Understanding silibinin's modes of action against HCV using viral kinetic modeling

Jeremie Guedj[1,@], **Harel Dahari**[1,2,@], **Ralf T. Pohl**[3], **Peter Ferenci**[4], and **Alan S. Perelson**[1,*]

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

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

[3]Rottapharm Madaus, Madaus GmbH, Colonia Allee 15, Cologne 51067, Germany

[4]Internal Medicine 3, Department of Gastroenterology and Hepatology, Medical University of Vienna, Austria

Abstract

Background & Aims—Legalon® SIL (SIL) is a chemically hydrophilized version of silibinin that has exhibited high antiviral effectiveness against hepatitis C virus (HCV). Its main mode of action (MOA) remains unclear, with contradicting in-vitro studies supporting either suppression of entry and cell-to-cell spread or suppression of viral RNA synthesis as the main MOA. We sought to provide new insight into SIL's MOA in genotype-1/4 patients receiving intravenous SIL monotherapy for 7 days via mathematical modeling.

Methods—Changes in HCV RNA in 25 patients receiving 10, 15 or 20 mg/kg/day of SIL were analyzed and modeled using viral kinetic methods.

Results—In 15 patients virus declined in a biphasic manner, in which a sharp drop between days 0 and 2 was followed by a slower second phase of decline. In 10 patients the initial decline was weaker and virus declined in a single phase over the 7 day period. The blocking production effectiveness, ε, was dose dependent with mean $\varepsilon=0.49$ and 0.89 in the 10 or 15 mg/kg/day and 20 mg/kg/day dosing groups, respectively (P=0.020). The effectiveness of blocking viral infection, η, was estimated as 0.60 with no significant differences across dosing groups. For all patients the mean rate of viral-load decline measured between days 2 and 7 was high (0.3 logIU/mL/day), i.e., 4 fold higher than typically observed during the 2nd phase of (pegylated)-interferon-α±ribavirin treatment.

Conclusions—Modeling HCV kinetics in vivo suggests that SIL may block both viral infection and viral production/release with its main dose-dependent effect being blocking viral production/release.

*Corresponding author; mailing address: MS-K710, Los Alamos National Laboratory, Los Alamos, NM 87545; telephone: 505-667-6829; fax: 505-665-3493; asp@lanl.gov.
@These authors contributed equally to the study

Introduction

Chronic hepatitis C virus (HCV) infection has a worldwide prevalence of about 3% [1]. Achieving a long-term sustained virologic response (SVR), defined as undetectable serum HCV RNA 24 weeks after the end of antiviral treatment, is the most effective way to prevent disease progression permanently [2]. Treatment outcome with pegylated interferon (peg-IFN) and ribavirin (RBV) administered for 48 weeks, is correlated with HCV genotype and SVR is only achieved in approximately 50% of HCV genotype 1 patients, the most prevalent genotype in western countries [3]. Direct acting antiviral (DAA) agents constitute a new stage in HCV therapy and the combination of either of the two protease inhibitors (telaprevir or boceprevir), which recently have been approved, with peg-IFN plus RBV (PEG/RBV), should result in a dramatic improvement of SVR rates in both treatment-naive and treatment-experienced patients [4–7]. However, there is still a pressing need for alternative treatment strategies, as limitations associated with the PEG/RBV remain true (side effects and poor response) and new issues related to DAA have emerged (drug resistance).

