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Quantifying antiviral activity optimizes drug combinations against hepatitis C virus infection

ONE-SENTENCES SUMMARY:

Cell culture study combing a mathematical model and computer simulation quantifies the anti-hepatitis C virus drug efficacy at any concentrations and any combinations in preclinical settings, and can obtain rich basic evidences for selecting optimal treatments prior to costly clinical trials.

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ABSTRACT (237/250)

With the introduction of direct-acting antivirals (DAAs), treatment against hepatitis C virus (HCV) has been rapidly improving. To eradicate this worldwide infectious disease, the “best” multidrug treatment is demanded based on scientific evidence. However, there is no method available that systematically quantifies and compares the antiviral efficacy and drug-resistant profiles of drug combinations. Based on experimental anti-HCV profiles in an HCV cell culture system, we quantified the instantaneous inhibitory potential (IIP), which is the logarithm of the reduction in viral replication events, for both single and multiple drug combination treatments. From the calculated IIP of 15 anti-HCV drugs, we found that interferon-alpha (IFN-α) and a nucleoside polymerase inhibitor, sofosbuvir (SOF), had the largest potential to inhibit viral replication events. Profiling of 52 double-combination treatments indicated that the combinations based on a protease inhibitor, simeprevir (SMV), achieved high IIP. Our modeling also predicted the treatment amount of SOF in a SOF plus SMV combination could be reduced to 41% in comparison to the amount of SOF needed when combined with ledipasvir. By taking into account clinical concentrations, different SMV-based double-DAA combination under clinical development showed the most desirable IIP score. Furthermore, quantification analysis of triple-DAA IFN-free combinations suggests that triple DAAs greatly enhanced antiviral activity and reduced the emergence of drug resistant virus compared with double-DAA treatments. Our novel framework presents basic evidence to consider in the strategy to optimize multidrug treatment and also to increase its cost-effectiveness.

INTRODUCTION

Hepatitis C virus (HCV) affects approximately 170 million people worldwide (*1-4*) and is a major cause of liver cirrhosis and hepatocellular carcinoma. The standard treatment has long been a combination therapy of interferon (IFN), IFN-α or pegylated IFN-α (peg-IFN-α), with ribavirin (RBV), but the sustained virologic response (SVR) rate by this treatment was limited to around 50% (*5*). Improvements in the SVR rate have been made by using anti-HCV agents that inhibit viral-derived factors or cellular factors that are essential for viral replication in cells: Agents inhibiting viral proteins, called direct acting antivirals (DAAs), typically target HCV NS3 protease, NS5A, and NS5B polymerase (*3*). Anti-HCV molecules that target cellular factors, so-called host-targeting antivirals (HTAs), include those inhibiting cyclophilins and microRNA-122, which are required for HCV replication in cells (*3*). These agents have been evaluated in clinical trials. In 2011, the protease inhibitors telaprevir (TPV) and boceprevir were approved by the FDA for use in combination with peg-IFN and RBV. These drug combinations achieved significantly improved clinical outcome attaining more than a 70% SVR rate (*5*). The second-generation protease inhibitor, simeprevir (SMV), was approved in 2013 and this drug has been widely used as one of the first choices of protease inhibitors, in combinations such as SMV&peg-IFN-α&RBV and SMV&sofosbuvir (SOF) (*4*). SOF is a nucleoside polymerase inhibitor that was approved in 2013, and is or has been used in combination with RBV, SMV, and ledipasvir (LDV) (*4*). NS5A inhibitors that are already approved include daclatasvir (DCV) and LDV, which can be used in combinations such as DCV&SOF, DCV&asunaprevir (ASV), a protease inhibitor, and most importantly SOF&LDV. Other treatment choices include a combination of paritaprevir (protease inhibitor), ombitasvir (NS5A inhibitor), dasabuvir (non-nucleoside polymerase inhibitor) and ritonavir (*6*). Additional drugs have just been and will eventually be approved for adding new combination choices (*7*). Moreover, anti-HCV treatment with triple DAA regimens has also been clinically examined for seeking more rapid response (*8-10*).

In an era of rapid progress for anti-HCV treatments, patients and clinicians select one combination treatment from the available choices, which are approved based on clinical trial results and practical issues such as insurance company reimbursement policies. Toward the ultimate goal to eradicate HCV infection, it is important to understand the intrinsic characteristics of each drug including its antiviral activity, drug resistance profile and its adverse effects when used both singly and in combination in order to determine the “best” combination treatment. Although the

intrinsic antiviral activity is the most fundamental factor for treatment, there has been no data available that systematically evaluates and compares the intrinsic anti-HCV activity of drugs that are currently available or that will be available in the future.

Another problem of a more practical nature is the huge cost of current HCV therapies. For example, SOF, one of the major choices for HCV treatment, costs US $84,000 per patient in a 12-week course of treatment (*11*). Optimization of HCV treatment, based on both experimental and theoretical evidence, may be able to suggest less costly but equally potent regimens or indicate the most potent regimens that might be tested for shorter duration therapies.

In this study, the anti-HCV activity of 15 clinically available or developmental-phase anti-HCV drugs were profiled in an HCV genotype 1 cell culture model (*12*). We analyzed these data by computing the instantaneous inhibitory potential (IIP) (*13-18*) of single and multiple combinations. The IIP is the logarithm of the reduction in intracellular viral replication at a given drug concentration. At doses exceeding the half-maximal inhibitory concentration (IC_{50}), we found the strongest inhibitors of replication were drugs including SOF and IFN-α. Furthermore, we searched for drug combinations that are effective at lower dosage (and therefore potentially lower cost) than existing combinations, by estimating the required critical concentration index (RCI), i.e., the normalized doses required for 95% reduction of HCV replication. Among the antiviral profiles of 52 double-combination treatments, certain drug combinations reduced viral replication by 95% at doses slightly over the IC_{50}; namely, SMV&IFN-α, SOF&SMV, TPV&IFN-α, and SOF&IFN-α. Interestingly, all of them have been popular choices for IFN-based or IFN-free DAA treatment in clinic (here, Peg-IFN-α&RBV replacing IFN-α) (*4, 5*). From this analysis, a possible reduction of dosage was calculated and discussed for the SOF&SMV combination (*19-21*). We also investigated 8 triple DAA-combination IFN-free treatments, which are possible candidates in the future for achieving rapid antiviral responses (*8-10*). Additionally, we calculated the risk of occurrence of drug resistance for 15 double-combination and 6 triple-DAA combination treatments at clinical drug concentrations.

The basic information provided in the present study should be useful for optimizing drug choices and dosages in preclinical settings, and improving the drug-resistance management and the cost-effectiveness of drug treatments. Our findings also potentially impact clinical strategies for multidrug treatment.

RESULTS

Evaluation of intrinsic antiviral activity of single HCV drugs

We evaluated the intrinsic antiviral activity of 15 anti-HCV agents of different classes (**Fig. 1a**) in a cell culture model for HCV genotype 1, the most prevalent HCV genotype worldwide. HCV replication was evaluated in the HCV replicon system rather than in the HCV infection system for three reasons: (I) HCV genotype 1 robustly replicates in the replicon system but its replication is inefficient in the infection system (*22, 23*), (II) the sensitivity and throughput of the replicon system carrying the luciferase gene (see **Methods**) is much higher than that of the infection system (*23*), (III) all of the tested drugs primarily inhibit polyprotein processing or the replication stage of the HCV life cycle (**Fig. 1a**); therefore, the replicon system enables one to evaluate the efficacy of these drugs to genotype 1 HCV, in a highly sensitive and high throughput manner (*22*). We treated an HCV subgenomic replicon (strain-NN) (*12, 23*) with each drug for 72 h and measured the HCV replication activity (**Fig. 1b** and **Methods**).

The typical dose-response curve (**Fig. 2a** and **Supplementary Fig. S1**) of a single antiviral drug can be analyzed by the following median effect model (*13-18*):

$$\log\left(\frac{1-f_u}{f_u}\right) = m\log\left(\frac{D}{IC_{50}}\right). \qquad (1)$$

Here, $1-f_u$ and f_u are the fractions of infection events affected and unaffected by the drug, respectively, D is the drug concentration, IC_{50} is the drug concentration that inhibits 50% inhibition of the activity, and m is the slope parameter reflecting the steepness of the dose-response curve (*13-18*). The log–log dose-response curves (**Fig. 2a**) were converted into median effect plots by transforming $\log f_u$ into $\log(1-f_u)/f_u$ (**Fig. 2b**). The IC_{50} and slope parameter m were estimated by linear regression of the data plotted in the median effect plot as the intercept with 0 and the slope, respectively (**Fig. 2c**; **Supplementary Fig. S2**, and **Table S1**). The IC_{50} is widely used to measure drug potency, but the slope parameter m, which can vary with the drug class (*14*), also substantially affects the antiviral activity (*13-18*). In this assay, we used interferon-α (IFN-α) instead of peg-IFN-α as an IFN-based drug. As the antiviral activity of these two drugs is equivalent in cell cultures (*24*), the intrinsic antiviral effect of peg-IFN-α can be interpreted from the data for IFN-α in this study. Interestingly, we found that past and present first-line anti-HCV drugs (*2, 11, 25*), namely, IFN-α, TPV and SOF, had relatively high m values (around 1.5 or higher; **Fig. 2c, right**), confirming the high anti-HCV potential of these drugs. Cyclophilin inhibitors

(CIs) such as cyclosporin A (CsA) and SCY-635 (SCY) exhibited similarly high m values. This implies that HTAs such as IFN-α and CIs achieve a high antiviral effect at concentrations only slightly above the IC_{50}. Thus, the antiviral activity at drug concentration D is determined not only by the IC_{50} but also by m, which is unique to each drug (*13-18*).

The antiviral activity of a drug can be expressed as the instantaneous inhibitory potential (IIP) (*13-18*), which is the number of logs by which a drug at concentration D inhibits HCV replication:

$$\mathrm{IIP} = \log\left(\frac{1}{f_u}\right) = \log\left[1 + \left(\frac{D}{IC_{50}}\right)^m\right]. \qquad (2)$$

Thus if a drug reduces HCV replication by 1 log then $f_u = 0.1$ and its $\mathrm{IIP} = 1$, whereas if it reduces replication by 2 logs, i.e. 100-fold, its $\mathrm{IIP} = 2$. Note that the IIP incorporates all three parameters of the dose-response curve; D, IC_{50} and m. Eq. (2) indicates that the higher the m of the drug, the higher the IIP at a given D and IC_{50}.

The IIP of the 15 tested antiviral drugs was calculated from the experimentally measured f_u by $\mathrm{IIP} = \log(1/f_u)$. As shown in **Fig. 2d**, the IIP of the 15 drugs widely varied. The log reductions in HCV replication in the replicon system were well predicted by the equation $\mathrm{IIP} = \log[1 + (D/IC_{50})^m]$ (**Supplementary Fig. S3**), using the parameters estimated from the median effect plot in **Fig. 2c**. Classifying the 15 drugs used into groups: protease inhibitors (PI), nucleotide and nonnucleotide polymerse inhibitors (NI and NNI, respectively), NS5A inhibitors, IFN and cyclophilin inhibitors (CI), we found that drugs in the same class all had similar IIPs when normalized by the drug's IC_{50} (**Fig. 2e**). We also determined the IIP_{100}, defined as the IIP when $D = 100 \times IC_{50}$, by extrapolation (**Fig. 2f**) to estimate the effects of high drug concentrations as clinical doses can range between $10{\sim}100$-fold above the IC_{50} (*26*).

We found that previous or current first-line drugs such as IFN-α, TPV, SMV, and SOF, and cyclophilin inhibitors can inhibit more than 99% of HCV replication in this concentration range ($\mathrm{IIP}_{100} > 2$). Moreover, we calculated the critical dose D_C of each antiviral drug at which the IIP reaches 1.3 (indicating 95% inhibition of viral replication; see **Table S1**). **Fig. 2g** groups the critical doses normalized by IC_{50} into drug classes or subclasses. Drugs with small D_C/IC_{50} values are more efficient inhibitors of HCV replication, and gain larger anti-HCV effect as the dose is increased beyond the IC_{50}, than drugs with high D_C/IC_{50}. Interestingly, high m tends to be associated with smaller D_C/IC_{50}. From a potential pharmaceutical cost perspective,

this information assists the search for drugs that achieve a certain critical antiviral inhibition level (e.g. 95%) but use lower amounts of drug (as discussed below).

Evaluation of intrinsic antiviral activity of double-combination anti-HCV drugs

We next investigated the antiviral activity of multidrug combinations (i.e., $\mathrm{IIP}^{\mathrm{com}}$). In clinical settings, ribavirin (RBV) augments the antiviral efficacy of IFN-based and DAA-based treatments (*25*). However, because clinically relevant doses of RBV lack sufficient anti-HCV activity in cell culture systems (*27, 28*), the antiviral efficacy of RBV was not evaluated. Using the replicon system, the inhibitory activity against HCV replication was evaluated for 52 double-combinations of antiviral drugs (**Fig. 3** and **Supplementary Fig. S4**). In this experiment, drugs were combined so that their initial concentrations were $D_{\mathrm{initial}} = 0.25 \times IC_{50}$ and then the drug concentrations were both increased up to a maximum of 16-fold. Their $\mathrm{IIP}^{\mathrm{com}}$ values were computed from Eq. (2): $\mathrm{IIP}^{\mathrm{com}} = \log(1/f_u^{\mathrm{com}})$, where f_u^{com} are the experimental measurement of a drug combination (**Fig. 3a**). We confirmed that the largest concentration ($4 \times IC_{50}$) of each combination sufficiently suppressed HCV replication without significant cytotoxicity. The combination effects at the largest concentration were categorized by their $\mathrm{IIP}^{\mathrm{com}}$ values, visually presented as the upper triangular elements (blue areas) in **Fig. 3b** (**Table S2**). In **Supplementary Fig. S5**, we predicted the $\mathrm{IIP}^{\mathrm{com}}$ of each combination from the measured effects (see **Supplementary Note 1**). We also evaluated the combinations for Loewe additivity (*29-31*) and Bliss independence (*30-33*), because the combined effects of drugs have been evaluated using these concepts (see **Fig. 3b** and **Supplementary Note 2**). Consistent with a previous report for HIV drug combinations (*17*), most of the drug combinations ($\sim 65\%$) exhibited neither Loewe additivity nor Bliss independence but rather had intermediate activity as judged by the Jilek *et al*. (*17*) degree of independence (DI); see **Supplementary Fig. S6** and **Table S2**.

