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A randomized, proof-of-concept clinical trial on repurposing chlorcyclizine for the treatment of chronic hepatitis C

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ABSTRACT

Background & aims: Chlorcyclizine HCl (CCZ) is a piperazine-class antihistamine with anti-hepatitis C virus (HCV) activity *in vitro* and *in vivo*. In a first-in-humans study for HCV, we evaluated the antiviral effects and safety of CCZ ± ribavirin (RBV), characterized pharmacokinetic (PK) and viral kinetic (VK) patterns, and provide insights into CCZs mode of action against HCV.

Methods: Chronic HCV patients were randomized to CCZ (75 mg twice daily) or CCZ + weight-based RBV (1000/1200 mg daily) for 28 days. Therapy started with a loading dose of CCZ 150 mg ± RBV. Serial assessments of safety, liver tests, PK and VK markers were obtained.

Results: 24 HCV patients were treated; 54% male, median age 56 years, median HCV RNA 6.30 log IU/ml, without baseline differences between groups. At the end of therapy, subjects treated with CCZ monotherapy did not show any significant or sustained reduction in viremia ($p = 0.69$), whereas 7/12 (58%) subjects treated with CCZ + RBV had a > 3-fold decline in HCV RNA. Subjects who responded demonstrated monophasic ($n = 2$), biphasic ($n = 2$) and triphasic ($n = 3$) VK responses. Contrary to historical RBV monotherapy response, CCZ + RBV demonstrated a continued viral decline suggesting a possible synergistic effect of CCZ + RBV. Mathematical modeling predicts a median effectiveness of CCZ + RBV in blocking viral production (ε) of 59% (Interquartile range, IQR: 50%) and blocking infection (η) of 78% (IQR: 23%). Adverse events (AEs) were mild-moderate without treatment discontinuations for AEs.

Conclusions: In this human pilot study, CCZ demonstrated some anti-HCV effects, mostly in combination with RBV. More potent CCZ derivatives with optimal PK features may be more suitable for future therapeutic development. ClinicalTrials.gov number: NCT02118012.

1. Introduction

Chronic hepatitis C virus (HCV) infection affects approximately 80 million people worldwide and when left untreated can lead to cirrhosis, hepatic failure and hepatocellular carcinoma (HCC) (Polaris Observatory, 2017; Gower et al., 2014). In the United States (U.S.), hepatitis C-related liver disease remains the leading cause for liver transplantation and HCV-related HCC continues to be the fastest-growing cause of cancer related death (Liang et al., 2000; Thomas, 2013; Kim et al., 2015). Given the importance of identifying those

Abbreviations: HCV, hepatitis C virus; CCZ, chlorcyclizine HCl; HCC, hepatocellular carcinoma; U.S., United States; IFN, peginterferon; RBV, ribavirin; DAA, direct-acting antiviral; HTS, high-throughput screening; NPC, NCGC pharmaceutical collection; VK, viral kinetic; PK, pharmacokinetic; MOA, mechanism of action; RT-qPCR, reverse transcription polymerase chain reaction; NIH, National Institutes of Health; RNA, ribonucleic acid; HIV, human immunodeficiency virus; FDA, Food and Drug Administration; EOT, end of treatment; IQR, interquartile range; ALT, alanine aminotransferase; AST, aspartate aminotransferase

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infected, the U.S. Centers for Disease Control has recommended screening of all “baby boomers” for HCV with the expectation that a large number of individuals from diverse socioeconomic backgrounds will be identified for treatment (Smith et al., 2012).

With the revolutionization of HCV treatment with all-oral IFN-free regimens, today's therapies demonstrate response rates > 90%, broad genotypic activity, once daily dosing, and minimal side-effects (Panel, 2015). However, despite the significant advances in HCV therapy, it is widely acknowledged that cost remains a major barrier in treating the populations that are most affected by HCV (Lo Re et al., 2016; Callaway, 2014). Thus, there still exists a need for affordable therapy with similar attributes and efficacy to the currently available DAAs.