Silymarin is a mixture of flavonolignans extracted from the milk thistle (Silybum marianum (L.) Gaertner) [8] that has been used since ancient times as a liver tonic [9]. Although the use of silymarin compounds (orally) is gaining popularity among HCV infected patients [10], the absence of well designed prospective studies on large populations precludes general conclusions on the effect of silymarin on serum transaminases, HCV RNA levels, or quality of life [9–12]. The major active component of silymarin is silibinin in a roughly 50:50 isomeric mixture of silibinin A and silibinin B. High doses of silibinin can be administered intravenously (IV) using its esterified modification silibinin-C-2′, 3-dihydrogen succinate, disodium salt (Legalon® SIL). Legalon® SIL (SIL) is currently authorized, in the name of Madaus GmbH (a company of the Rottapharm|Madaus Group), and marketed since the mid-1980s for the treatment of hepatic intoxication by Amanita phalloides mushrooms ("death cap"). Surprisingly, a strong antiviral effectiveness of SIL against HCV genotype 1 or 4 was shown in a pivotal study [13]. In the aftermath of this study, a case of SVR was reported in a patient treated with SIL monotherapy for one week, followed by PEG/RBV+SIL in the second week and continued with PEG/RBV for 15 weeks [14]. Interestingly, this patient achieved SVR in spite of having not responded to PEG/RBV previously, having the unfavorable T/T *rs12979860* IL28B, being co-infected with HIV and receiving a short (16 week) duration of PEG/RBV therapy [15].

In spite of these encouraging clinical results, SIL's mode of action (MOA) against HCV remains unknown, and results from in vitro experiments are controversial [16]. In particular, Ahmed-Belkacem et al. have experimentally shown that SIL mainly inhibits the HCV NS5B RNA-dependent RNA polymerase (RdRp) [17], while Wagoner et al. have presented results suggesting that SIL has a profound effect on HCV entry and cell-to-cell spread with only marginal suppression of HCV-RdRp activity [18, 19]. Other MOAs such as inhibition of T-cell inflammatory cytokines and hepatocyte NF-κB signaling have been suggested as well [18, 20].

While viral dynamic modeling has provided valuable insights by quantifying the effects of (peg)-IFN, ribavirin and HCV protease inhibitors and estimating its antiviral effectiveness *in vivo* (reviewed in [21]), it has not been used to analyze the effects of SIL treatment. Here, we analyzed and modeled HCV RNA kinetics from 25 patients, infected with HCV genotype 1 or 4, who were treated for 7 days with monotherapy of 10, 15 or 20 mg/kg/day of SIL. Using such an approach, we sought to provide new insights into SIL's *in vivo* mode of action.

Patients & Methods

Patients and study protocol

All patients had failed prior treatment to full dose peg-IFN-α and ribavirin therapy. Treatment failure was defined by the lack of a >2 log-drop in viral load after 12 weeks of treatment and/or by not achieving SVR. The patients' baseline characteristics are given in Table 1.

Patients received 5 (N=3), 10 (N=3), 15 (N=5), 20 (N=17) mg/kg/day SIL infused over 4 hours for 7 days as monotherapy. During this period, HCV RNA was measured daily. The study protocol can be found in [13] (see "Protocol 2"). Compared to the initial study report [13], 8 patients in the 20 mg/kg/day silibinin were added to the present study (Table 1, patients numbered ≥ 40).

Data analysis

Patients receiving 5 mg/kg/day did not show a significant antiviral effect [13] and were excluded in our analysis. Further, given the small study populations and the fact that the viral responses of the 10 and 15 mg/kg/day dosing groups were largely comparable, we analyzed these groups together (termed 10+15 mg/kg/day dosing group).

Empirical analysis of the viral decline

Let $\Delta V_{i-j} = \log(V_i) - \log(V_j)$ be the difference in log viral load between days i and j, and s_{i-j} the slope of the log viral decline between days i and j, calculated by linear regression.

HCV RNA during therapy typically declines in a biphasic manner, where a rapid drop during the first 2 days of treatment, s_{0-2}, is followed by a slower but sustained viral decline slope, s_{2-7} [21]; thus, during a typical two-phase decline the decline rate during the first phase is much larger than the second phase decline rate, i.e., $s_{0-2} \gg s_{2-7}$. However, in some patients, viral declines during SIL treatment do not exhibit two clearly separated phases but rather decline in a single phase [16]. Since viral load was only measured daily, it was not possible to accurately determine s_{0-2} and we used $\max(\Delta V_{0-1}, \Delta V_{1-2})$ as a proxy for the first phase decline slope s_{0-2}. Then we defined a viral decline as monophasic if $\max(\Delta V_{0-1}, \Delta V_{1-2}) < 2s_{2-7}$, i.e., if the two typical phases of viral decline have slopes that differ by less than a factor of 2. As shown in Figure 1 and Table 1, in this case the two slopes (s_{0-2} and s_{2-7}) cannot be distinguished empirically and only one single phase of viral decline is visible (termed monophasic).