Characterization of cost-effectiveness of double-combination anti-HCV drugs

Although DAAs such as SOF-based combination regimens are highly effective against HCV, with $> 90\%$ SVR rates in treatment-naïve patients (*19, 20*), their medical costs at least in developed countries are prohibitively high. For example, in the USA, a 12-week course of SOF costs US $84,000 per patient (*11*). Thus, reducing the medical cost with a robust antiviral outcome is highly desired (*34*). To this end, we searched for regimens that can achieve relevant antiviral activity at relatively low

dosage, which potentially could reduce the cost of treatment. The required critical concentration index ($\mathrm{RCI} = \widetilde{D_C}/D_{initial}$) that achieves 95% reduction in HCV replication (i.e., IIP^{com}=1.3) is shown for each drug combination in **Fig. 3c** (see also **Supplementary Note 1**). Note that, for example, in drug combination A and B, $\widetilde{D_C}$ of drug A and $\widetilde{D_C}$ of drug B, which are the amount of drug A and that of B achieving IIP^{com}=1.3 in the combination, have different values (see **Table S2**). The RCI varies among the drug combinations. SMV&IFN-α yielded the lowest RCI value among the tested combinations, meaning that this combination requires only a small-fold increase of concentration above the IC_{50} to achieve critical (95%) antiviral activity. Intriguingly, its corresponding clinical combination, a triple combination of SMV, peg-IFN-α and RBV, has been one of the first-line treatments since 2013, especially for HCV genotype 1b (*25, 35*). Moreover, the combinations yielding the 10 lowest RCI (< 8.0) include most of the past or present first-line treatment options, such as SMV&IFN-α, SOF&SMV, TPV&IFN-α, and SOF&IFN-α (**Fig. 3c**). Therefore, these treatments will likely achieve high and relevant anti-HCV effects at lower dosages over IC_{50}. IFN-α (or peg-IFN-α) has been a standard anti-HCV agent for a long time before the development of DAAs. TPV and SMV are PIs that have been developed early and have constituted a main choice for HCV treatment, especially early in the era of DAA usage.

SOF is currently recognized as a strong first-line choice for an anti-HCV agent. It is interesting that combinations associated with these mainstream drugs are characterized by low RCI. In contrast, SOF&ledipasvir (LDV), which shows $> 90\%$ SVR and is a first line standard combination (*19-21*), showed a relatively high RCI value (~ 11.6). Because the intrinsic antiviral activity of SOF&SMV is higher than that of SOF&LDV (**Fig. 3b**), SOF&SMV even with less SOF content can achieve the same antiviral activity as SOF&LDV. Comparing the RCI values (**Fig. 3c**: $\mathrm{RCI}_{\mathrm{SOF\&SMV}} = 6.84 < \mathrm{RCI}_{\mathrm{SOF\&LDV}} = 11.6$), we find that 224.5 nM SOF combined with 2.53 nM SMV achieves the same 95% reduction in HCV replication as 378.9 nM SOF combined with 2.31 nM LDV based on our assay. Therefore, the amount of SOF in SOF&SMV can be reduced 41% and still yield the same antiviral activity as SOF&LDV, following the calculation $378.9 - 224.5 = 154.5$ nM, or $\frac{(\mathrm{RCI\ for\ SOF\&LDV}) - (\mathrm{RCI\ for\ SMV\&SOF})}{(\mathrm{RCI\ for\ SOF\&LDV})} \approx 0.41 = 41\%$ for possible reduction of SOF. This suggests that SOF&SMV could show a significant antiviral effect even with a reduced SOF dosage. If the drug cost is determined proportionally to the dosage of drug, this could achieve a cost-effective treatment.

Unfortunately, the ASV&DCV combination, which is already approved in Japan and Korea as the first IFN-free therapy, yielded an even higher RCI value ($\sim$19.15), suggesting that this treatment has little opportunity for seeking a better cost-effectiveness. Thus, especially high-ranking drug combinations with low RCI values are candidates for cost reduction, because their doses might be able to be reduced to the level that presents the anti-HCV effect equivalent to that of high-RCI combinations.

Profiling of triple-combination anti-HCV drugs

Currently, triple-DAA IFN-free combinations have been developing clinically to seek more rapid and efficacious elimination of HCV, including SOF&NS5A&PI and SOF&NS5AI&NNI (*8, 36-38*). However, it has not been clear what triple DAA combination is the most potent and cost-effective. We here quantified the anti-HCV activity of 8 candidate combinations of triple DAA treatment; SOF with NS5AI (DCV, LDV) plus PI (SMV, ASV) or NNI (VX, DSV) (**Fig. 4a**, **Table S3** and **Supplementary Fig. S7**). Interestingly, we found that these triple DAAs greatly enhanced antiviral activity (i.e., 5-fold IIP^{com} at the maximum) compared with double-DAA treatments in **Fig. 4a**, and that these drug combinations exhibited an intermediate activity compared to Loewe additivity and Bliss independence (**Supplementary Fig. S8**). Especially, SOF&LDV&SMV, SOF&DCV&DSV, and SOF&DCV&SMV achieved high IIP^{com} values (see also **Table S3**). Our analysis clearly supports a clinical advantage for triple DAA-based IFN-free treatments as discussed in (*8-10*). Consistently, these three combinations achieved low RCI values, with SOF&LDV&SMV yielding the lowest RCI value ($\sim$4.83) among the tested candidates for triple-DAA IFN-free regimens (**Fig. 4b**). Thus, it was indicated that the addition of SMV to SOF&LDV ($\mathrm{RCI} = 11.55$) or SOF&DCV ($\mathrm{RCI} = 11.42$) combinations produced much preferable RCIs (4.83 for SOF&LDV&SMV and 5.24 for SOF&DCV&SMV) (see also **Table S2** and **S3**).

Calculation of risk for HCV drugs resistance emergence

A large number of HCV virions ($= 10^{12}$) is produced per day within a patient (*39*). With some DAA-combination treatments, the emergence of HCV drug resistance is one of the major causes leading to treatment failure (*25, 40*). There are at least two possible mechanisms underlying the emergence of drug resistance in DAA-combination treatments: (I) HCV variants that are resistant to a drug already exist in the HCV quasispecies before treatment and are selected to become the major population under the treatment pressure, (II) mutations that confer drug resistance are

introduced by the error-prone polymerase during HCV replication and viruses carrying these mutations expand to be the major population. To minimize the emergence or selection of drug resistant virus during treatment, multidrug combination is the key treatment strategy. Using the mutation-estimating approach developed previously (*41*), we calculated the risk of emerging drug resistance for clinically important multidrug combinations at clinical drug concentrations (**Fig. 5**).

First, we estimated the anti-HCV effect of each drug combination at their clinical concentrations by applying a drug combination theory, Bliss independence (*29, 31-33*) (see **Supplementary Fig. S5**). Bliss independence assumes that each drug acts on different targets, and is defined as follows for double-combinations:

$$f_u^{Bcom} = f_u^A \times f_u^B, \qquad (3)$$

where f_u^{Bcom}, f_u^A and f_u^B are the fractions of HCV replication events unaffected by the combined drugs A and B, single drug A and single drug B, respectively (see also **Supplementary Note2** for triple-combinations). According to our results, most of multi-drug combinations show anti-HCV activity intermediate between Loewe additivity and Bliss independence (see **Supplementary Fig. S6**, **Table S2** and **S3**). Thus, we here assumed the anti-HCV effects of drug combinations calculated by Bliss independence to be the upper limit of their effectiveness. Using Eq. (3), we determined the fractions of production events unaffected by the combined drugs A and B from that of the single drugs based on the estimated values of IC_{50} and *m*, and clinical concentrations of each drug (*42, 43*) (see **Table S4**). The fractions of unaffected production events and IIP^{Bcom} of each double-drug and triple-drug combination are shown in **Fig. 5a** and **b**, respectively. Among the current clinically relevant double DAA-combinations (SOF&SMV, DCV&SOF, DCV&ASV, and LDV&SOF), SOF&SMV at clinical concentration showed the highest IIP^{Bcom} (and lowest f_u^{Bcom}, which was followed by DCV&SOF, DCV&ASV, and LDV&SOF (**Fig. 5a**). Interestingly, the DCV&SMV combination, which is under clinical development (*44*), presents the highest IIP^{Bcom} and the lowest f_u^{Bcom} among the 15 possible combinations. This suggests that the combination of DCV&SMV is the most effective drug combination to suppress HCV production among the current choices of double-DAA combinations. Among triple DAA combinations, SOF&DCV&SMV showed further improvement in IIP^{Bcom}. This triple combination achieved the highest IIP^{Bcom} and the lowest f_u^{Bcom} among the 8 triple-combinations (**Fig. 5b**).

As previously reported in Rong *et al. (41)*, since the number of newly produced virions per day is higher than that of all possible single and double mutations, all

possible one-nucleotide and two-nucleotide mutants are predicted to be produced multiple times each day and preexist before treatment (**Fig. 5c**, **d** and **Supplementary Note 3**). In **Fig. 5c** and **d**, the Y-axis show the number of all possible one-nucleotide and two-nucleotide mutants (i.e., 2.9×10^4 and 4.1×10^8, respectively). Thus blue and red bars for "No therapy" face to the right (**Fig. 5c** and **d**). Based on the estimated antiviral activity of the above clinically major multidrug combinations at clinical concentrations, we next calculated the expected number of newly produced virions carrying one-nucleotide or two-nucleotide mutations after one day of treatment in **Fig. 5c** and **d** (see also **Supplementary Note 3**). Note that, if blue or red bar faces to the left for a drug combination, it means that the expected number of newly produced mutants is below the number of all possible mutants under the corresponding treatment, suggesting drug resistant mutants are unlikely to occur. DCV&SMV presented the lowest chance for mutant viruses to emerge, stressing an advantage of this combination. The combination of SMV&SOF shows a relatively low number of emerging mutants within the 15 considered drug combinations, which is consistent with our cell culture analyses of IIP^{com} (**Fig. 3a** and **b**). This result explains the excellent clinical performance of SMV&SOF (>90% SVR) in both treatment naïve patients and non-responders to IFN-based therapy as well as in liver transplant recipients (*45, 46*).

Notice there is still a chance of producing all possible one-nucleotide mutants after the first day of therapy for the majority of the double-drug combination treatments (**Fig. 5c**), although many of those mutants are expected to be lethal (or could not grow under the double-combination treatments) and have lower fitness than wild-type virus. In contrast, the triple-drug combinations significantly decrease the number of newly produced mutants with one-nucleotide substitutions (**Fig. 5d**). Except for SOF&LDV&ASV, the triple-combinations are likely to mitigate the risk of emerging drug resistance. For example, a possible clinical choice for triple DAA combination, SOF&LDV&SMV, showed a much lower risk of emergent mutants compared with SOF&LDV. Interestingly, treatment with any of the double-DAA and triple-DAA combinations can decrease the newly produced mutants with two-nucleotide substitutions below the level covering all the patterns of possible two-nucleotide mutants (**Fig. 5c** and **d**, red bars: left to Y-axis). Thus, these combinations effectively reduce the probabilities that two-nucleotide mutants occur coincidently during treatment, and therefore, the probabilities to generate drug resistance. However, a previous study suggests that insufficient plasma concentrations of ASV&DCV allow drug resistance to occur and lead to viral breakthrough (*47*). Furthermore, there is a

chance of generating two-nucleotide mutants by making a one-nucleotide substitution to variants that already contain a one-nucleotide substitution. We address this point further in the **Discussion**.

DISCUSSION

As a series of HCV drugs have recently been or will soon be approved for clinical use, the clinical outcome of HCV treatment has been dramatically improved. To achieve the final goal to eradicate HCV infection worldwide, it is essential to understand the characteristics of each drug and to choose the optimal drug combination based on scientific evidence. The practical choice of drug depends on many factors; side effects of the drug, the genotypes of HCV and patient, the diversity of HCV in the patient and the patient's treatment history. Among these, one of the primary and fundamental factors to be considered for treatment optimization is the magnitude of antiviral activity and the emergence of drug resistance. Until now, however, the intrinsic anti-HCV activity achieved by mono- and combination-treatments has not been systematically quantified, and the difference in characteristics of each anti-HCV drug has not been tabulated. In this study, we evaluated the anti-HCV activity in an HCV genotype 1 replicon cell culture system (**Fig. 1b**). Although some anti-HCV drugs block multiple steps including viral assembly/secretion (*48*), the primary target of all the drugs used in this study is the genome replication step, which prompted us to use the replicon system to evaluate drug effectiveness. This system supports efficient replication of genotype 1 HCV, and thus enables one to measure the intrinsic antiviral effects of any drug combination and at any concentration of the component drugs in a highly sensitive manner and with high throughput. The experimental data were analyzed by calculating the instantaneous inhibitory potential (IIP), which is the log reduction in HCV replication, caused by drugs singly and in combination at a particular concentration (*13-18*).

By profiling the anti-HCV activity of 15 clinically available and currently developmental-phase drugs (**Fig. 1a**), we found that the dose-response curve slope and thus the IIP value varied among drugs (**Fig. 2**). Interestingly, IIP values depended on the subclass of antiviral agent. TPV showing high IIP is a linear ketoamid-type PI, while all the other PIs that had relatively low IIP (DPV, ASV, and SMV) are macrocyclic PIs (*49*). Among polymerase inhibitors, SOF, a NI, and VX, an allosteric polymerase inhibitor that binds to site 2 of the thumb domain of the polymerase, showed high IIP. In contrast, all the palm domain-targeting NNIs (DSV, NSV, and TGV) had low IIP values (*50*). Agents that target NS5A (DCV and LDV) had low IIPs, but those inhibiting cyclophilins (CsA and SCY) had consistently high IIPs. Thus, IIP values tended to depend on the subclass of antiviral agent. In this study, IIP values for IFN-λ were irregularly low; it is possible that the expression level or function of IFN-λ receptors,

either IFN-λR1 or IL10R2, or both are low in the cells used in this study (*51*). The molecular basis for determining IIP value remains to be understood, but the agents that have multiple modes of action for antiviral activity, including IFN-α and CIs (IFN-α induces numerous antiviral factors; CIs inhibit multiple cyclophilins involved in HCV replication (*52, 53*)), tended to show high IIP values. High IIPs were achieved by agents including SOF and also HTAs, implying that these drugs inhibit the largest number of HCV replication events when administered at doses above their IC_{50} (**Fig. 2e** and **f**). This result adds a favorable characteristic to the already-known advantages of HTAs; pan-genotypic antiviral effect, high barrier to drug resistance, and relatively low cost (*25*). However, given the current trends in HCV therapy - the replacement of IFN-α-based regimens by all-oral, IFN-free therapies - evaluating DAA-combinations is a timely issue of debate.

