Supplementary Online Material

Rapid emergence of protease inhibitor resistance in hepatitis C virus

Libin Rong[1,2], Harel Dahari[3], Ruy M. Ribeiro[1], Alan S. Perelson[1,*]

[1]Theoretical Biology and Biophysics, Los Alamos National Laboratory, Los Alamos, NM 87545

[2]Department of Mathematics and Statistics and Center for Biomedical Research, Oakland University, Rochester, MI 48309

[3]Department of Medicine, University of Illinois at Chicago, Chicago, IL 60612

*Correspondence should be addressed to A.S. Perelson (asp@lanl.gov)

Materials and Methods

Steady states of Eq. (1) and basic reproductive ratios

Considering the predominance of wild-type virus before treatment and resistance-associated loss of fitness (*S1*), the solutions of Eq. (1) in the absence of treatment ($\varepsilon_s = \varepsilon_r = 0$) converge to the steady state in which both drug-sensitive (wild-type) and drug-resistant strains coexist. The coexistence steady state is ($\overline{T}$, $\overline{I_s}$, $\overline{I_r}$, $\overline{V_s}$, $\overline{V_r}$), where

$$\overline{T} = \frac{c\delta}{(1-\mu)\beta p_s},\ \overline{V_s} = \frac{s + \rho_T \overline{T}(1 - \frac{\overline{T}+N}{T_{\max}}) - d\overline{T}}{\beta\overline{T}\frac{1-r}{1-\mu-r}(1+\frac{\rho_T\overline{T}}{\delta T_{\max}})},\ \overline{V_r} = \frac{\mu}{1-\mu-r}\overline{V_s},\ \overline{I_s} = \frac{\beta}{\delta}\overline{V_s}\,\overline{T},\ \overline{I_r} = \frac{\beta}{\delta}\overline{V_r}\,\overline{T}.$$

Here $r = R_r / R_s = p_r / p_s$ is the relative fitness of the mutant to wild-type virus. R_s and R_r are the basic reproductive ratios of drug-sensitive and drug-resistant strains, respectively, and are

given by $R_s = \beta p_s T_0 /(c\delta)$ and $R_r = \beta p_r T_0 /(c\delta)$, where

$T_0 = T_{\max}\left(\rho_T(1-N/T_{\max}) - d + \sqrt{(\rho_T(1-N/T_{\max}) - d)^2 + 4\rho_T s/T_{\max}}\right)/(2\rho_T)$ is the target cell level in the absence of viral infection. The frequency of the pre-existing mutant virus in the total virus population is $\Gamma = \overline{V_r}/(\overline{V_s} + \overline{V_r}) = \mu/(1-r)$.

Further, we can calculate that the frequency of preexisting *i*-mutants (mutants with *i* substitutions) is proportional to μ^i. Because μ is small, the frequency of *i*-mutants (for example, $i \geq 3$) is very low. In this scenario, stochastic effects may play an important role and the frequency of *i*-mutants may not obey the steady state distribution.

The basic reproductive ratio can be defined as the ratio of secondary virions produced in a host by one virion at the beginning of infection. To derive the basic reproductive ratio of drug-sensitive strain, R_s, we suppose one virion infects cells in a host with a target cell level of T_0. During its lifetime, the virion will infect $\beta T_0 \cdot \frac{1}{c}$ cells, where *1/c* is the viral lifespan. These infected cells will produce $\beta T_0 \cdot \frac{1}{c} \cdot p_s \cdot \frac{1}{\delta}$ virions, where p_s is the viral production rate and $1/\delta$ is the lifespan of infected cells. Thus, $R_s = \beta p_s T_0 /(c\delta)$ is the basic reproductive ratio of drug-sensitive strain. Similarly, we can obtain the basic reproductive ratio of drug-resistant strain, $R_r = \beta p_r T_0 /(c\delta)$.

During therapy, the reproductive ratios for the two strains are $R_s' = (1-\varepsilon_s)R_s$ and $R_r' = (1-\varepsilon_r)R_r$, where ε_s and ε_r are the drug efficacies against the drug-sensitive and drug-resistant strains, respectively.