Statistical analysis

Statistical comparisons were performed with SPSS V.18 (Chicago, IL). Continuous variables were compared using a 2-tailed Mann-Whitney U-test; to compare categorical variables we used a 2-tailed Fisher exact test. In all cases a p-value <0.05 was considered significant.

Viral kinetic modeling

The changes in viral load over time, $V(t)$, were modeled using the standard model of HCV kinetics [22], which predicts that after therapy is initiated:

$$\begin{cases} V(t) = V_0 \left(A e^{-\lambda_1(t-t_0)} + (1-A) e^{-\lambda_2(t-t_0)} \right), A = \frac{\varepsilon c - \lambda_2}{\lambda_1 - \lambda_2}, \\ \lambda_{1,2} = \frac{1}{2} \left[(c+\delta) \pm \sqrt{\left((c-\delta)^2 + 4(1-\varepsilon)(1-\eta)c\delta \right)} \right] \end{cases} , \qquad \text{Eq. (1)}$$

where V_0 is the HCV RNA level at baseline, i.e., at $t \leq t_0$, ε and η are the drug's effectiveness in blocking viral production/release and viral infection, respectively, and c and δ are the clearance rates of serum HCV RNA and HCV-infected cells, respectively, and t_0 is the time after initiation of therapy that drug starts having an effect on viral load (see supplementary material).

Parameter identifiability, parameter estimation and model selection

The absence of frequent sampling during the first two days of treatment made it impossible to accurately estimate the viral clearance rate and therefore c was fixed at 6 day^{-1}, consistent with values found in patients treated with IFN or PEG/RBV [22, 23]. The antiviral effectiveness in blocking infection, η, cannot be uniquely determined by fitting Eq. 1 to the viral load data [22] on a patient by patient basis but can be estimated using mixed-effect modeling (see supplementary material). Once parameters were estimated using Monolix 3.2© (http://software.monolix.org) [24], models were selected using the Akaike information criterion (AIC), which allows one to compare the ability of models with different numbers of parameters to fit experimental data [25]..

Results

Viral kinetic descriptive analysis

The drop in viral load levels during the first two days of treatment, ΔV_{0-2}, was significantly associated with the dosing group, with mean ΔV_{0-2}=0.73 logIU/ml vs 1.33 logIU/ml in the 10+15 mg/kg/day and in the 20 mg/kg/day dosing groups, respectively (P=0.048), and was highly variable among patients (Table 1). The subsequent slope of viral decline, s_{2-7}, corresponded to rapid viral decay in all patients and was between 0.15 logIU/ml/day and 0.65 logIU/ml/day, and was not significantly correlated with the dosing group (mean s_{2-7}=0.28 logIU/ml/day vs 0.31 logIU/ml/day in the 10+15 mg/kg/day and the 20 mg/kg/day, respectively, P=0.29). Interestingly, 40% (10/25) of the patients did not show the typical biphasic viral decline (Table 1), but rather exhibited a monophasic (see Methods) viral decline throughout the seven days of SIL monotherapy (Figure 1). This pattern was not dosing group dependent (as was seen in N=4/8 vs 6/17 patients in the 10+15 mg/kg/day and the 20 mg/kg/day dosing groups, respectively, P=0.67). Compared to patients with a biphasic viral decline, monophasic viral decline patients were associated with a significantly lower viral decline from baseline to day 2 (mean ΔV_{0-2}=0.59 logIU/ml vs 1.50 logIU/ml in the monophasic and biphasic viral decline patients, respectively, P<10^{-4}), but had a similar rate of second phase viral decline (mean s_{2-7}= 0.30 logIU/ml/day in both groups).