Among the DAA double-combinations in this study, SOF combinations yielded desirably high IIP^{com} values. SOF is one of the strong candidates for a constituent in the future standard of care multidrug treatment (*25*). Our IIP and IIP^{com} analyses show that even a small increase in the concentration on SOF can present a dramatic gain of antiviral effect, and the potential antiviral effect of SOF combinations is much higher compared with other drug combinations that show low IIP^{com} values. In antiviral profiles of 52 double-combination treatments based on the required critical concentration index (RCI), which indicates the doses required for 95% reduction of HCV replication, high-scoring drug combinations include SOF&SMV as a double-DAA IFN-free combination better than SOF&LDV or SOF&DCV. By comparing the RCI of SOF&SMV and SOF&LDV, for example, the amount of SOF in the SOF&SMV combination is theoretically reducible by 41% (relative to the amount of SOF in the existing drug combination SOF&LDV).

Clinically, both SOF&SMV and SOF&LDV are administered for 12 weeks (*35*), while the treatment duration is generally different among drug combinations and a matter of consideration for the choice of drug; In SMV&Peg-IFN-α&RBV treatment, SMV is administered for 12 weeks while Peg-IFN-α&RBV is for 24 or 48 weeks; with SOF&Peg-IFN-α&RBV, all three drugs are administered for 12 weeks (*35*). Although these treatment durations should impact the choice of the best multidrug treatment, we have established a platform for quantifying the intrinsic drug efficacy of combinations of different DAA and HTA classes against HCV which could impact their needed amount and duration.

Triple-DAA IFN-free combinations have been under clinical development with the hope of achieving a rapid, better and universal cure of HCV, although it is not yet understood whether triple-DAA is more advantageous than double-DAA treatment, and which triple combination will give the best treatment outcome (*8, 36-38*). Here, triple-DAA treatment showed much higher IIPs and lower RCI, and the combinations of SOF&DCV&SMV and SOF&LDV&SMV showed the highest IIPs and the lowest RCI (**Fig. 4**). We also showed that an advantage of triple-DAA combinations over double-DAA treatment was that treatment with triple-DAA greatly reduced the possible emergence of mutant viruses (**Fig. 5c** and **d**, see below). Actually, even under treatments with most double-DAA combinations except for DCV&SMV and SMV&DSV, there is still a chance for one-nucleotide drug resistant mutants to emerge (**Fig. 5c**, blue bars: right to Y-axis). In contrast, triple combinations except for SOF&LDV&ASV showed an even lower probability for drug resistance to emerge with a one-nucleotide mutation. Thus, we quantified the advantage of triple DAA combinations over double DAA treatment. This may be especially important in cases where resistance-associated HCV variants pre-exist in patients. For example, protease inhibitor-resistant variants generally are seen with low frequency (0.1-3%) in untreated patients, however, the Q80K mutation in NS3, that generates weak resistance to SMV, has been observed in 9-48% of patients infected with HCV genotype 1a, but at much lower frequency in genotype 1b (*54-56*). L31M and Y93H in NS5A, conferring resistance to NS5A inhibitors, have high frequency in ~30% of treatment-naïve patients infected with HCV genotype 1b (*57, 58*). Pre-existence of these resistant variants against anti-HCV agents such as SMV, DCV, or LDV limits treatment efficacy (*47*). Our analysis showed the advantage of triple-DAA treatments over double-DAA combinations, and suggested SOF&DCV&SMV would have the highest barrier to resistance of any combinations tested.

Our experimental evidence-based mathematical analysis is useful for optimizing drug usage, as it computes drug antiviral activity at various concentrations in a preclinical setting, thereby providing basic information for designing more cost-effective drug treatments with a high barrier to drug resistance. This study used a *in vitro* model of genotype 1 HCV, the most prevalent HCV genotype worldwide. Given that the antiviral efficacy of most DAAs varies among the HCV genotypes, optimizing drug combinations that target other genotypes should be investigated in future work. Our framework is also useful for quantifying the antiviral activity of drugs and for identifying better multidrug treatments against multiple HCV genotypes.

MATERIALS AND METHODS

In this study, HCV replication was evaluated in the HCV replicon system. We used LucNeo#2 cells, which carry an HCV subgenomic replicon including open reading frames for a fusion protein of firefly luciferase–neomycin phosphotransferase and the NS3–NS5B region of an HCV of genotype 1b (strain NN) (*12, 23*). LucNeo#2 cells were seeded at 7×10^3 cells per well, incubated for 24 h, and treated with each compound at the indicated concentration. After incubation for 72 h, the cells were lysed and their luciferase activity was measured with a Luciferase Assay System according to the manufacturer's protocol (Promega, Madison, WI) (*23*). Simultaneously, cell viability was measured at 72 h post-treatment with a Cell Proliferation Kit II, XTT, as recommended by the manufacturer (Roche, Basel, Switzerland) (*59*).

In the mono-treatment study, we evaluated the intrinsic anti-HCV activity of 15 anti-HCV drugs (**Fig. 1**): direct-acting antivirals (DAAs) that directly inhibit a viral-derived factor, and host-targeting antivirals (HTAs) that inhibit HCV replication by targeting cellular factors. The DAAs included protease inhibitors [PIs: telaprevir (TPV), danoprevir (DPV), asunaprevir (ASV), and simeprevir (SMV)], nucleoside type polymerase inhibitors [NI: sofosbuvir (SOF)] and non-nucleoside type polymerase inhibitors [NNIs: VX-222 (VX), dasabuvir (DSV), nesbuvir (NSV), and tegobuvir (TGV)], and NS5A inhibitors [NS5AIs: daclatasvir (DCV) and ledipasvir (LDV)]. The HTAs comprised interferons [IFNs: IFN-α (IFN-α) and IFN-λ1 (IFN-λ)] and cyclophilin inhibitors [CIs: cyclosporin A (CsA) and SCY-635 (SCY)]. In the co-treatment experiment, we treated cells with the indicated combinations of drugs and measured their HCV replication activity as described above. We confirmed that no toxicity was observed in any of drug combination. SMV, ASV, DSV, NSV, TGV, and LDV were purchased from MedChem Express (Monmouth Junction, NJ). TRV, DPV, SOF, VX, and DCV were from Selleckchem (Houston, TX). IFN-α was obtained from MSD (Kenilworth, NJ). IFNλ was purchased from R&D systems (Minneapolis, MN). CsA was purchased from Sigma-Aldrich (St. Louis, MO), and SCY was kindly provided by Scynexis, Inc (Research Triangle Park, NC).

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AUTHOR CONTRIBUTIONS

YK, SI and KW designed the research. SN, HO and KW conducted the experiments. YK and SI carried out the computational analysis. ASP, SI and KW supervised the project. YK, YT, TW, ASP, SI and KW wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing interests.

FIGURE LEGENDS

Figure 1 | Schematics of the anti-HCV drug targets and the experimental system: **(a)** HCV life cycle and drug targets. After entry into the host cell, HCV genomic RNA is translated into viral precursor polyprotein and processed into functional proteins (C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). HCV RNA replicates inside the isolated membrane compartments derived from the endoplasmic reticulum (ER), and assembles into viral particles on the lipid droplets, which traffic through the Golgi body and are released outside of the cell. Protease inhibitor (PI: TPV, DPV, ASV, and SMV) inhibits the processing step, and drugs such as nucleoside type polymerase inhibitor (NI: SOF), non-nucleoside type polymerase inhibitor (NNI: VX, DSV, NSV, and TGV), NS5A inhibitor (NS5AI: DCV and LDV), and cyclophilin inhibitor (CI: CsA and SCY) target the replication. IFN (IFN-α and IFNλ) supposedly inhibits at least the step(s) of translation and replication. **(b)** HCV replication activity was evaluated using a HCV subgenomic replicon (genotype 1b, strain NN) carrying a fusion of the firefly luciferase gene (Luc) with the neomycin phosphotransferase (Neo^r). The replicon autonomously and persistently replicates in Huh-7 cells. Cells treated with drugs were incubated for 72 h and then harvested for luciferase assay. Inhibition of HCV replication was measured by the luciferase activity in drug-treated cells, relative to activity in DMSO-treated cells.

Figure 2 | Quantification of the instantaneous inhibitory potential (IIP) of single HCV drugs: (a) Log–Log plots of dose-response curves normalized by IC_{50}, determined from the replicon assay, of protease inhibitors (TPV, DPV, SMV, ASV: red), the nucleoside polymerase inhibitor (SOF: blue), non-nucleoside polymerase inhibitors (VX, DSV, NSV, TGV: orange), NS5A inhibitors (DCV, LDV: green), interferons (IFN-α, IFN-λ: cyan), and cyclophilin inhibitors (CsA, CSY: purple). Each point represents the mean of three experiments. **(b)** Median effect plots of the normalized dose-response curves from (a). **(c)** IC_{50} and m value for each drug, estimated by fitting Eq. (1) to the corresponding median effect plot, are grouped into drug classes or subclasses. Unit of IC_{50} is nM; exceptions are VX and DCV (pM), IFN-α (IU/ml), IFN-λ (ng/ml), CsA (μg/ml), and SCY (μM). **(d)** IIP of each drug at the indicated concentration D, calculated from the experimentally measured f_u by Eq. (2). Unit of D is nM; exceptions are VX and DCV (pM), IFN-α (IU/ml), IFN-λ (ng/ml), CsA (μg/ml), and SCY (μM). **(e)** IIP of classes or subclasses of antiviral drugs, normalized

by IC_{50}. **(f)** IIP values at drug concentration $D = 100 \times IC_{50}$ (IIP_{100}) determined by extrapolation. **(g)** The critical doses of each antiviral drug D_C for which IIP = 1.3 (corresponding to 95% inhibition of virus replication) are normalized by IC_{50} and grouped by drug class or subclass. Note that D_C/IC_{50} of IFN-λ is 7442.61.

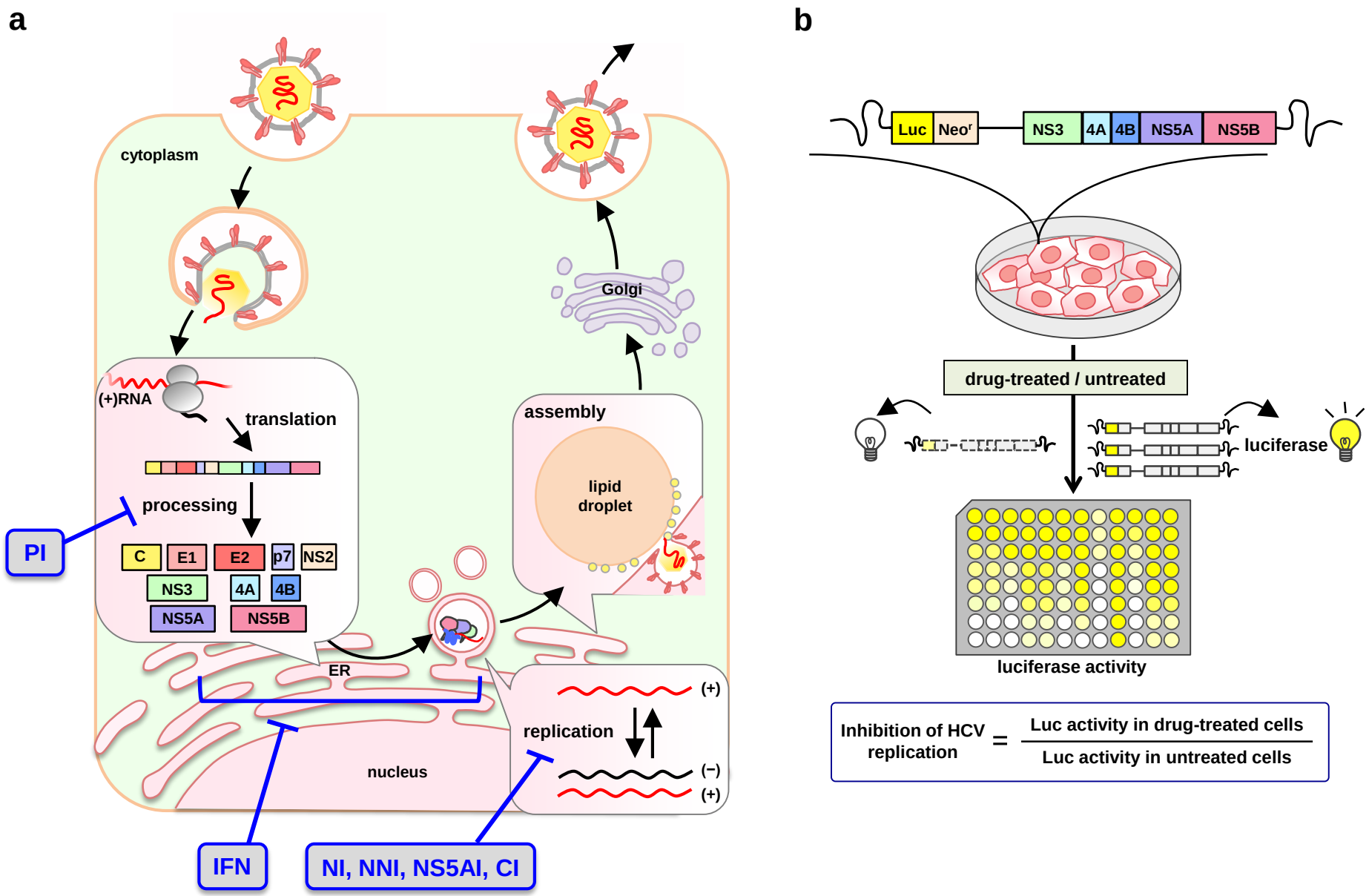
Figure 3 | Quantification of inhibitory potential of anti-HCV drug double-combinations: (a) IIP^{com} of antiviral drug double-combinations was calculated from the measured f_u^{com} by Eq. (2). 52 double-combinations of inter-class (or subclass) antiviral drugs were analyzed using the HCV replicon assay. Each point represents the mean of three experiments. Drugs were concentrated at constant ratio from their initial concentrations $D_{\mathrm{initial}} = 0.25 \times IC_{50}$, where the IC_{50} values were determined in separate single drug experiments. **(b)** Lower triangular elements show the expected combination effects based on the binding-site criterion. Upper triangular elements show the observed combination effects categorized by IIP^{com} values at the final concentration $4 \times IC_{50}$. ND, not done. **(c)** Expected critical doses $\widetilde{D_C}$ that achieve IIP^{com}=1.3, normalized by D_{initial}. The 52 drug combinations are colored by their IIP^{com} values at their final concentrations $(4 \times IC_{50})$ as in (b).

