9669 was defined as minor synergy whereas we observed strong synergy, as this combination yielded the highest alpha in our study ($\alpha = 5.96$) and 2) LDV + SOF was identified as additive but

we observed synergy. LDV + VDV was additive in both studies. Overall, antagonism was not observed in either study and the concordance between results was quite good. Although both mathematical approaches have their strengths and weaknesses, it is important to note that the use of different analytical methods can derive similar conclusions.

There are some limitations to our study. First, our experimental approach evaluates antiviral activity at a single time-point after only three days of treatment. It is more than likely that the antiviral effect of DAAs as well as drug-drug interactions will change over a longer evaluation period. Additionally, as mentioned above, we are only evaluating monotherapy and combination regimens on the basis of inhibiting HCV replicon replication and are not considering resistance emergence. Resistance emergence is a significant challenge for HCV therapy and often results in treatment failure. Therefore, optimal combination regimens must prevent viral replication and suppress resistance emergence. We are currently conducting antiviral studies in our laboratory that address both of these limitations using the BelloCell system (Brown et al., 2012). This system allows for the evaluation of replicon replication and resistance emergence over time through serial sampling for at least 14 days.

In summary, we have shown that DAAs, regardless of drug class, are potent against GT1a and GT1b replicons. Moreover, antiviral activity is enhanced when DAAs from different classes are used in combination. All combinations are not considered equal, as some are more effective than others. Here, we show a comprehensive evaluation of all possible two-drug combinations consisting of DAAs from different classes against genotype 1a and 1b replicons. Additionally, we describe an analytical method to rank-order the combinations based on the ability of each regimen to inhibit viral replication. This information can aid in the decision as to which combinations should move forward in further preclinical evaluations.

Acknowledgments

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Highlights

- We evaluated the effectiveness of direct acting antiviral agents (DAAs) in combination against genotype 1a and 1b HCV.
- We employed a mathematical model to define drug-drug interactions for antiviral effect for all combinations of DAAs.
- We rank ordered the combinations based on the interaction (i.e. synergy/additivity) between compounds for HCV suppression.
- These methods are a rationale strategy for selecting and ranking DAA combinations for further preclinical development.

Table 1
Antiviral Activities of Direct Acting Antiviral agents against Hepatitis C Virus Genotype 1a and 1b replicons

Direct Acting Antivirals (DAAs)	Drug Class	Units	EC50 values [95% CI]	
			HCV Genotype 1a	HCV Genotype 1b
GS-9669	Non-Nucleoside NS5B Inhibitor	ng/ml	25.67 [20.40,30.94]	4.574 [3.832,5.316]
Ledipasvir (LDV)	NS5A Inhibitor	pg/ml	38.24 [32.11,44.37]	1.421 [0.979,1.862]
Sofosbuvir (SOF)	Nucleoside NS5B Inhibitor	ng/ml	309.4 [264.7,354.0]	217.0 [203.6,230.5]
Vedoprevir (VDV)	NS3/4A Protease Inhibitor	ng/ml	73.88 [58.20,89.56]	22.04 [10.74,33.33]

Table 2

The mean parameter estimates from the Greco URSA model for each combination of DAAs against GT1a and GT1b HCV replicons^a

Antiviral Combination Regimen ^b																	
Parameter ^c	Units	GS-9669 + LDV				LDV + VDV		SOF + GS-9669				SOF + LDV		SOF + VDV		VDV + GS-9669	
		1a	1b	1a	1b	1a	1b	1a	1b	1a	1b	1a	1b	1a	1b		
<i>r</i> ²	--	0.949	0.932	0.941	0.915	0.975	0.974	0.945	0.954	0.970	0.962	0.967	0.938				
Econ	Log ₁₀ RLU	6.27	6.25	7.09	6.56	6.60	6.27	6.53	6.36	7.01	6.40	7.42	6.26				
EC _{50,D1}	ng/ml	20.25	8.10	0.089	0.004	120.10	325.80	80.14	202.90	82.89	120.50	59.04	73.58				
EC _{50,D2}	ng/ml	0.168	0.014	50.60	36.11	21.70	7.42	0.115	0.009	72.93	58.56	19.24	6.86				
B	Log ₁₀ RLU	3.50	2.71	4.38	4.35	3.63	3.17	4.15	3.65	4.18	4.01	4.20	3.57				
α d[95% C.I.]	--	5.96 [0.12, 11.81]	1.32 [-0.76, 3.22]	2.27 [-0.07, 4.61]	1.08 [-0.80, 2.96]	1.48 [0.42, 2.53]	0.342 [-0.27, 0.95]	3.21 [0.04, 6.38]	2.54 [0.03, 5.04]	2.22 [0.78, 3.65]	0.214 [-0.31, 0.73]	2.32 [0.63, 4.01]	2.52 [-0.51, 5.55]				

^aThe data represent one of two independent experiments.

^bThe first compound listed in the regimen corresponds to D1 and the second compound corresponds to D2

^cH1 and H2 were fixed to 1.0.

^dBolded values indicate synergy