Duration of the first-phase decline of drug-sensitive virus is longer than that of drug-resistant virus (t_s>t_r in Fig. 2B,D)

Assuming a constant target cell concentration and ignoring the term $\mu(1-\varepsilon_s)p_s I_s$, we can solve Eq. (1). The solution is $V_s(t) = C_1 e^{\lambda_1 t} + C_2 e^{\lambda_2 t}$ and $V_r(t) = C_3 e^{\lambda_3 t} + C_4 e^{\lambda_4 t}$, where

$$\lambda_{1,2} = -\frac{c+\delta \pm \sqrt{\Delta_1}}{2},\ \lambda_{3,4} = -\frac{c+\delta \pm \sqrt{\Delta_2}}{2},\ C_{1,2} = \frac{\sqrt{\Delta_1} \mp [c(1-2\varepsilon_s)+\delta]}{2\sqrt{\Delta_1}} V_s(0),$$

$$C_{3,4} = \frac{\sqrt{\Delta_2} \mp [c(1-2\theta)+\delta]}{2\sqrt{\Delta_2}} V_r(0) \text{ with } \Delta_1 = (c+\delta)^2 - 4\varepsilon_s c\delta,\ \Delta_2 = (c+\delta)^2 - 4\theta c\delta, \text{ and}$$

$$\theta = 1 - \frac{(1-\varepsilon_r)R_r}{(1-\mu)R_s}.$$

Because t_s represents the time at which the second-phase decline of wild-type virus begins, it is the time at which two curves $\log_{10}(C_1 e^{\lambda_1 t})$ and $\log_{10}(C_2 e^{\lambda_2 t})$ intersect. Thus, we have

$t_s = \frac{\ln \frac{C_1}{C_2}}{\sqrt{\Delta_1}}$. Similarly, $t_r = \frac{\ln \frac{C_3}{C_4}}{\sqrt{\Delta_2}}$. Calculating the difference between C_1/C_2 and C_3/C_4, we have

$$\frac{C_1}{C_2} - \frac{C_3}{C_4} = \frac{-c(1-2\varepsilon_s) - \delta + \sqrt{\Delta_1}}{c(1-2\varepsilon_s) + \delta + \sqrt{\Delta_1}} - \frac{-c(1-2\theta) - \delta + \sqrt{\Delta_2}}{c(1-2\theta) + \delta + \sqrt{\Delta_2}}.$$ Using the common denominator, we obtain the numerator, which can be simplified to $4c(\varepsilon_s\sqrt{\Delta_2} - \theta\sqrt{\Delta_1}) - 2(c+\delta)(\sqrt{\Delta_2} - \sqrt{\Delta_1})$.

Because drug resistant virus is more fit than wild-type virus during therapy, we have $(1-\varepsilon_s)R_s < (1-\varepsilon_r)R_r$, leading to $\theta < \varepsilon_s$. Therefore, the numerator satisfies

$$\begin{aligned}
&4c(\varepsilon_s\sqrt{\Delta_2} - \theta\sqrt{\Delta_1}) - 2(c+\delta)(\sqrt{\Delta_2} - \sqrt{\Delta_1}) \\
&> 4c(\varepsilon_s\sqrt{\Delta_2} - \varepsilon_s\sqrt{\Delta_1}) - 2(c+\delta)(\sqrt{\Delta_2} - \sqrt{\Delta_1}) \\
&= 2(\sqrt{\Delta_2} - \sqrt{\Delta_1})[2c\varepsilon_s - (c+\delta)] \\
&> 0
\end{aligned}$$

The last inequality holds because ε_s is close to 1 and $c > \delta$ (*S2*). Thus, $C_1 / C_2 > C_3 / C_4$. Also considering that $\sqrt{\Delta_2} > \sqrt{\Delta_1}$, we have $t_s > t_r$, which implies that wild-type virus undergoes a longer first-phase decline than drug-resistant virus during therapy.