Viral kinetic modeling

Next, we used the standard model of HCV infection and treatment (Eq. 1) to get a better understanding of the kinetics of viral decline and to infer SIL's mode of action.

A model assuming that SIL only acts by blocking viral infection cannot reproduce a biphasic pattern of viral decline (see Methods and [22]), and therefore gave a poor description of the viral load data of patients with a biphasic viral decline (Figure 1, dotted lines in upper panels). The estimated treatment effectiveness in blocking infection was low (mean η=0.40), with no difference across dosing groups (P=0.25), and the estimate of the loss rate of infected cells was unrealistically high (mean δ=3.36 day^{-1}), partially due to the poor fit of this model to the data.

A model assuming that SIL only acts by blocking viral production/release gave a very good description of the viral load data (see reduced AIC in Table 2), which clearly establishes that SIL's main mode of action is blocking viral production/release rather than blocking viral

infection. Using this model the effectiveness in blocking viral production/release was significantly associated with the dosing regimen (mean $\varepsilon=0.69$ vs 0.91 in the 10+15 mg/kg/day and in the 20 mg/kg/day dosing groups, respectively, P=0.031). The estimated loss rate of infected cells, although lower than in the model assuming no effect in blocking viral production (mean $\delta=0.91$ day^{-1}), remained larger than previous estimates [22, 23]. The rate of viral decline between day 2 and day 7, s_{2-7}, is the net balance between the loss rate of infected cells and the rate of new cell infections. Contrary to biphasic viral decline patients, monophasic viral decline patients do not have their viral load levels rapidly fall by 1–2 logs, and thus a significant number of new cell infections can still occur. As s_{2-7} was similar in both groups, this can be either attributed to a higher δ in monophasic viral decline patients (mean $\delta=1.10$ day^{-1} vs $\delta=0.83$ day^{-1}, respectively, P=0.002), or to the fact that SIL can also block new cell infections.

Indeed assuming that SIL had an effect in blocking both viral infection and viral production/release yielded an improvement in the fit to the data (Table 2), suggesting that SIL may indeed have a significant effect in blocking infection (P=0.0016, likelihood ratio test). Further, the estimated value of δ was lower (mean 0.77 day^{-1}) with no differences between biphasic and monophasic viral decline patients (P=0.15).. A precise estimate of the distribution of the effectiveness in blocking viral infection in the sample population, η, could not be obtained and thus we assumed this parameter to be the same for all patients. The best-fit to the data was obtained with $\eta=0.60$ (CI$_{95\%}$=[0.24;0.96]). Compared to the model assuming no effectiveness in blocking viral infection (i.e., $\eta=0$), the estimates of the treatment effectiveness in blocking viral production remained largely unchanged (mean $\varepsilon=0.49$ and 0.89 in the 10+15 mg/kg/day and in the 20 mg/kg/day dosing groups, respectively, P=0.020).

Accounting for pharmacokinetic related changes in treatment effectiveness

One could argue that the use of a model that assumes constant drug effectiveness over time, such as Eq. 1, is incorrect and makes the parameter estimates unreliable. To test this, we used data from 7 patients treated with 20 mg/kg/day for whom both the viral load and the SIL A concentration were frequently measured. Using a model that allows the treatment effectiveness in blocking viral production or viral infection to change according to the drug concentration [27, 28], we could estimate the viral kinetic parameters accounting for pharmacokinetic/pharmacodynamic effects. We found that the parameter estimates arrived at using this method and the constant effectiveness model were very close for all 7 patients (see supplemental material).