One strategy for achieving affordability and facilitating pharmaceutical development involves the evaluation of existing drugs for new therapeutic indications (Collins, 2011). Various studies have reported drugs used for other purposes that have antiviral activity against various viral infections (Simanjuntak et al., 2015; Boonyasuppayakorn et al., 2014; Clouser et al., 2010; Sainz et al., 2012; Rolt et al., 2018). Recently, via high-throughput screening (HTS) of pharmaceutical collections, we and others discovered that the antihistamine chlorcyclizine HCl (CCZ) has anti-HCV activity (Huang et al., 2011; He et al., 2016; Chamoun-Emanuelli et al., 2014). CCZ is a first-generation over-the-counter piperazine class antihistamine that demonstrated high anti-HCV activity *in vitro* and *in vivo* with high liver distribution, synergy with various approved anti-HCV drugs and no evidence of drug resistance (He et al., 2015, 2016). The anti-HCV mechanism of action identified in cell culture was inhibition of late-stage HCV entry while not affecting viral replication or assembly/secretion (Chamoun-Emanuelli et al., 2014; He et al., 2015).

In this proof-of-concept study, we examined the antiviral effect of CCZ (with and without ribavirin (RBV)) in patients with chronic HCV infection and evaluated its safety and tolerability. We characterized the pharmacokinetic (PK) parameters and viral kinetic (VK) patterns of CCZ ± RBV, and further used mathematical modeling to provide insights into the viral-host interactions, as well as CCZ + RBV efficacy and its mechanism of action (MOA) in humans. Finally, we performed a kinetic and modeling analysis in a previously described *in vivo* mouse model of CCZ monotherapy (He et al., 2015) and describe the similarities and differences in response patterns compared to human subjects in this study.

2. Methods

2.1. Patients

Patients ≥18 years of age with chronic HCV and quantifiable HCV RNA by reverse transcription polymerase chain reaction (RT-qPCR) in serum ≥ 10,000 IU/mL were enrolled between April 2014 and September 2016 at the National Institutes of Health (NIH) Clinical Center. Full eligibility criteria are provided in the supplementary section. All patients provided written informed consent.

2.2. Study design

In this single-center, randomized clinical trial, patients received orally administered CCZ or CCZ + RBV twice daily. Enrolled patients were randomized into one of two dosing groups which consisted of CCZ 75 mg twice daily in group 1 and CCZ 75 mg with weight-based ribavirin twice daily in group 2 (Fig. 1). At the initiation of therapy, patients received a one-time loading dose of 150 mg of CCZ with or without weight-based RBV and then followed their assigned doses for 28 days. Regardless of HCV genotype, RBV was dosed via a weight-based regimen of 1000 mg daily < 75 kg and 1200 mg daily ≥75 kg. At the conclusion of therapy, patients were followed for 3 months for post-treatment monitoring. Additional details of the study design are described in the supplementary methods section.

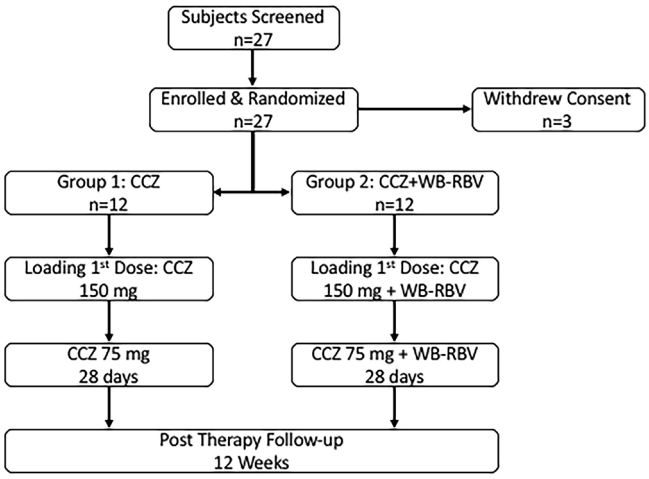

Fig. 1. Study flow diagram. Of the 27 patients screened and enrolled in this study, 24 patients were dosed. This study shows the patients in the study and the number of subjects that were dosed in the CCZ monotherapy group and the CCZ + RBV group. Abbreviations: CCZ, Chlorcyclizine; WB, weight based; RBV, ribavirin.