Model with combination therapy of PEG-IFN-α-2a and telaprevir

We modified Eq. (1) by incorporating the effect of combination PEG-IFN-α-2a and telaprevir. Assuming that interferon lowers the viral production rate by a factor $(1-\varepsilon_{IFN})$, where ε_{IFN} is the effectiveness of interferon (*S2, S3*), Eq. (1) changes to

$$\begin{aligned}
dT/dt &= s + \rho_T T(1-\frac{T+I_s+I_r+N}{T_{\max}}) - dT - \beta V_s T - \beta V_r T \\
dI_s/dt &= \beta V_s T - \delta I_s \\
dI_r/dt &= \beta V_r T - \delta I_r \\
dV_s/dt &= (1-\mu)(1-\varepsilon_s)(1-\varepsilon_{IFN})\, p_s I_s - cV_s \\
dV_r/dt &= \mu(1-\varepsilon_s)(1-\varepsilon_{IFN})\, p_s I_s + (1-\varepsilon_r)(1-\varepsilon_{IFN})\, p_r I_r - cV_r
\end{aligned} \qquad \text{Eq. (S1)}$$

Defining $\varepsilon^s_{total} = 1-(1-\varepsilon_s)(1-\varepsilon_{IFN})$ and $\varepsilon^r_{total} = 1-(1-\varepsilon_r)(1-\varepsilon_{IFN})$, ε^s_{total} and ε^r_{total} represent the total drug effectiveness against drug-sensitive and drug-resistant virus, respectively. Because $\varepsilon_r < \varepsilon_s$, we have $\varepsilon^r_{total} < \varepsilon^s_{total}$. Notice that the modified model is the same as the original model [Eq. (1)] except that ε_s and ε_r are replaced with ε^s_{total} and ε^r_{total}, respectively.

Model with proliferation of both uninfected and infected hepatocytes. Triphasic viral declines have been observed (*S4*). Dahari et al. (*S5*) have shown that a model including proliferation of both uninfected and infected cells can account for a triphasic HCV RNA decay. We extended our two-strain model [Eq. (1)] by incorporating proliferation of both uninfected and infected hepatocytes, and obtained

$$
\begin{aligned}
dT/dt &= s + \rho_T T(1-\frac{T+I_s+I_r+N}{T_{\max}}) - dT - \beta V_s T - \beta V_r T \\
dI_s/dt &= \rho_I I_s(1-\frac{T+I_s+I_r+N}{T_{\max}}) + \beta V_s T - \delta I_s \\
dI_r/dt &= \rho_I I_r(1-\frac{T+I_s+I_r+N}{T_{\max}}) + \beta V_r T - \delta I_r \\
dV_s/dt &= (1-\mu)(1-\varepsilon_s) p_s I_s - c V_s \\
dV_r/dt &= \mu(1-\varepsilon_s) p_s I_s + (1-\varepsilon_r) p_r I_r - c V_r
\end{aligned}
\qquad \text{Eq. (S2)}
$$

Here ρ_I is the maximum proliferation rates of infected cells. The other parameters are the same as in Eq. (1). When we fit Eq. (S2) to viral load data from patients on combination therapy, we replace ε_s and ε_r with ε_{total}^s and ε_{total}^r, respectively.

We fitted Eq. (S2) to the viral load data of patient 3011 (Fig. S3). Because a triphasic decline occurs only in patients in which a majority of hepatocytes are infected before therapy (*S5*), we fixed N=0 for this patient. In fact, the model provides an excellent fit when N is chosen to be small (e.g., $<10^5$ cells/mL), relative to $T_{max}=1.3\times10^7$ cells/mL. The fit shows that the two-strain model with proliferation of both uninfected and infected cells can predict a triphasic viral decay after drug treatment. The root mean square (RMS) of the difference between data and fit is 0.19, which is smaller than the value (RMS=0.55) using Eq. (S1). Using an F-test to compare the fitting results of Eq. (S1) to those of Eq. (S2) that includes an additional parameter (ρ_I), we find that there is a statistical trend (P=0.09) to support Eq. (S2) when fitting the viral load data of patient 3011.

We also fitted Eq. (S2) to viral load data obtained from patients on telaprevir monotherapy using the same procedure as described in Fig. 3. We find that Eq. (S2) also provides good fits (not shown), but the estimated proliferation rate of infected cells, ρ_I, is very small (<0.001 day^{-1}) for these patients. This is in agreement with the observation that no triphasic decline of either drug-sensitive or drug-resistant virus was seen except for patient 3011. Therefore, to minimize

the number of parameters, we employed Eq. (1) in the main text to fit patient data, without considering proliferation of infected cells.