Discussion

Viral kinetics during IFN based therapy and protease inhibitor therapy is typically characterized by a rapid drop in HCV RNA level during the first 2 days of treatment, followed by a slower but sustained rate of viral decline afterwards [21]. Patterns of viral kinetics during SIL monotherapy were substantially different, with 40% (N=10/25) of patients showing a single phase of viral decline, consistent with other SIL studies [14, 29]. In all patients, the rate of viral load decline measured between days 2 and 7 was high with a mean value of 0.30 log IU/mL/day. Assuming this decline would be sustained beyond day 7, this value translates into a decline of 2.1 log IU/mL/week, which is about four times more rapid than what has typically been observed with PEG/RBV in HCV genotype 1 treatment-naive patients (c.f. [30, 31]).

Using the standard model of viral kinetics, we showed that SIL's major mode of action is blocking viral production, which supports Ahmed-Belkacem et al.'s in vitro findings [17]. The effectiveness in blocking viral production/release was significantly higher in the 20 mg/

kg/day than in the 10+15 mg/kg/day dosing group (mean ε=0.89 vs 0.49, P=0.02), and was nearly as high as values found in naïve patients treated with high daily doses of IFN [22]. Assuming that SIL also blocks viral infection gave a statistically significant improvement to the model's ability to fit the patient viral load data (P=0.0016). We estimated SIL's effectiveness in blocking infection to be moderate with η=0.60 in the population sample, however this estimate should be taken with caution as the confidence interval was rather large (CI$_{95\%}$=[0.24;0.96]). Therefore our in vivo analysis supports, in part, the in vitro findings of Wagoner at al. [18, 19], which suggested SIL may have an effect on HCV entry and cell-to-cell spread, and thus may have played a role in the prevention of HCV infection after liver transplantation [32, 33]. However, this effect is moderate; using our estimated parameters and simulating the viral decline assuming SIL only blocks production/release reduces the mean viral decline at the end of the 7 day treatment period of 0.53 log$_{10}$ (mean decline of 2.15 log$_{10}$ vs 2.68 log$_{10}$).

The origin of the monophasic viral decline observed in 40% of the patients irrespective of the dose of drug received remains to be understood. It is related to having a weak first phase decline, i.e. relatively low value of ε, such that subsequent viral declines continue at approximately the same rate. We recently reported a similar monophasic decline pattern in some patients treated with mericitabine (RG7128), a potent NS5B polymerase inhibitor. For the case of mericitabine, which needs to be phosphorylated intracellularly, we showed that a monophasic decline could arise from a progressive increase in the drug effectiveness over time [34]. However accounting for the change in SIL A concentration over time did not substantially affect the parameter estimates provided by our model assuming constant effectiveness (Table S1). This supports the hypothesis that monophasic viral decline with SIL is not related to pharmacokinetic issues. However, our analysis was done only using SIL A concentrations in a limited number of patients, and further PK/PD analysis including both SIL A and SIL B need to be performed to confirm this hypothesis. Furthermore, such analyses will still be limited by the fact that drug concentrations are measured in the serum and not within liver cells.

Whatever the pattern of viral decline, all patients treated with SIL exhibited a rapid viral decline between days 2 and 7. The standard model of viral kinetics attributes this to a high loss rate of infected cells, δ, estimated here to be 0.77 day^{-1}. Interestingly, this estimate is much more rapid that what is typically found during IFN-based treatment [22, 23], but is on the order of values found with telaprevir-based therapy [35]. Recently, Guedj and Perelson [35] showed that the large second phase slopes observed during telaprevir therapy were correlated with a high effectiveness in blocking viral production (ε). This example with SIL shows that one can have a high second phase slope without having an extremely high value of ε. Thus high drug effectiveness may be sufficient to generate a high second phase slope but may not be necessary.

The origin of the high δ seen with SIL remains to be elucidated. One possibility is that the high value of δ is due to an immunomodulatory effect of SIL [9], but no increase in aminotransferase (ALT) levels, a marker of cell necrosis, was reported during the dosing period [13]. Another possibility suggested by other viral dynamics models is that the second phase of HCV RNA decline is enhanced by loss of intracellular viral RNA [36] or blocking hepatocyte proliferation [37].