Figure 4 | Quantification of inhibitory potential of anti-HCV drug triple-combinations: (a) IIP^{com} of antiviral drug triple-combinations was calculated from the measured f_u^{com} by Eq. (2). 8 triple-combinations of antiviral drugs were analyzed using the HCV replicon assay. Each point represents the mean of three experiments. Drugs were concentrated at constant ratio from their initial concentrations $D_{\mathrm{initial}} = 0.25 \times IC_{50}$, where the IC_{50} values were determined in separate single drug experiments. **(b)** Expected critical doses $\widetilde{D_C}$ that achieve IIP^{com}=1.3, normalized by D_{initial}, for 8 triple-drug combinations.

Figure 5 | Quantification of risk of HCV drug resistance: The fraction of unaffected HCV replication events f_u^{Bcom} and the IIP^{Bcom}s of each **(a)** double-drug and **(b)** triple-drug combination at clinical concentrations. The expected number of newly produced mutants with one-nucleotide (blue) and two-nucleotide (red) substitutions after the first day of **(c)** double-drug and **(d)** triple-drug combination treatment. Each number is calculated by multiplying the number of newly produced mutants per day and the fraction of production events unaffected by a drug combination as follows: $10^{12} \times P_1 \times f_u^{com}$ and $10^{12} \times P_2 \times f_u^{com}$, where P_1 and P_2 are the probability of 1 and 2 mutations

occurring in the HCV genome after one replication event. The Y-axis show the number of all possible one-nucleotide and two-nucleotide mutants (i.e., 2.9×10^4 and 4.1×10^8, respectively).

Fig.1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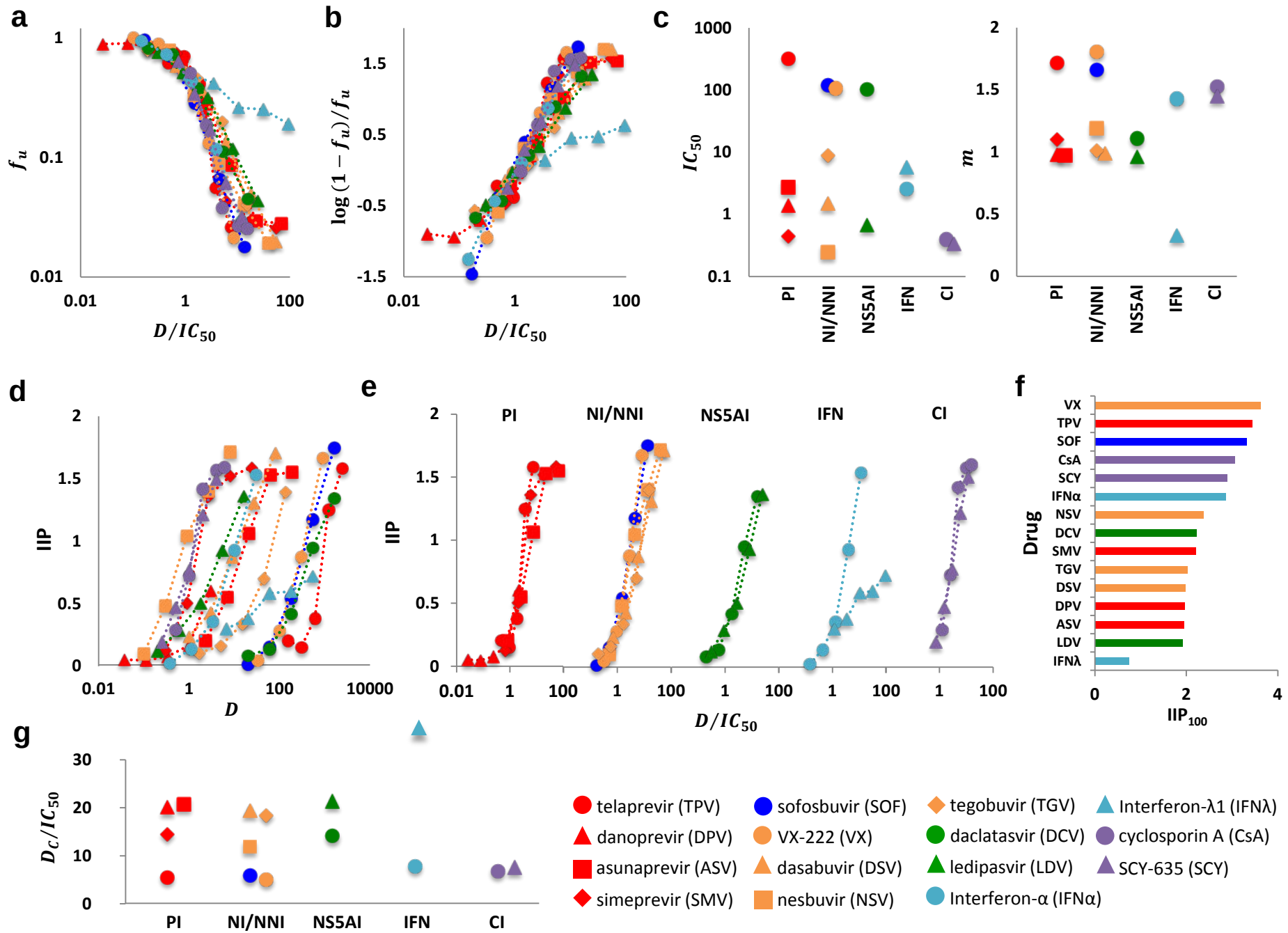
4B
NS5A
NS5B
ER
nucleus
replication
(+)
(−)
(+)
IFN
NI, NNI, NS5AI, CI
b
Luc
Neor
NS3
4A
4B
NS5A
NS5B
drug-treated / untreated
luciferase
luciferase activity
Inhibition of HCV replication = Luc activity in drug-treated cells / Luc activity in untreated cells

Fig.2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b
c
d
e
f
g
f_u
D/IC_{50}
$\log(1-f_u)/f_u$
IC_{50}
m
PI
NI/NNI
NS5AI
IFN
CI
IIP
D
Drug
VX
TPV
SOF
CsA
SCY
IFNα
NSV
DCV
SMV
TGV
DSV
DPV
ASV
LDV
IFNλ
IIP_{100}
D_C/IC_{50}
telaprevir (TPV)
sofosbuvir (SOF)
tegobuvir (TGV)
Interferon-λ1 (IFNλ)
danoprevir (DPV)
VX-222 (VX)
daclatasvir (DCV)
cyclosporin A (CsA)
asunaprevir (ASV)
dasabuvir (DSV)
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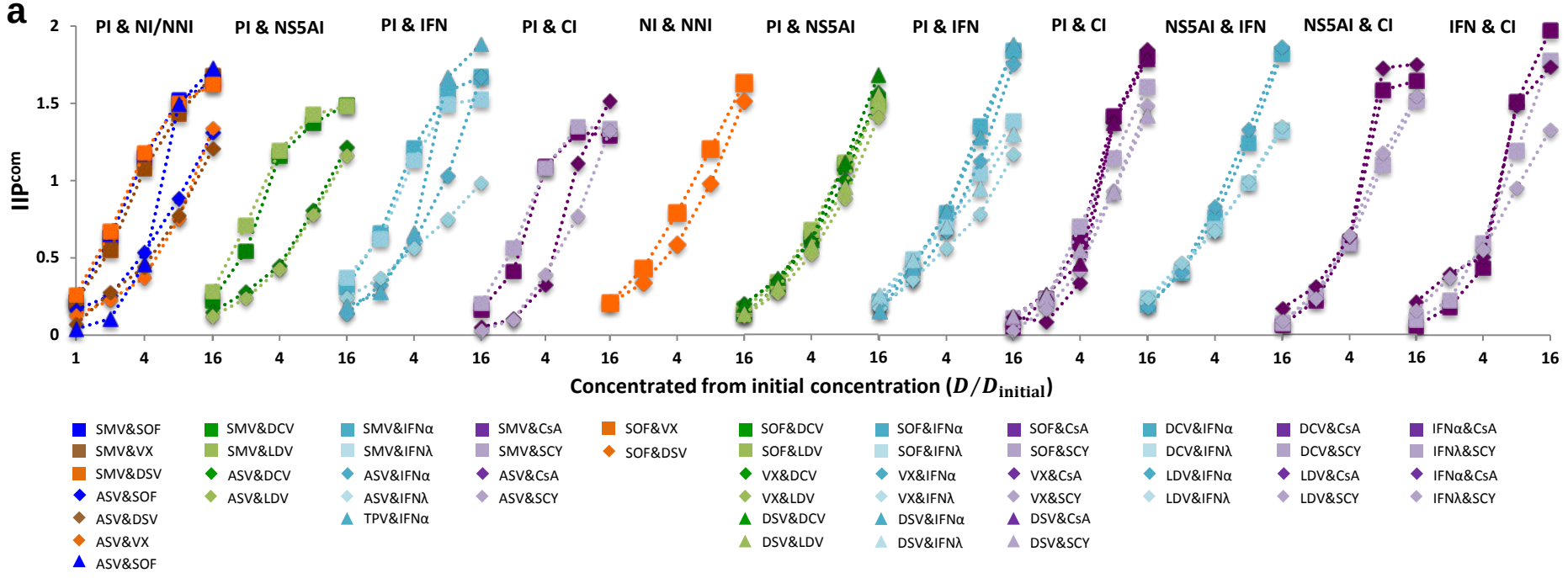
SCY-635 (SCY)
simeprevir (SMV)
nesbuvir (NSV)
Interferon-α (IFNα)

Fig.3


a
PI & NI/NNI
PI & NS5AI
PI & IFN
PI & CI
NI & NNI
PI & NS5AI
PI & IFN
PI & CI
NS5AI & IFN
NS5AI & CI
IFN & CI
IIPcom
Concentrated from initial concentration (D/Dinitial)
SMV&SOF
SMV&VX
SMV&DSV
ASV&SOF
ASV&DSV
ASV&VX
ASV&SOF
SMV&DCV
SMV&LDV
ASV&DCV
ASV&LDV
SMV&IFNα
SMV&IFNλ
ASV&IFNα
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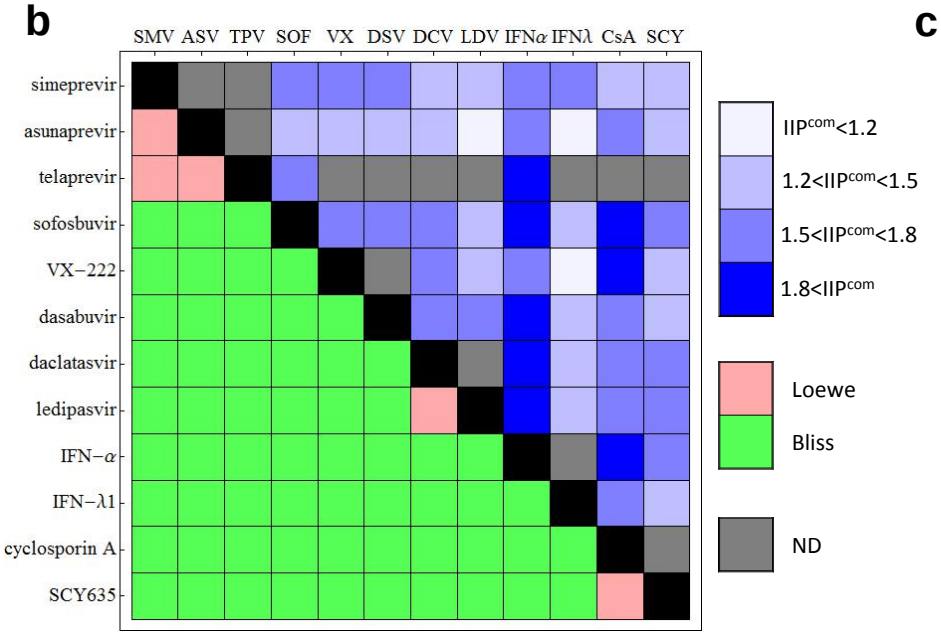
b
SMV ASV TPV SOF VX DSV DCV LDV IFNα IFNλ CsA SCY
simeprevir
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VX−222
dasabuvir
daclatasvir
ledipasvir
IFN−α
IFN−λ1
cyclosporin A
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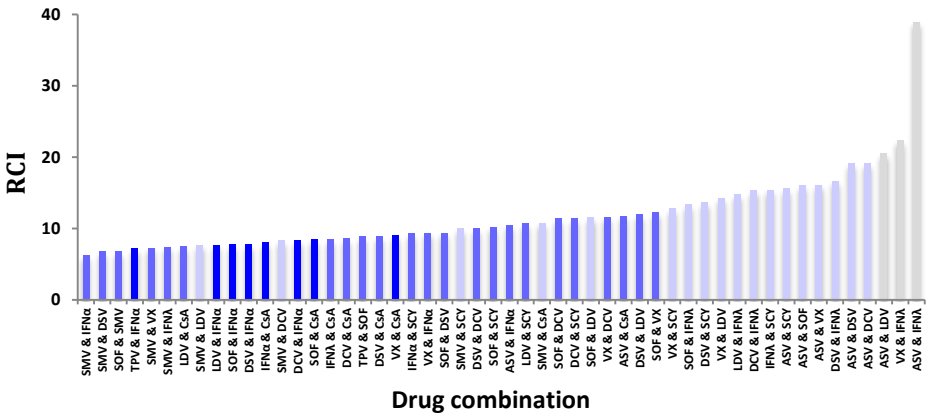
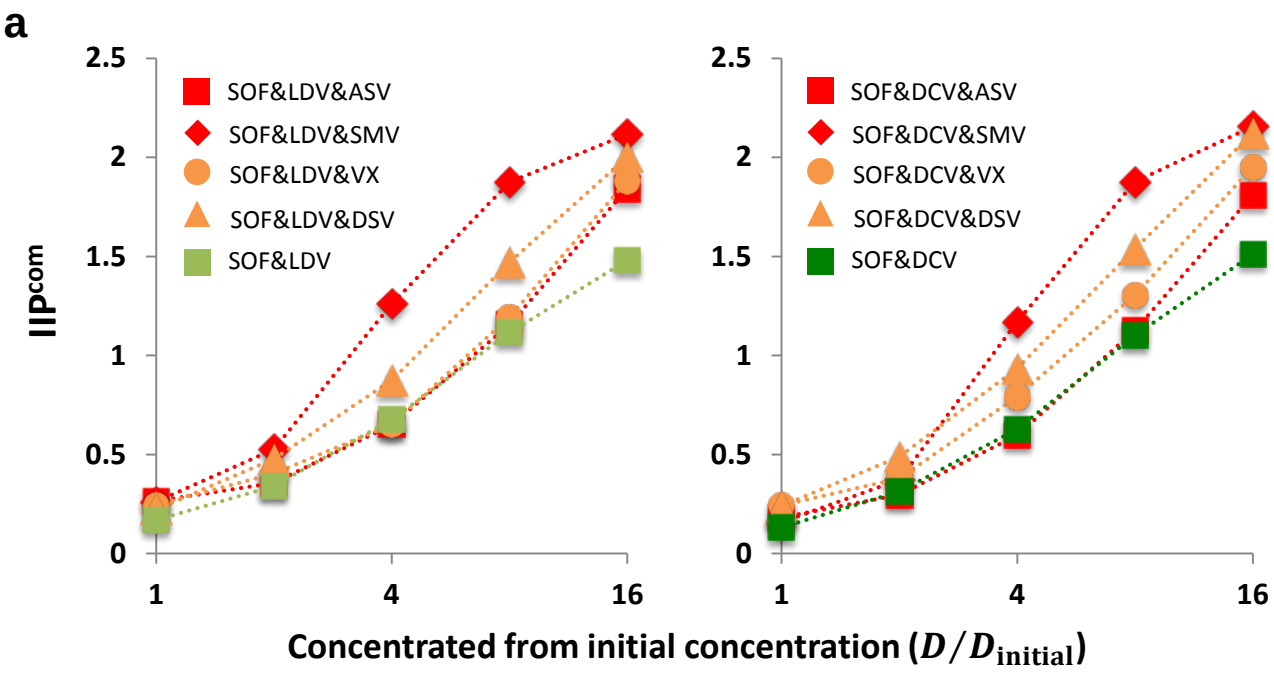