Average waiting time before a specific mutant is generated

The probability of having a specific *i*-mutant (a mutant with specific changes at *i* specific nucleotides simultaneously, i=1,2,3, …) is $p_i=(\mu/3)^i$, and the probability of not having these substitutions at the same time is $q_i=1-p_i$, where $\mu/3$ is the probability a nucleotide mutates to a specific one of the three possible other nucleotides. Thus, the probability that a specific *i*-mutant is generated after n virions are produced is $p_i q_i^n$ (that is, the $(n+1)$th copy has that *i*-nucleotide-change mutation, while the previous n copies do not). The expected number of virions required for that mutation to be generated is $\sum_{n=0}^{\infty} n p_i q_i^n = \frac{q_i}{p_i} = \frac{1-(\mu/3)^i}{(\mu/3)^i} \approx \frac{3^i}{\mu^i}$. Suppose M virions are produced in one day, it will take on average $3^i/(\mu^i M)$ days for that particular *i*-mutant to be generated.

Figures

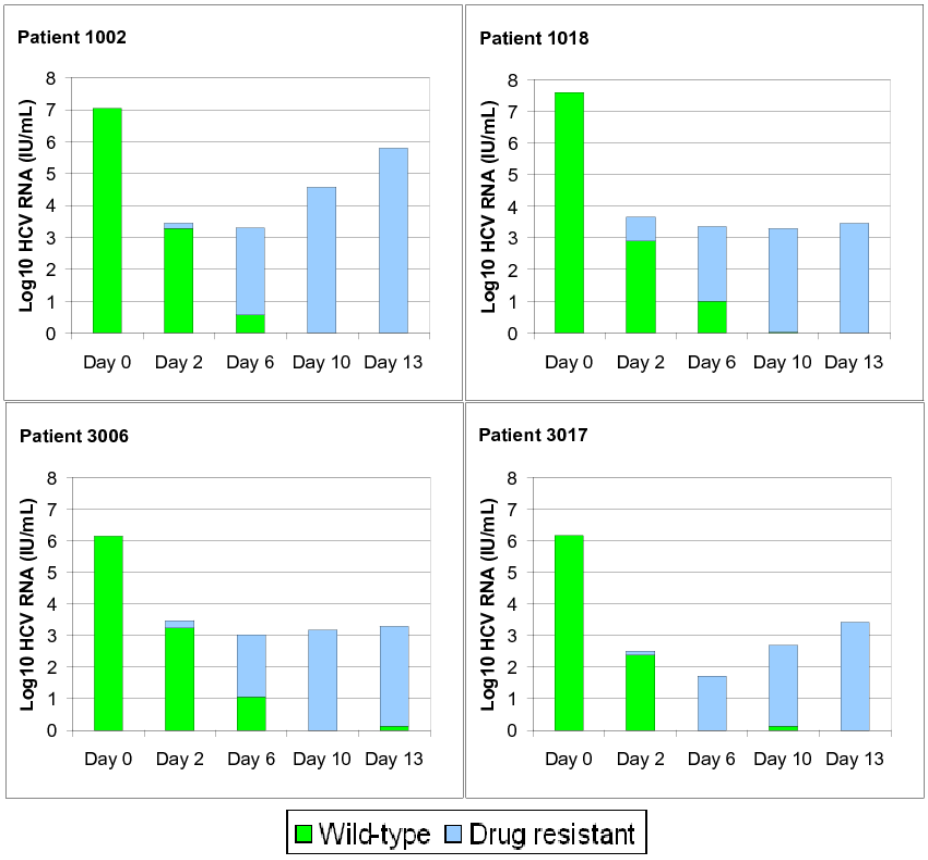

Figure S1. Drug resistance profiles during telaprevir monotherapy. We plot the plasma HCV RNA levels and their composition (drug-sensitive + drug-resistant) in four patients who received only telaprevir and had viral breakthrough during the 14-day dosing period (*S6*). All HCV variants (single and double mutants) were lumped into one drug-resistant strain. The limit of detection for the sequencing assay is 100 IU/mL and the limit of HCV RNA detection is 10 IU/mL. Sensitivity of the sequencing assay is down to ~5% with a 95% confidence interval. Note here day 0 is the time of initiation of telaprevir therapy, whereas in the original study (*S6*, *S7*) telaprevir therapy was started at day 2.