In summary, viral kinetics analyses show that SIL may block both viral infection and viral production/release with its main dose-dependent effect being blocking viral production/release. Further, SIL induces a high rate of viral decline between days 2 and 7 in all patients, which is about 4-fold higher than typically observed during (peg)-IFN±RBV treatment. Together, these results suggest that SIL could be a valuable candidate for combination with

direct antiviral agents against HCV. HCV kinetic studies with SIL monotherapy that include frequent viral load sampling at the beginning of treatment along with frequent ALT measurements are needed to further understand SIL's modes of action against HCV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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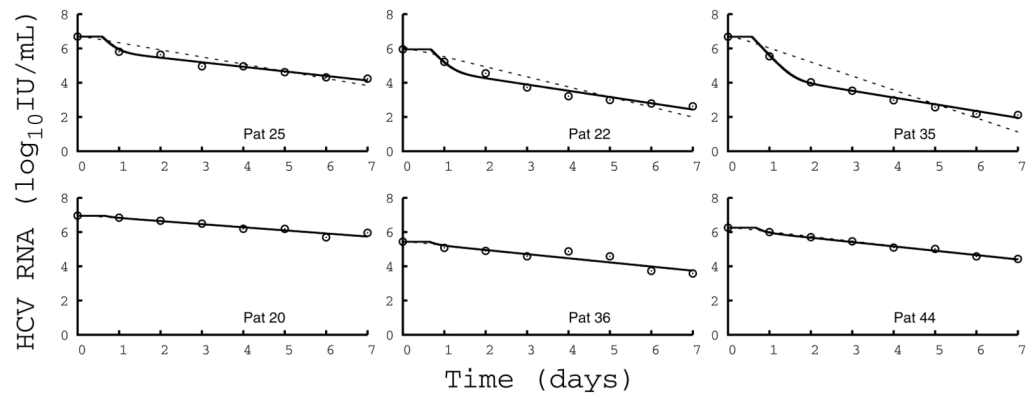
Figure 1.
Fit of the model (Eq.1) to HCV RNA kinetics. Comparison of measured HCV RNA (circle) and best-fit prediction assuming SIL blocks viral production and viral infection (solid line) or blocks only infection (dotted line) in 6 representative patients dosed at (left) 10mg/kg/day, (center) 15mg/kg/day, and (right) 20 mg/kg/day. For each dosing group, one patient with a biphasic (upper row) and one with a monophasic (lower row) decline are shown. Parameter values used to generate the fits are given in Table S1.

Table 1

Baseline characteristics and empirical viral kinetic parameters

Patient No.	Age [yr]	GT	Fibrosis stage	SIL Dose	V_0	ΔV_{0-1}	ΔV_{1-2}	ΔV_{0-2}	s_{2-7}	VK pattern[*]
25	48	1b	4	10	6.69	0.17	0.89	1.06	0.27	B
20	59	1a	4	10	6.95	0.18	0.11	0.26	0.17	M
28	56	1a	2	10	7.12	0.69	0.20	0.89	0.24	B
Mean (SD)	**54**				**6.92 (0.22)**	**0.35 (0.30)**	**0.40 (0.43)**	**0.75 (0.42)**	**0.23 (0.05)**	
21	57	1b	3	15	6.65	0.44	0.03	0.41	0.15	B
22	49	4	0	15	5.95	0.67	0.73	1.40	0.36	B
29	58	1a	2	15	6.91	0.26	0.08	0.35	0.18	M
30	67	1a	2	15	5.75	0.65	0.25	0.90	0.65	M
36	41	1b	2	15	5.43	0.18	0.36	0.54	0.27	M
Mean (SD)	**54**				**6.14 (0.62)**	**0.44 (0.22)**	**0.28 (0.28)**	**0.72 (0.44)**	**0.32 (0.20)**	
31	24	1b	2	20	6.44	1.07	1.25	2.31	0.27	B
32	33	1a	n.a.	20	6.98	1.15	1.04	2.19	0.50	B
33	48	1a	1	20	6.04	1.31	0.42	1.73	0.33	B
34	60	1b	4	20	6.50	0.57	0.39	0.96	0.18	B
35	49	1b	n.a.	20	6.68	1.52	1.14	2.66	0.40	B
26	60	1a	4	20	6.61	0.45	0.27	0.71	0.39	M
37	67	1b	1	20	6.07	0.50	0.31	0.81	0.34	M
38	68	1b	4	20	6.30	0.30	0.15	0.45	0.19	M
39	50	4	n.a.	20	5.77	0.39	0.65	1.04	0.27	B
40	69	1b	3	20	6.32	−0.32	0.53	0.21	0.19	M
41	50	1b	4	20	6.27	0.63	0.74	1.36	0.24	B
43	30	1b	2	20	5.65	0.59	0.41	1.00	0.26	B
44	55	1b	2	20	6.25	0.30	0.26	0.56	0.26	M
45	52	1a	2	20	7.66	0.30	0.75	1.06	0.35	M
46	68	4	n.a.	20	6.50	1.31	1.05	2.36	0.45	B
47	63	1	4	20	6.86	1.15	0.39	1.54	0.19	B