c
RCI
Drug combination

Fig.4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SOF&DCV
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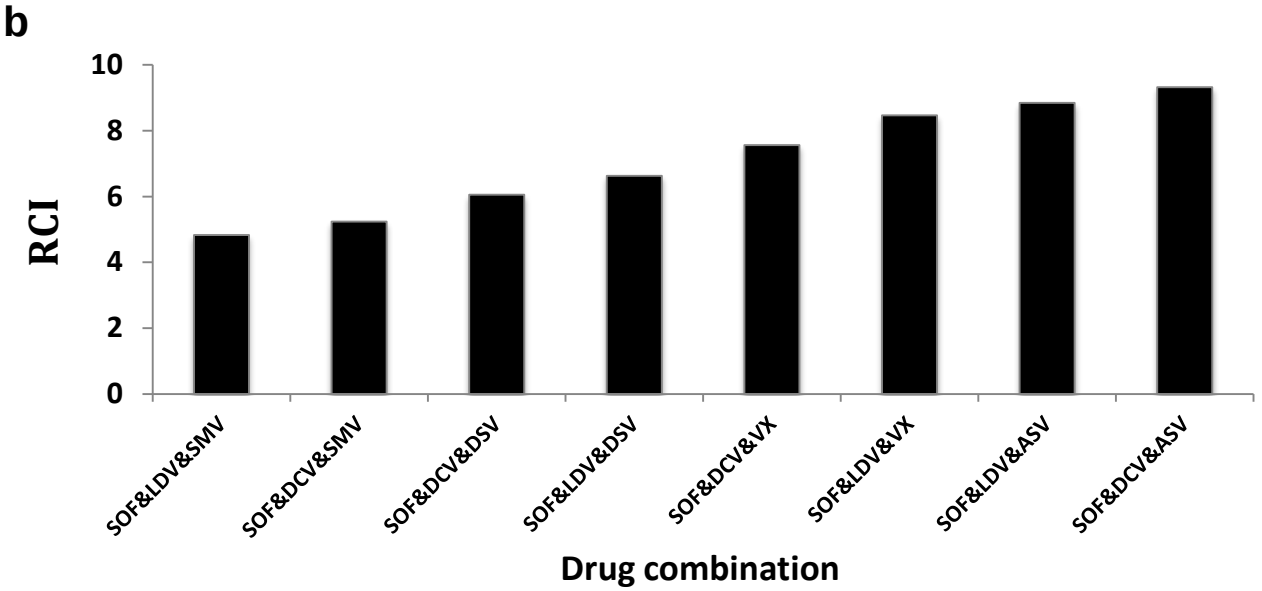
4
16
Concentrated from initial concentration (D/Dinitial)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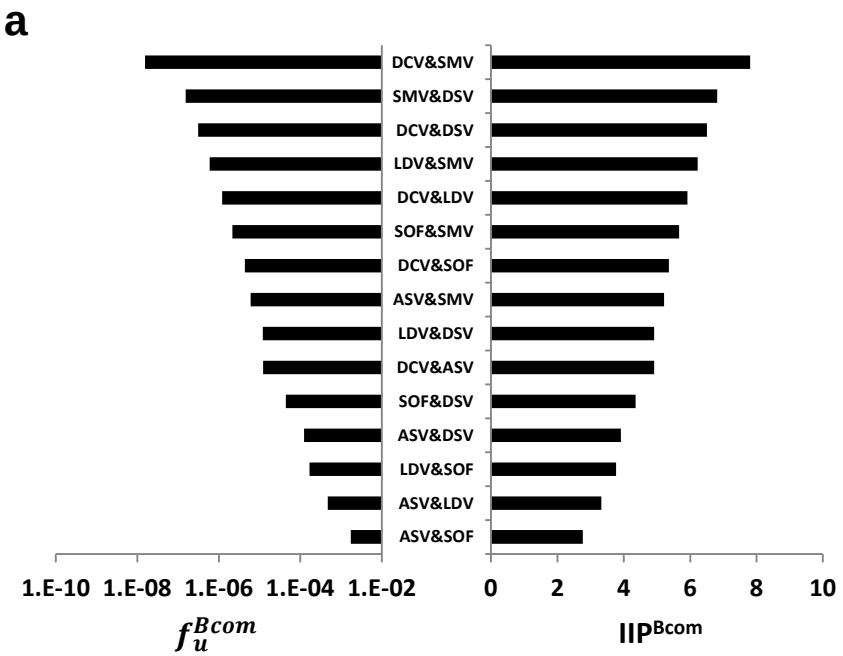
SOF&DCV&ASV
Drug combination

Fig.5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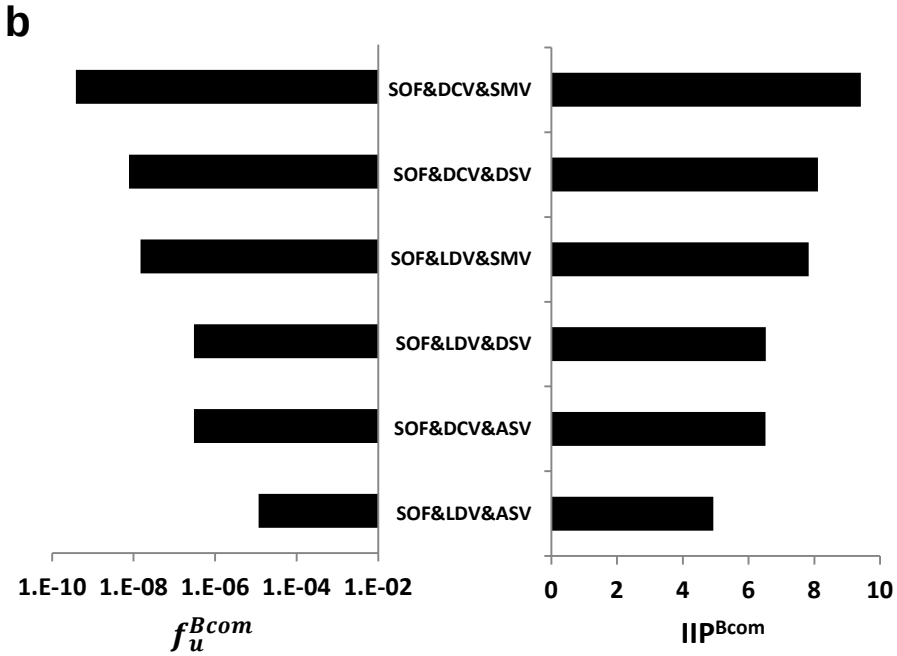
IIP^Bcom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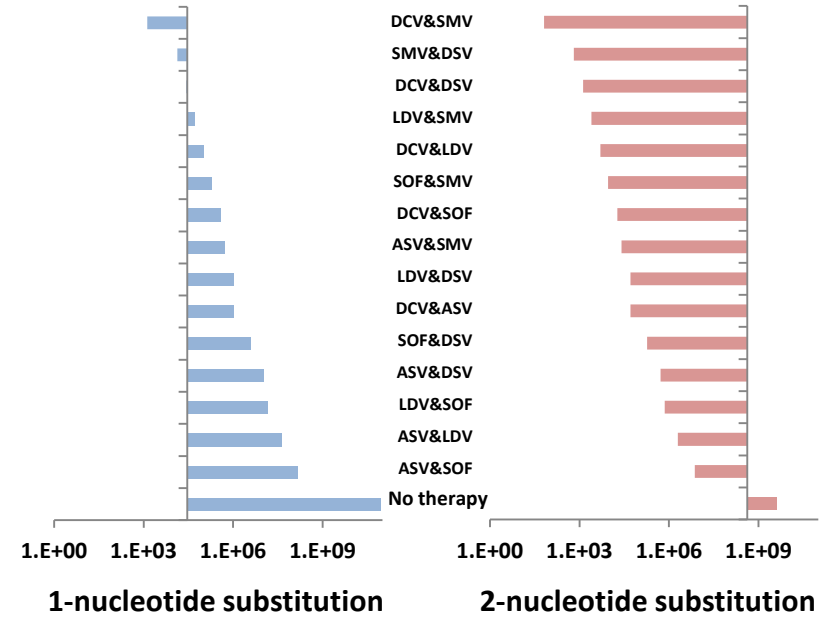
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DCV&DSV
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DCV&ASV
SOF&DSV
ASV&DSV
LDV&SOF
ASV&LDV
ASV&SOF
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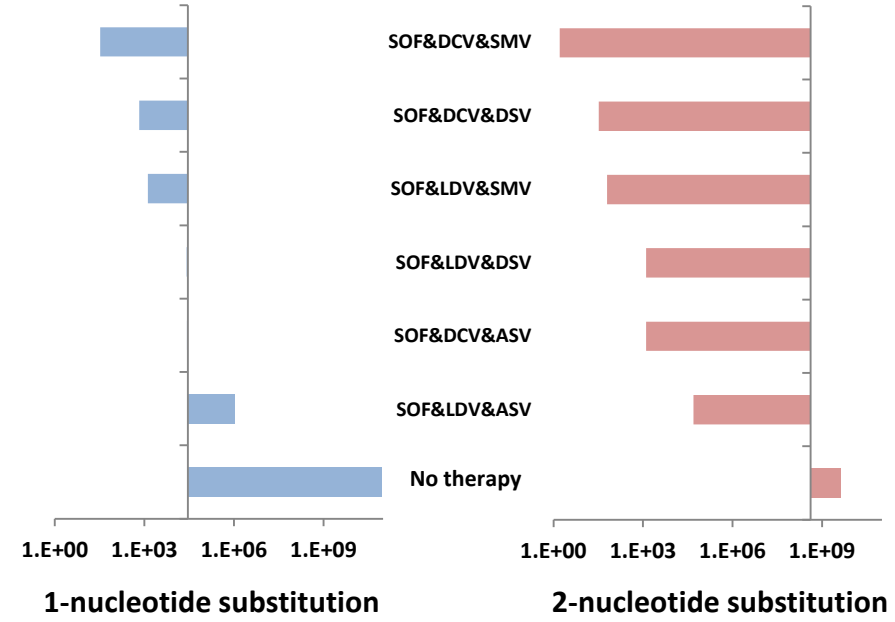
1-nucleotide substitution
1.E+00 1.E+03 1.E+06 1.E+09
2-nucleotide substitution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SOF&DCV&DSV
SOF&LDV&SMV
SOF&LDV&DSV
SOF&DCV&ASV
SOF&LDV&ASV
No therapy
1.E+00 1.E+03 1.E+06 1.E+09
1-nucleotide substitution
1.E+00 1.E+03 1.E+06 1.E+09
2-nucleotide substitution

Supplementary Information

Quantifying antiviral activity optimizes drug combinations against hepatitis C virus infection