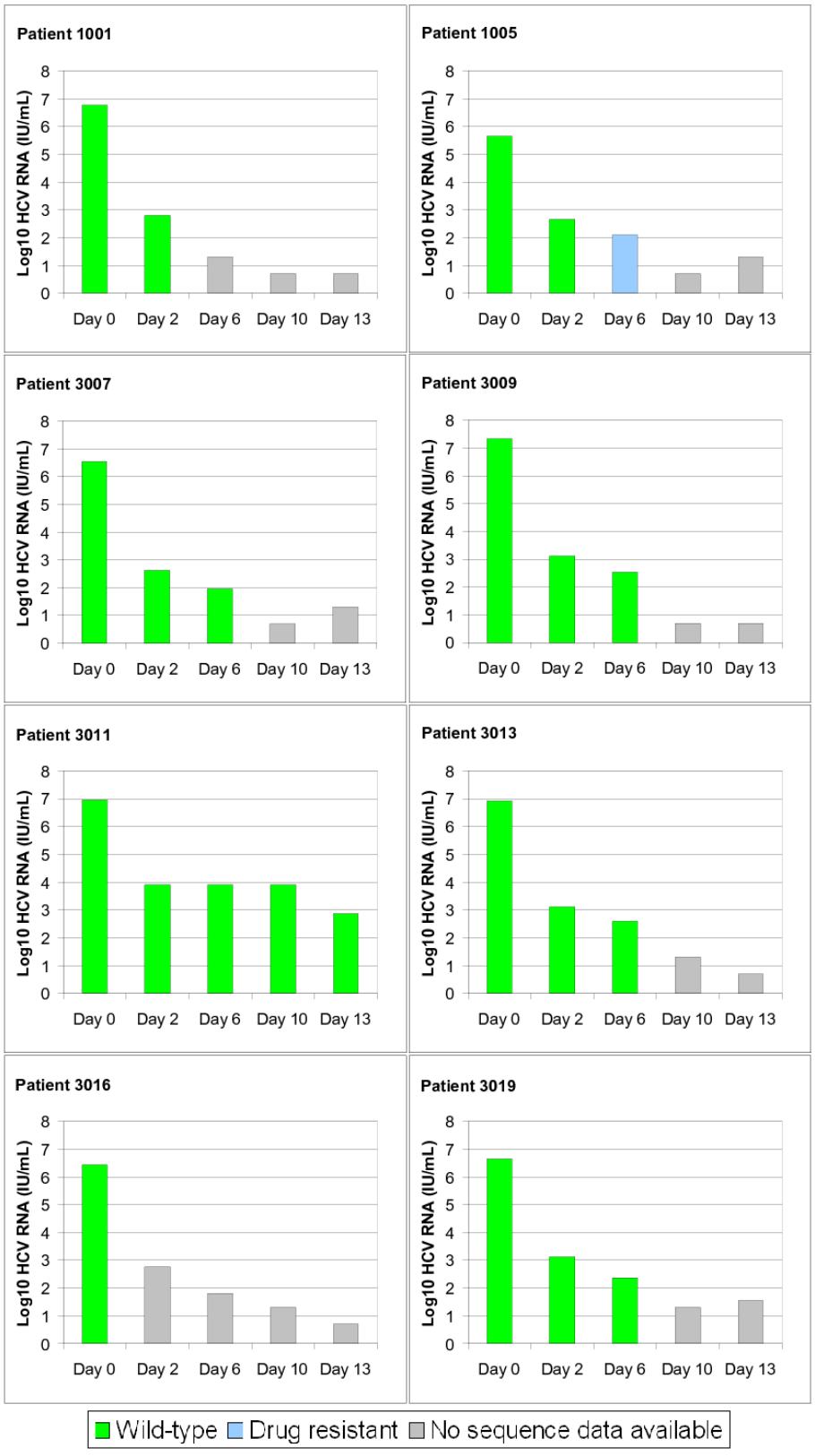
Patient 1001
Patient 1005
Patient 3007
Patient 3009
Patient 3011
Patient 3013
Patient 3016
Patient 3019
Log10 HCV RNA (IU/mL)
0
1
2
3
4
5
6
7
8
Day 0
Day 2
Day 6
Day 10
Day 13
Wild-type
Drug resistant
No sequence data available

Figure S2. Drug resistance profiles during combination therapy of telaprevir and PEG-IFN. We plot the plasma HCV RNA concentrations and their composition in patients who received both telaprevir and PEG-IFN-α-2a and had continued antiviral response during the 14-day treatment (*S6*). Note that in refs (*S6*, *S7*) day 1 denoted the initiation of PEG-IFN therapy and day 2 denoted the initiation of telaprevir therapy.