Patient No.	Age [yr]	GT	Fibrosis stage	SIL Dose	V_0	ΔV_{0-1}	ΔV_{1-2}	ΔV_{0-2}	s_{2-7}	VK pattern[*]
48	45	1a	n.a.	20	6.32	1.12	0.49	1.61	0.45	B
Mean (SD)	**52**				**6.42 (0.47)**	**0.73 (0.49)**	**0.60 (0.34)**	**1.33 (0.73)**	**0.31 (0.10)**	
P-value[&]	**0.93**				**0.64**	**0.09**	**0.03**	**0.04**	**0.29**	**0.67[$]**

[*] VK, viral kinetic pattern: B= biphasic and M=monophasic; s_{i-j}, HCV decline slope between days i and j (logIU/mL/day); ΔV_{i-j}, HCV decline between days i and j (logIU/mL); SIL dose (mg/kg/day); GT, HCV genotype; n.a., not available; V_0, baseline viral load (logIU/mL); SD, standard deviation;

[&] and [$]P-value to reject the null hypothesis that the distribution between 10+15 and 20 SIL dosing groups is the same (Mann-Whitney test and Fisher's exact test, respectively).

Table 2

Mean viral kinetic parameters estimated using a population approach

Model		t_0 [day]	ε		δ [day^{-1}]	η	AIC
			10+15 mg/kg/day dosing group	20 mg/kg/day dosing group			
Blocking infection only	Pop. parameters	0**	0	0	3.36	0.40*	232.7
	SE				1.30	0.072	
	Q1–Q3				2.0–5.81	--	
Blocking production only	Pop. parameters	0.68	0.69	0.91	0.91	0	26.3
	SE	0.036	0.13	0.034	0.076		
	Q1–Q3	0.65–0.73	0.43–0.81	0.72–0.98	0.78–1.03		
Blocking production and infection	Pop. parameters	0.66	0.49	0.89	0.77	0.60*	16.7
	SE	0.11	0.19	0.049	0.075	0.18	
	Q1–Q3	0.63–0.70	0.24–0.80	0.66–0.98	0.65–0.90		

*
Parameter estimated assuming no inter-individual variations; Pop. parameters, parameters estimated using mixed-effect modeling (see Methods);

**
t_0 was fixed to zero; SE, standard error; Q1–Q3: first and third quartiles of the distribution of the individual a posteriori estimates (see Supp. Materials); t_0, HCV delay before decline begins; ε, SIL effectiveness in blocking viral production/release; δ, HCV-infected cell loss rate; η, SIL effectiveness in blocking viral infection; AIC, Akaike information criterion. The model with the lowest AIC is considered the best.