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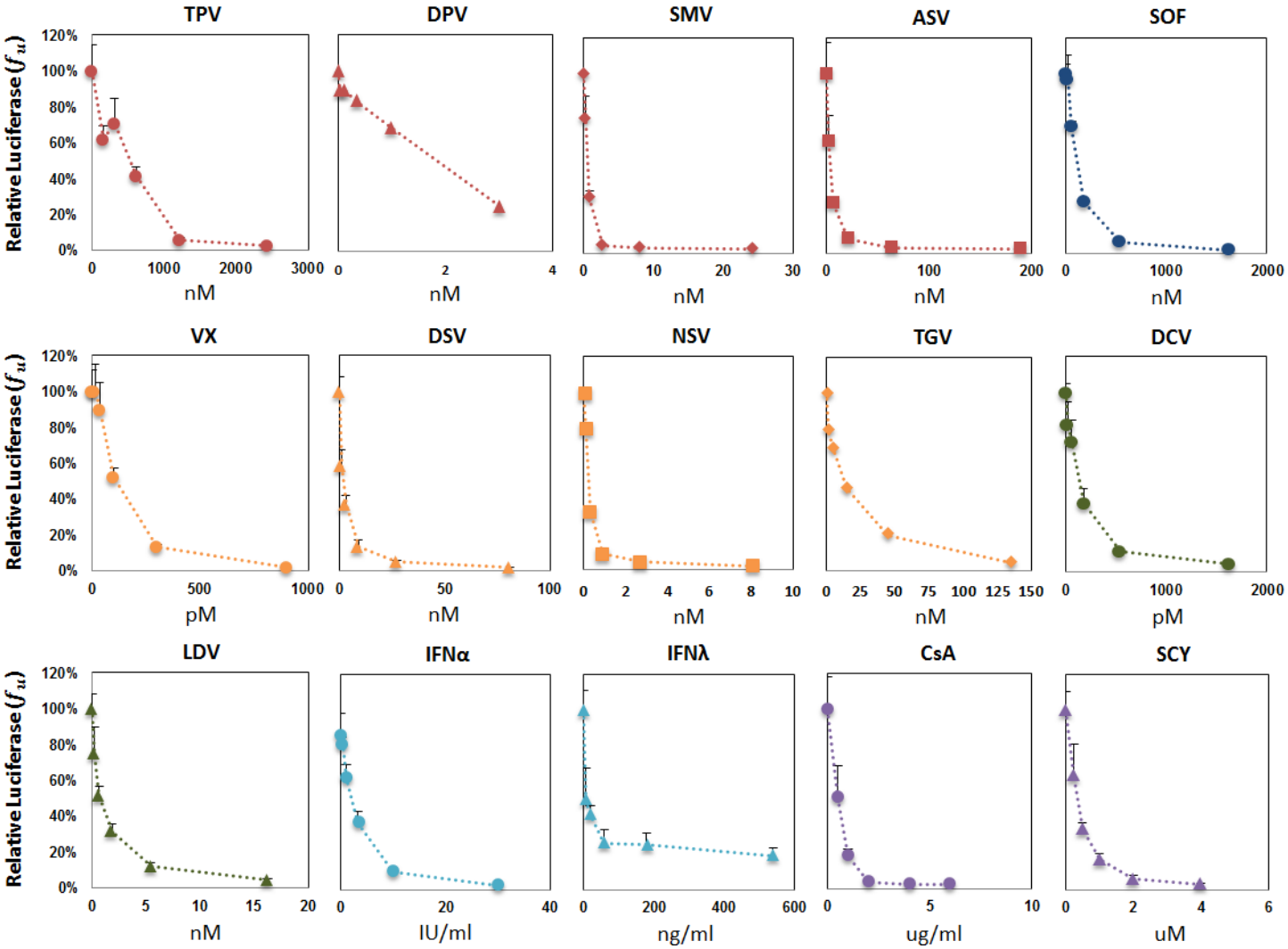

Figure S1. Dose-response curves of PIs (TPV, DPV, SMV, ASV: red), NI (SOF: blue), NNIs (VX, DSV, NSV, TGV: orange), NS5AIs (DCV, LDV: green), IFNs (IFN-α, IFN-λ: cyan), and CIs (CsA, SCY: purple), obtained by HCV replicon assay. Each point represents the mean ± standard deviation (s.d.) of three experiments.

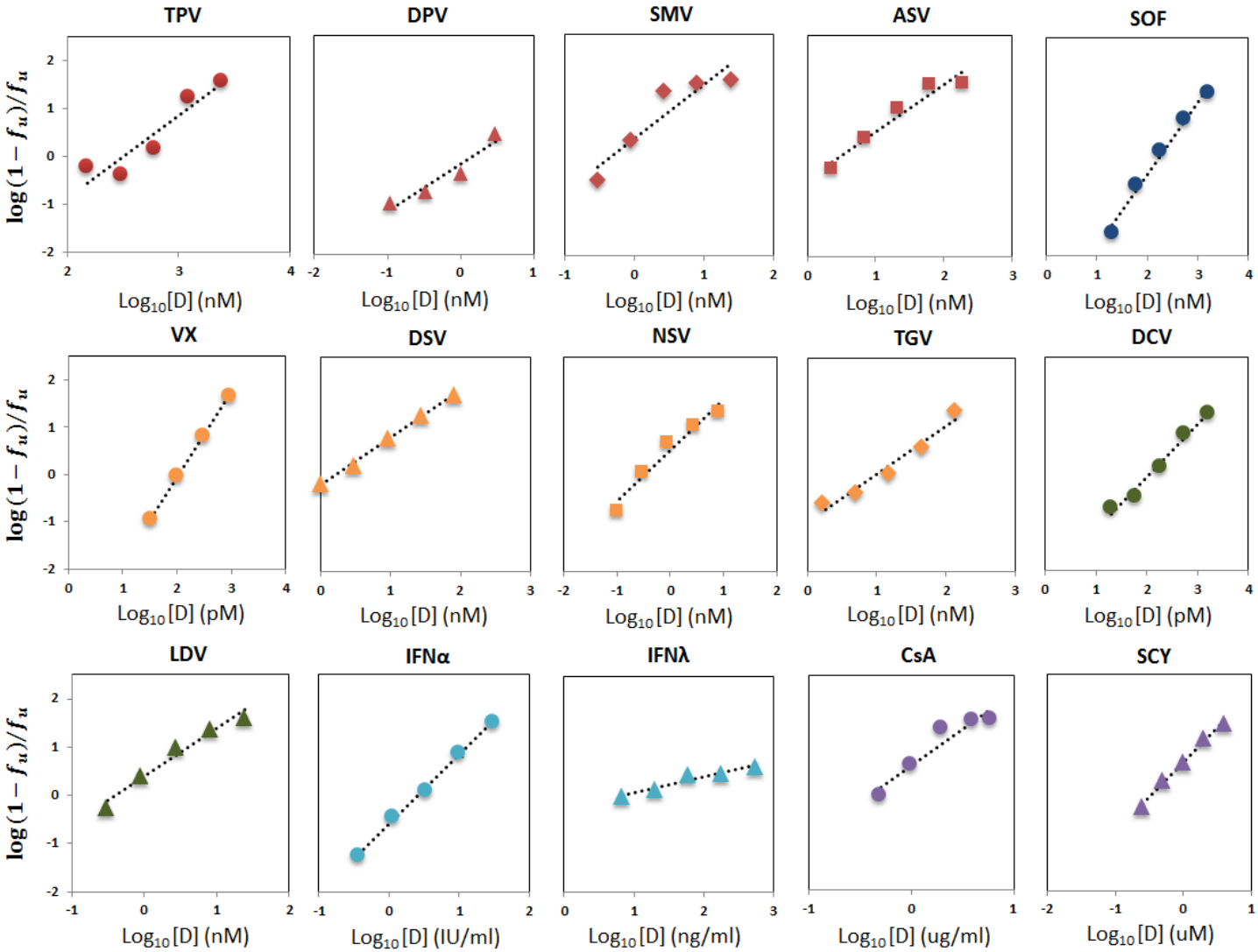

Figure S2. Median effect plots for PIs (TPV, DPV, SMV, ASV: red), NI (SOF: blue), NNIs (VX, DSV, NSV, TGV: orange),NS5AIs (DCV, LDV: green), IFNs (IFN-α, IFN-λ: cyan), and CIs (CsA, SCY: purple), obtained by HCV replicon assay. Each point represents the mean of three experiments. The dashed lines are predicted from $m \log(D/IC_{50})$ in Eq. (1) using the best-fitted parameters.

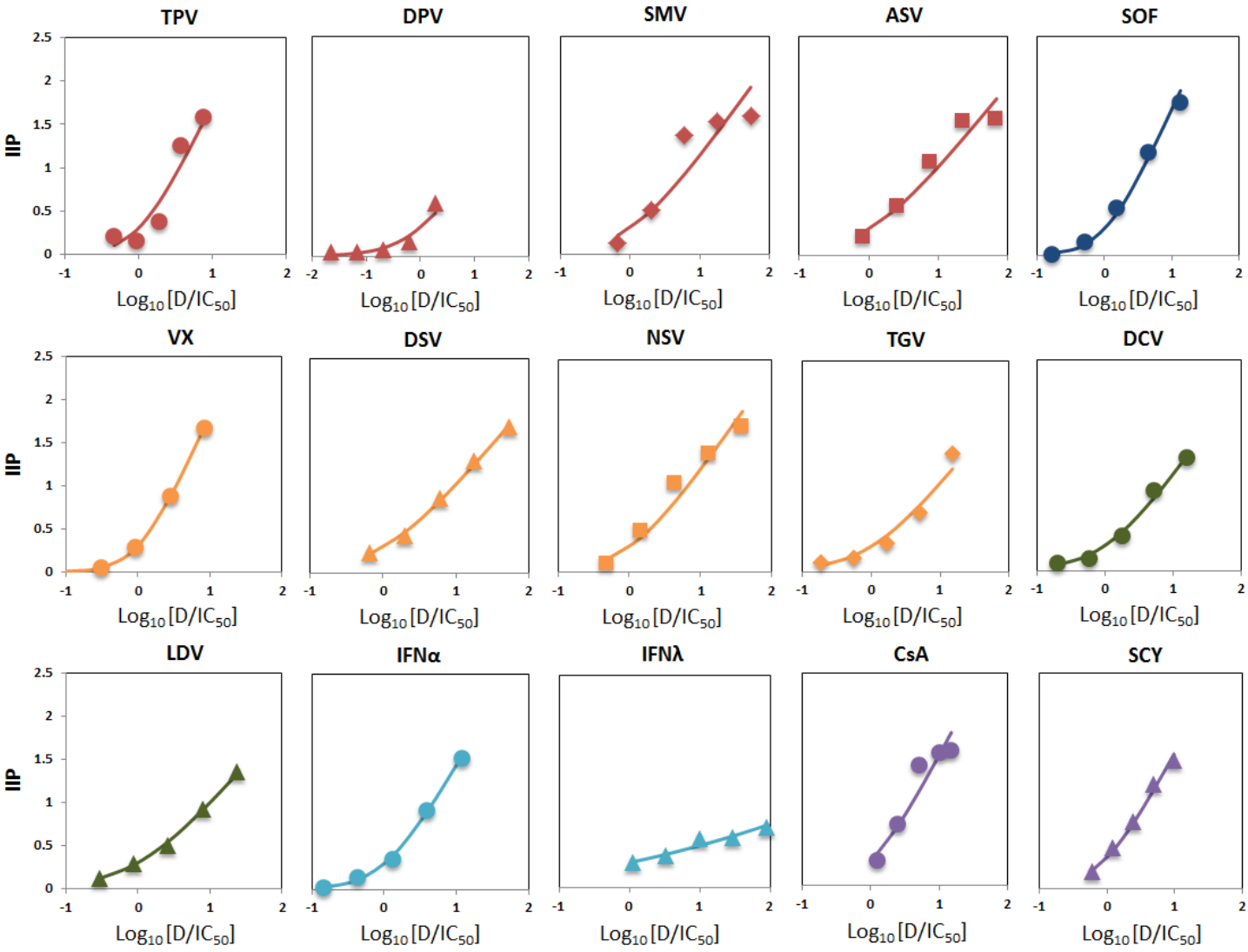

Figure S3. Instantaneous inhibitory potential (IIP) of PIs (TPV, DPV, SMV, ASV: red), NI (SOF: blue), NNIs (VX, DSV, NSV, TGV: orange), NS5AIs (DCV, LDV: green), IFNs (IFN-α, IFN-λ: cyan), and CIs (CsA, SCY: purple), obtained by HCV replicon assay. Each point represents the mean of three experiments. The solid lines are predicted from $\log[1 + (D/IC_{50})^m]$ in Eq. (2) using the parameters estimated from the median effect plots (Fig. S2).

Figure S4. Dose-response curves of the 52 double-combinations of inter-class (sub-class) antiviral drugs selected for the study, obtained by HCV replicon assay. Each point represents the mean $\pm$ s.d. of three experiments. Drugs were concentrated by constant ratios from their initial concentrations $D_{\text{initial}} = 0.25 \times IC_{50}$ to a maximum concentration of $4 \times IC_{50}$.