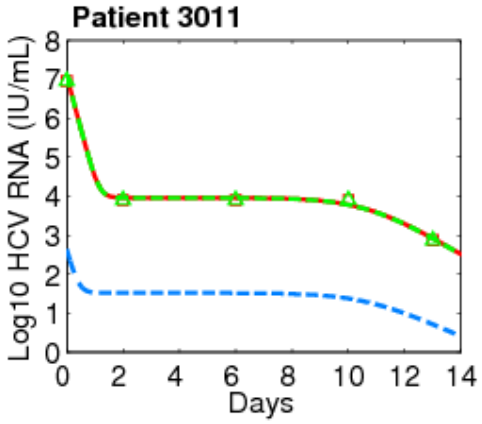

Figure S3. Comparison between predictions of Eq. (S2) and viral load data of Patient 3011. Patient 3011 received combination therapy of PEG-IFN-α-2a and telaprevir for 14 days. The symbols used are the same as those in Fig. 3. *N* was fixed to be 0 (see *Materials and Methods* in Supplementary Online Materials). The parameter values based on the fit are: $\beta = 7.94\times10^{-8}$ mL day^{-1} virion^{-1}, $\delta = 1.00$ day^{-1}, $\rho_T = 1.55$ day^{-1}, $\rho_I = 1.54$ day^{-1}, $\varepsilon_s = 0.99928$, $\varepsilon_r = 0.92$, $p_s = 17.82$ virions cell^{-1} day^{-1}. Note that in this case, ε_s and ε_r represent the effectiveness of combination therapy against drug-sensitive and drug-resistant virus, respectively.

Tables

Patient	Uninfected cells, T (10^6 cells/mL)		Cells infected with drug-sensitive virus, I_s (cells/mL)		Cells infected with drug-resistant virus, I_r (cells/mL)		Total hepatocytes, $T+I_s+I_r+N$ (cells/mL)	
	Before therapy	End of therapy	Before therapy	End of therapy	Before therapy	End of therapy	Before therapy	End of therapy
1002	1.71	6.28	1.54×10^6	2.02×10^3	20	1.10×10^5	9.75×10^6	1.29×10^7
1018	3.56	6.43	1.85×10^6	9.73×10^3	87	2.03×10^2	1.17×10^7	1.29×10^7
3006	4.29	6.44	1.34×10^6	2.16×10^3	267	2.33×10^3	1.21×10^7	1.29×10^7
3017	1.69	6.32	1.54×10^6	2.47×10^4	19	3.52×10^3	9.73×10^6	1.28×10^7

Table S1. Changes of the numbers of uninfected, infected, and total hepatocytes during 14-day telaprevir monotherapy based on data fits in Fig. 3.

Patient	Uninfected cells, T (10^6 cells/mL)		Cells infected with drug-sensitive virus, I_s (cells/mL)		Cells infected with drug-resistant virus, I_r (cells/mL)		Total hepatocytes, $T+I_s+I_r+N$ (cells/mL)	
	Before therapy	End of therapy	Before therapy	End of therapy	Before therapy	End of therapy	Before therapy	End of therapy
1001	1.54	6.23	4.59×10^5	3	17	2	8.50×10^6	1.27×10^7
1005	4.26	5.63	4.72×10^5	1.14×10^4	17	1	1.12×10^7	1.21×10^7
3007	1.34	6.30	1.31×10^6	1.93×10^4	47	12	9.15×10^6	1.28×10^7
3009	4.52	6.42	1.00×10^6	9.12×10^2	36	0.05	1.20×10^7	1.29×10^7
3011	3.91	6.10	1.48×10^6	1.42×10^5	53	7	1.19×10^7	1.27×10^7
3013	4.84	6.44	9.86×10^5	1.42×10^3	36	0.07	1.23×10^7	1.29×10^7
3016	2.27	6.37	1.31×10^6	3.43×10^3	47	4	1.01×10^7	1.29×10^7
3019	1.18	6.40	1.34×10^6	4.95×10^3	48	19	9.02×10^6	1.29×10^7

Table S2. Changes of the numbers of uninfected, infected, and total hepatocytes during 14-day combination therapy based on data fits in Fig. 4.

References

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