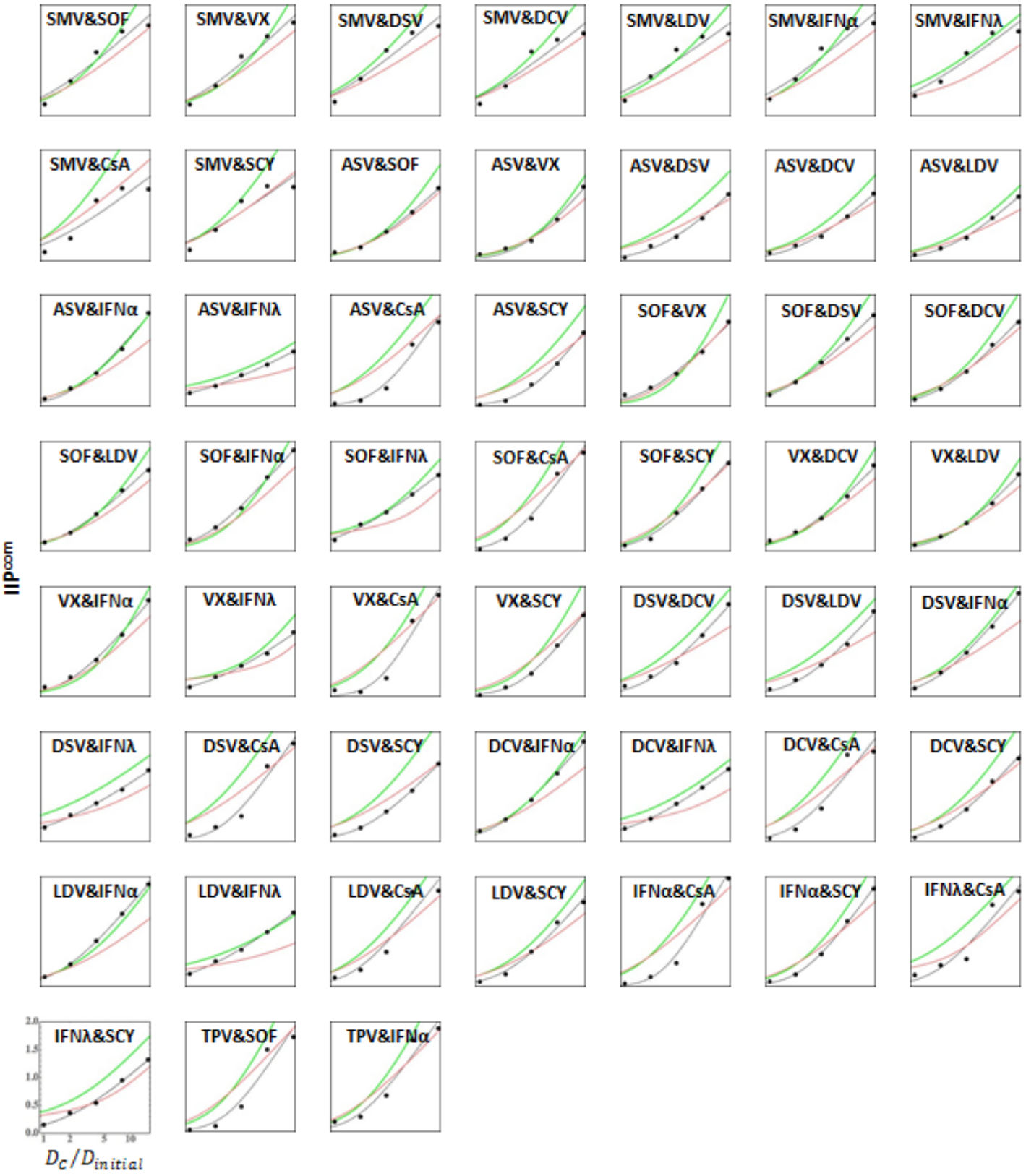

Figure S5. Instantaneous inhibitory potentials of the 52 tested drug combinations (IIP^{com}), calculated as $\log(1/f_u^{com})$ of the experimentally determined f_u^{com} values (black dots). The pink and green lines are the IIP^{com}s predicted by Loewe additivity and Bliss independence, respectively. The black lines are the theoretical predictions of $\log\{1+[(D_c/D_{initial})/IC_{50}^{com}]^{m^{com}}\}$ using the best-fitted parameters.

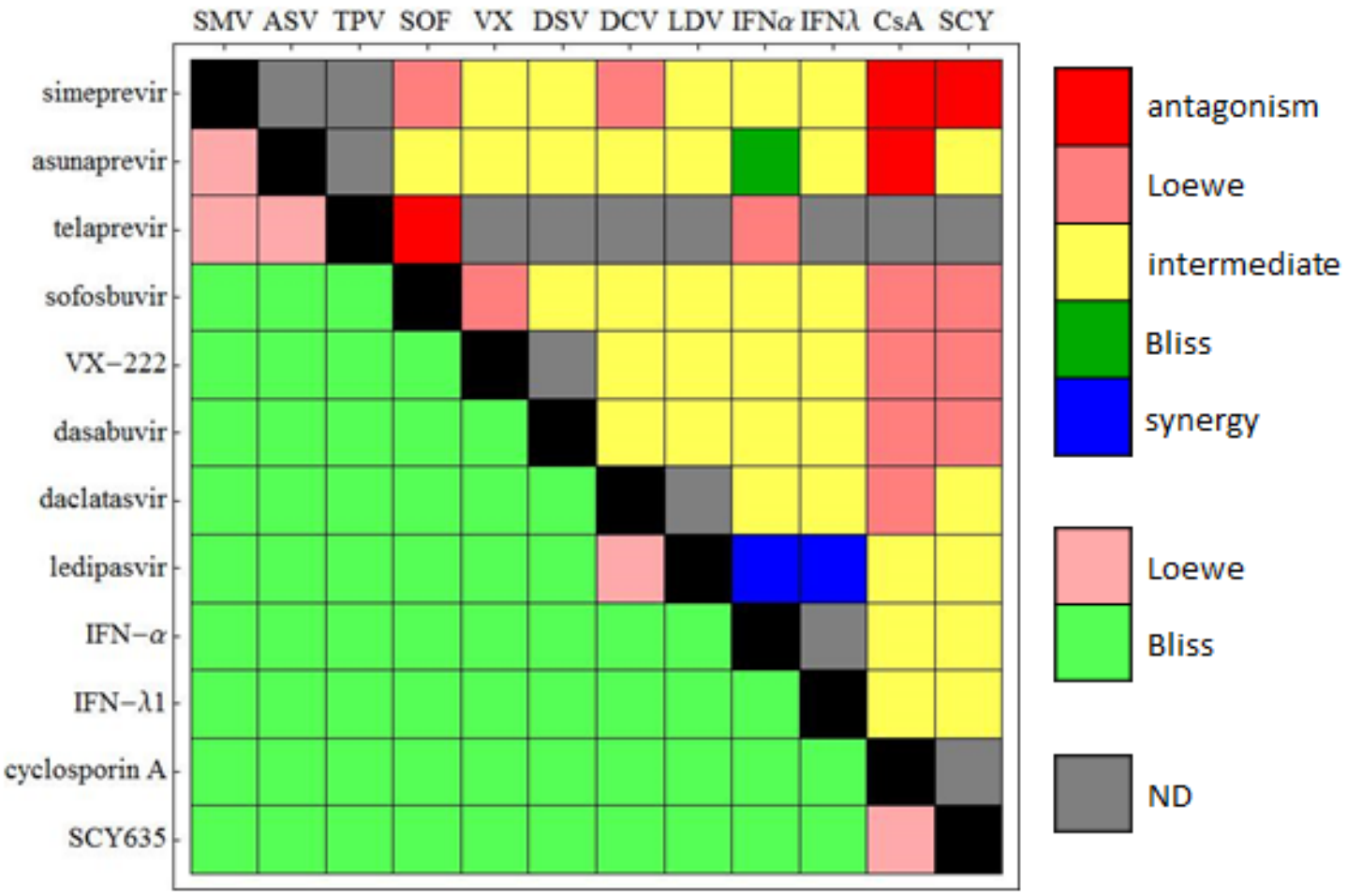

Figure S6. Lower triangular elements show the expected combination effects based on the binding-site criterion. Upper triangular elements show the observed combination effects categorized by DI values at $4 \times IC_{50}$: antagonism, DI < −0.1; Loewe, −0.1 < DI < 0.1; intermediate, 0.1 < DI < 0.9; Bliss, 0.9 < DI < 1.1; synergy, 1.1 < DI. ND, not done. Among the 52 combinations of inter-class (sub-class) antiviral drugs, 65% showed intermediate activity.

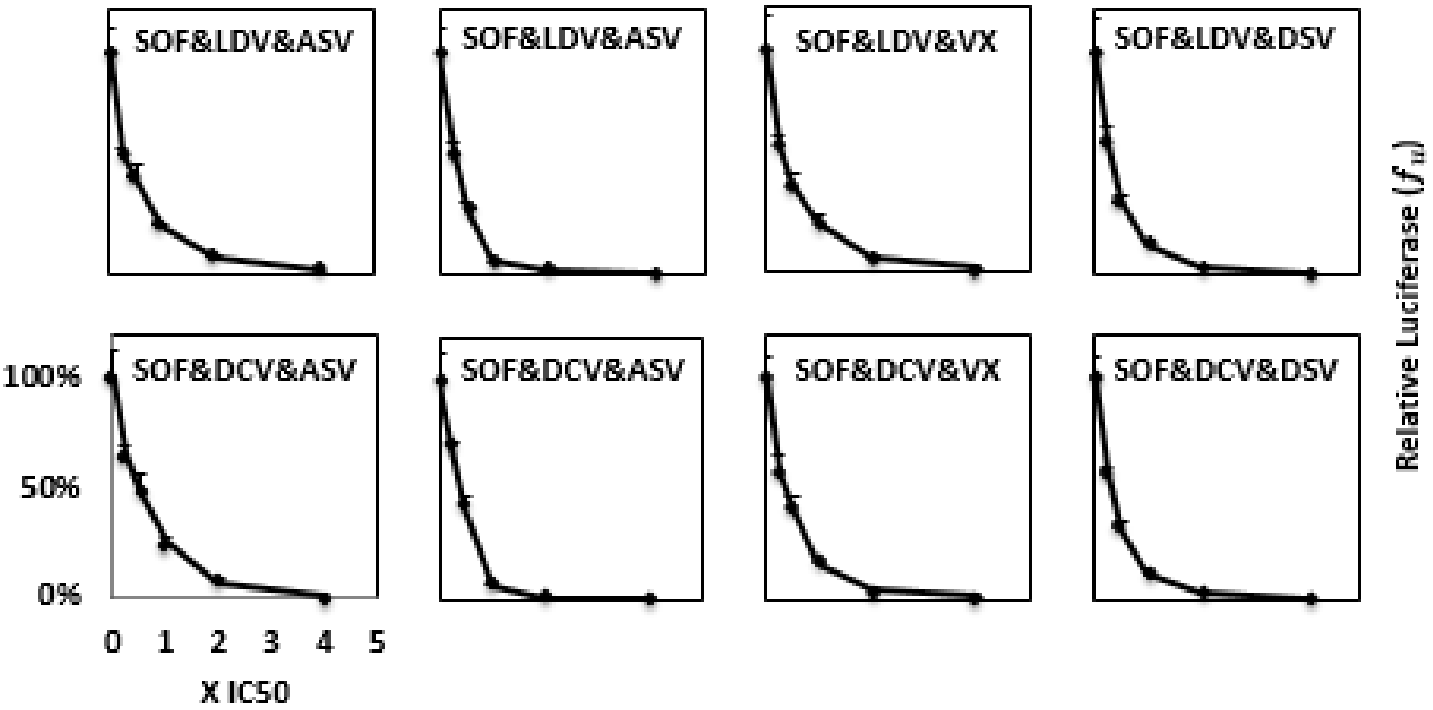

Figure S7. Dose-response curves of the 8 triple-combinations of inter-class (sub-class) antiviral drugs selected for the study, obtained by HCV replicon assay. Each point represents the mean $\pm$ s.d. of three experiments. Drugs were concentrated by constant ratios from their initial concentrations $D_{\text{initial}} = 0.25 \times IC_{50}$ to a maximum concentration of $4 \times IC_{50}$.

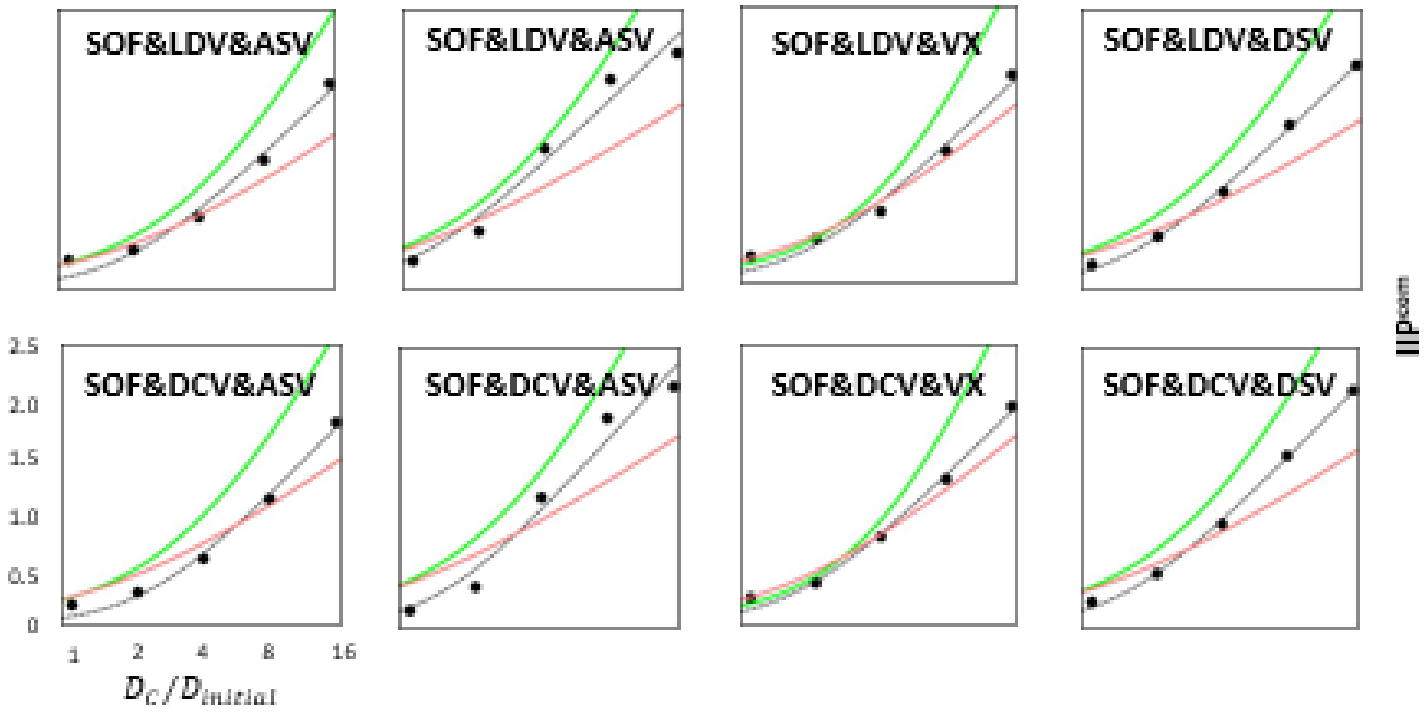

Figure S8. Instantaneous inhibitory potentials of the 8 tested drug combinations (IIP^{com}), calculated as $\log(1/f_u^{com})$ of the experimentally determined f_u^{com} values (black dots). The pink and green lines are the IIP^{com}s predicted by Loewe additivity and Bliss independence, respectively. The black lines are the theoretical predictions of $\log\{1 + [(D_c/D_{initial})/IC_{50}^{com}]^{m^{com}}\}$ using the best-fitted parameters.

Table S1 | Estimated characteristic parameters of the tested antiviral drugs

Drug (unit)	Class	IC_{50}	m	D_c
TPV (nM)	PI	323.79	1.72	1790.56
DPV (nM)	PI	1.40	0.98	28.18
SMV (nM)	PI	0.45	1.10	6.53
ASV (nM)	PI	2.75	0.97	57.09
SOF (nM)	NI	120.48	1.66	708.42
VX (pM)	NNI	107.58	1.81	546.51
DSV (nM)	NNI	1.50	0.99	29.28
NSV (nM)	NNI	0.25	1.19	2.96
TGV (nM)	NNI	8.92	1.01	164.22
DCV (pM)	NS5AI	103.84	1.11	1470.37
LDV (nM)	NS5AI	0.67	0.96	14.35
IFNα (IU/ml)	IFN	2.56	1.43	20.02
IFNλ (ng/ml)	IFN	5.80	0.33	43167.14
CsA (μg/ml)	CI	0.40	1.53	2.74
SCY (μM)	CI	0.34	1.45	2.59

Table S2 | Estimated characteristic parameters of the antiviral drug combinations

Drug combinations (Drug A and B)	IIP of final concentration	DI of final concentration	IC_{50}^{com} of combination	m^{com} of combination	$\mathrm{RCI} = \widetilde{D_C}/D_{initial}$	$\widetilde{D_C}$ of Drug A	$\widetilde{D_C}$ of Drug B
SMV & IFNα	1.671	0.192	0.689	1.337	6.224	2.302	4.979
SMV & DSV	1.620	0.218	0.712	1.306	6.774	2.506	5.893
SMV & SOF	1.631	0.074	0.808	1.377	6.843	2.532	224.4
TPV & IFNα	1.883	0.071	1.838	2.144	7.250	1225.	5.800
SMV & VX	1.678	0.253	0.960	1.453	7.275	2.691	143.3
SMV & IFNλ	1.526	0.493	0.614	1.181	7.405	2.740	44.43
LDV & CsA	1.748	0.170	1.714	1.991	7.514	1.502	1.953
SMV & LDV	1.481	0.199	0.577	1.137	7.675	2.839	1.535
LDV & IFNα	1.863	1.150	1.527	1.819	7.690	1.538	6.152
SOF & IFNα	1.841	0.529	1.463	1.766	7.739	253.8	6.191
DSV & IFNα	1.879	0.761	1.582	1.838	7.840	6.821	6.272
IFNα & CsA	1.971	0.225	2.688	2.671	8.088	6.470	2.102
SMV & DCV	1.490	0.078	0.801	1.257	8.319	3.078	326.9
DCV & IFNα	1.819	0.770	1.607	1.789	8.325	327.1	6.660
SOF & CsA	1.800	-0.048	2.196	2.183	8.451	277.2	2.197
IFNλ & CsA	1.737	0.308	1.807	1.906	8.462	50.77	2.200
DCV & CsA	1.641	-0.071	1.990	2.016	8.562	336.4	2.226
TPV & SOF	1.726	-0.191	2.568	2.365	8.909	1505.	292.2
DSV & CsA	1.785	0.099	2.380	2.228	8.910	7.751	2.316
VX & CsA	1.847	0.071	3.065	2.710	9.076	178.8	2.359
IFNα & SCY	1.779	0.373	2.346	2.135	9.303	7.442	1.209
VX & IFNα	1.754	0.632	1.884	1.830	9.395	185.0	7.516
SOF & DSV	1.633	0.326	1.375	1.529	9.407	308.5	8.184
SMV & SCY	1.334	-0.290	0.759	1.142	9.970	3.688	1.296
DSV & DCV	1.679	0.631	1.872	1.750	10.05	8.744	394.9
SOF & SCY	1.608	0.039	1.995	1.799	10.23	335.6	1.330
ASV & IFNα	1.673	1.096	2.117	1.835	10.51	5.362	8.411
LDV & SCY	1.540	0.379	2.064	1.785	10.73	2.146	1.394
SMV & CsA	1.292	-0.502	0.938	1.204	10.78	3.991	2.804
SOF & DCV	1.510	0.189	1.895	1.637	11.42	374.8	449.1
DCV & SCY	1.515	0.148	2.132	1.751	11.43	449.3	1.486
SOF & LDV	1.480	0.362	1.668	1.520	11.55	378.8	2.310
VX & DCV	1.565	0.502	1.873	1.612	11.61	228.8	456.6
ASV & CsA	1.513	-0.181	3.186	2.250	11.77	6.006	3.062
DSV & LDV	1.551	0.674	2.175	1.722	12.00	10.44	2.400
SOF & VX	1.515	0.036	1.886	1.567	12.32	404.2	242.8
VX & SCY	1.483	-0.052	3.057	2.054	12.80	252.1	1.664
SOF & IFNλ	1.388	0.527	1.237	1.236	13.34	437.8	80.09
DSV & SCY	1.419	-0.010	2.278	1.640	13.68	11.90	1.779
VX & LDV	1.405	0.499	2.358	1.632	14.29	281.5	2.858
LDV & IFNλ	1.349	1.126	1.264	1.196	14.79	2.959	88.77
DCV & IFNλ	1.323	0.724	1.307	1.196	15.29	601.1	91.77
IFNλ & SCY	1.327	0.281	1.813	1.375	15.39	92.39	2.001
ASV & SCY	1.322	0.106	3.502	1.967	15.62	7.969	2.031
ASV & SOF	1.309	0.184	2.177	1.474	16.01	8.165	525.1
ASV & VX	1.338	0.612	3.099	1.783	16.12	8.224	317.7
DSV & IFNλ	1.299	0.534	1.125	1.092	16.64	14.47	99.84
ASV & DSV	1.207	0.206	2.604	1.475	19.13	9.759	16.64
ASV & DCV	1.216	0.350	2.361	1.405	19.14	9.764	752.4
ASV & LDV	1.161	0.598	2.610	1.427	20.51	10.46	4.102
VX & IFNλ	1.169	0.461	1.756	1.156	22.35	440.4	134.1
ASV & IFNλ	0.983	0.660	1.396	0.884	38.92	19.84	233.5

Table S3 | Estimated characteristic parameters of the antiviral drug combinations

Drug combinations (Drug A, B and C)	IIP of final concentration	DI of final concentration	IC_{50}^{com} of combination	m^{com} of combination	$\mathrm{RCI} = \widetilde{D_C}/D_{initial}$	$\widetilde{D_C}$ of Drug A	$\widetilde{D_C}$ of Drug B	$\widetilde{D_C}$ of Drug C
SOF&LDV&ASV	1.843	0.457	1.848	1.879	8.843	290.1	1.769	4.510
SOF&LDV&SMV	2.117	0.336	1.025	1.899	4.827	158.3	0.965	1.786
SOF&LDV&VX	1.883	0.261	1.794	1.896	8.470	277.8	1.694	166.9
SOF&LDV&DSV	2.003	0.382	1.388	1.881	6.632	217.5	1.326	5.770
SOF&DCV&ASV	1.809	0.310	2.189	2.031	9.321	305.7	366.3	4.753
SOF&DCV&SMV	2.157	0.298	1.328	2.144	5.235	171.7	205.7	1.937
SOF&DCV&VX	1.952	0.233	1.632	1.919	7.557	247.9	297.0	148.9
SOF&DCV&DSV	2.123	0.385	1.360	1.970	6.057	198.7	238.0	5.270

Table S4 | Clinical concentrations of drugs

Drug	Concentration (nM)	References
ASV	40	(*1*)
SMV	2200	(*1*)
SOF	1100	(*1*)
DSV	900	(*2*)*
DCV	250	(*1*)
LDV	120	(*1*)

* The unit of clinical concentration in (*2*), 666ng/ml, is converted to nM.

Supplementary Note 1. Calculating the critical doses $\mathbf{RCI} = \widetilde{\boldsymbol{D_c}}/\boldsymbol{D_{initial}}$ in the multiple-drug combinations

To produce the black solid lines in **Supplementary Fig. S5** and **S8**, we fitted Eq. (S1) to the corresponding experimental data of 52 two and 8 three-drug combinations, respectively:

$$\mathrm{IIP}^{\mathrm{com}} = \log\left[1 + \left(\frac{\widetilde{D_c}/D_{initial}}{IC_{50}^{\mathrm{com}}}\right)^{m^{\mathrm{com}}}\right], \qquad (S1)$$

where IC_{50}^{com} is the normalized concentration of the combined drugs that inhibits the HCV replication by 50%, and m^{com} is the Hill coefficient. Estimated parameter values are listed in **Table S2** and **S3**. To identify RCI ($\widetilde{D_c}/D_{initial}$) for which $\mathrm{IIP}^{\mathrm{com}}$ = 1.3 (replication inhibition = 95%) in the two- and three-drug combinations, we rearranged Eq. (S1) as follows:

$$\widetilde{D_c}/D_{initial} = IC_{50}^{\mathrm{com}}\left(10^{\mathrm{IIP}^{\mathrm{com}}} - 1\right)^{\frac{1}{m^{\mathrm{com}}}}. \qquad (S2)$$

Substituting the estimated parameters IC_{50}^{com} and m^{com}, and setting $\mathrm{IIP}^{\mathrm{com}}$ = 1.3 in Eq. (S2), we calculated the critical doses of antiviral drugs, RCI ($\widetilde{D_c}/D_{initial}$), required for 95% inhibition of HCV replication (**Fig. 3c** and **4b**).

Supplementary Note 2: Anti-viral activity in multiple-drug combinations assessed by the DI index

Pharmacologists assess the combined effect of drugs by two fundamental indices; the Loewe additivity (*3*-*5*) and Bliss independence (*4*-*7*). The Loewe additivity for two (or three) drug A and B (and C) assumes that each drug affects similar targets or pathways, and is expressed as follows:

$$\frac{D_A^*}{D_A}+\frac{D_B^*}{D_B}\left(+\frac{D_C^*}{D_C}\right)=1, \qquad (S3)$$

where D_A^* and D_B^* (and D_C^*) are the concentrations of the drugs when combined, and D_A and D_B (and D_C) are the concentrations of the single drugs required to produce the antiviral activity of the combined drugs. Substituting the dose response curve $1-f_u^{com}=D_A^{m_A}/\left(D_A^{m_A}+IC_{50_A}^{m_A}\right)$ or $D_B^{m_B}/\left(D_B^{m_B}+IC_{50_B}^{m_B}\right)$ (or $D_C^{m_C}/\left(D_C^{m_C}+IC_{50_C}^{m_C}\right)$) into D_A and D_B (and D_C) in Eq. (S3), the additive effects of the drug combination are determined as follows:

$$\frac{D_A^*}{IC_{50_A}\left(\frac{1-f_u^{com}}{f_u^{com}}\right)^{\frac{1}{m_A}}}+\frac{D_B^*}{IC_{50_B}\left(\frac{1-f_u^{com}}{f_u^{com}}\right)^{\frac{1}{m_B}}}\left(+\frac{D_C^*}{IC_{50_C}\left(\frac{1-f_u^{com}}{f_u^{com}}\right)^{\frac{1}{m_C}}}\right)=1. \qquad (S4)$$

We numerically solved Eq. (S4) for f_u^{com}, and thereby predicted the additive effects of the drug combinations (see **Supplementary Fig. S5**).

Bliss independence assumes that each drug acts on different targets, and is defined as:

$$f_u^{com}=f_u^A\times f_u^B(\times f_u^C), \qquad (S5)$$

where f_u^{com}, f_u^A and f_u^B (and f_u^C) are the fractions of infection events unaffected by the combined drugs A and B (and C), single drug A and single drug B (and drug C), respectively. Using Eq. (S5), we determined the anti-viral effects of combined drugs A and B (and C), $1-f_u^{com}$, from the anti-viral effects of the single drugs (see **Supplementary Fig. S5**).

To characterize the independence of each drug in experimental data, Jilek et al. (*8*) proposed a new index called the degree of independence (DI):

$$DI=\frac{F_E-F_L}{F_B-F_L}, \qquad (S6)$$

where F_E, F_B and F_L denote the logarithmic drug effects $(\log[(1-f_u^{com})/f_u^{com}])$ of experimental data, Bliss independence and Loewe additivity, respectively. Note that this index incorporates both Bliss independence and Loewe additivity, and categorizes the experimental data of combination effects. From the DI values calculated by Eq. (S6), we assessed the anti-HCV effects of drug combinations (**Supplementary Fig. S5** and **Table S2**).

Supplementary Note 3: Emergence probability of HCV having nucleotides mutants

Each HCV RNA of 9600 nucleotides is synthesized by the NS5B polymerase with an error rate of $\sim 10^{-5}$ per copied nucleotide (*9*). According to the binomial distribution or its Poisson approximation, Rong *et al.*, estimated the probability of x mutations occurring in the HCV genome after one replication event as follows:

$$P_x = \binom{9600}{x} \times (10^{-5})^x \times (1 - 10^{-5})^{9600-x}. \quad (S6)$$

Multiplying Eq. (S6) and the total number of HCV virions produced within a patient per day at baseline viral load, ~ 10^{12} (*10*), we estimated the expected number of newly produced virions per day carrying one-nucleotide substitution, 8.7×10^{10} (i.e., $P_1 \times 10^{12}$). Similarly, the expected number of newly produced virions per day carrying two-nucleotide substitutions is calculated to be 4.2×10^9 (i.e., $P_2 \times 10^{12}$). Because mutation can change a nucleotide to any of three other nucleotides, the number of all possible one-nucleotide and two-nucleotide changed mutants is $\binom{9600}{1} \times 3^1 = 2.9 \times 10^4$ and $\binom{9600}{2} \times 3^2 = 4.1 \times 10^8$, respectively. Since the number of newly produced virions per day is higher than that of all possible mutations, all possible one-nucleotide and two-nucleotide mutants seem to be produced multiple times each day and preexist before treatment (*9, 10*) (**Fig. 5c** and **d**). In addition, based on the estimated antiviral activity of the clinically major multidrug combinations (i.e., 15 double-combinations and 6 triple-combinations) under the clinical concentrations, we calculated the expected number of newly produced virions carrying one-nucleotide or two-nucleotide mutations after one day of treatment in **Fig.5c** and **d** (i.e., $f_u^{com} \times P_1 \times 10^{12}$ and $f_u^{com} \times P_2 \times 10^{12}$, respectively).

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