2.3. Study assessments and oversight

The primary therapeutic endpoint of the study was defined as a ≥3-fold decrease of HCV RNA viral titer in serum from pre-treatment levels, as measured by RT-qPCR. The primary safety endpoint of the study was the ability to tolerate the drug at the prescribed dose for the full 4-week duration. The secondary endpoints included changes in ALT levels, symptom scales, and safety questionnaire responses. At the conclusion of the study, measurements of plasma CCZ and nor-CCZ concentration was performed using liquid chromatography-mass spectrometry. The study was registered in ClinicalTrials.gov (#NCT02118012) and all study materials were approved by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) institutional review board at the NIH Clinical Center. All authors had access to the study data and reviewed and approved the final manuscript.

2.4. Safety

Safety was assessed at each time-point based on laboratory analysis and study participant reports. Monitoring of adverse events was performed from the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 with modification for patients with liver disease. No dosing reduction was planned in this study.

2.5. Statistical analysis

In each group, analysis of baseline and end of therapy HCV RNA and laboratory markers was performed via paired Student's t-test. Comparison of baseline continuous variables between groups were analyzed with a Mann-Whitney test. All statistical tests were two-sided and a p value of less than 0.05 was considered to be statistically significant. All statistical analysis was performed with SAS 9.3 (SAS, Inc., Cary, NC), GraphPad Prism V6.0 (GraphPad Software, Inc., La Jolla, CA) or IBM SPSS (Version 23).

2.6. Kinetic analysis and mathematical modeling in patients

Serum HCV kinetic data was analyzed from group 1 and 2 patients who received therapy for 28 days. Mathematical modeling (Supplemental Eq. S(1)) was used to assess the dynamics of HCV infection in CCZ + RBV treated patients, based on a previous model (Dahari et al., 2007), with further details provided in the

Table 1
Demographic and clinical characteristics of the subjects at baseline.

Patient Demographics	All (N = 24)	Group 1 (N = 12)	Group 2 (N = 12)	P Value
Male Sex – no. (%)	13 (54)	7 (58)	6 (50)	1.00
Median Age – Yr (IQR)	56 (50–60)	57 (54–61)	56 (47–60)	0.27
Race – no. (%)				0.17
Caucasian	14 (58)	7 (58)	7 (58)	
Asian	5 (21)	1 (8)	4 (34)	
Black	5 (21)	4 (34)	1 (8)	
HCV Genotype				
1A/1B/2/3/4/6	11/7/1/3/1/1	4/4/1/2/1/0	7/3/0/1/0/1	0.68
Clinical Characteristics Median (IQR)				
Alkaline Phosphatase U/L	77 (61–93)	74 (61–93)	78 (63–86)	0.92
Alanine Aminotransferase U/L	53 (34–88)	50 (36–106)	58 (34–80)	0.86
Aspartate Aminotransferase U/L	50 (34–71)	52 (35–88)	48 (31–62)	0.41
Total Bilirubin mg/dl	0.4 (0.4–0.6)	0.6 (0.4–0.8)	0.4 (0.4–0.4)	0.07
Platelets 10^3/L	204 (153–236)	194 (129–235)	203 (160–253)	0.58
Hemoglobin g/dl	14.1 (12.9–14.9)	14.2 (12.7–14.9)	14.1 (13.3–14.7)	0.74
Vibration Controlled Transient Elastography kPa	9.5 (5.8–20.0)	7.3 (4.2–24.8)	10.4 (8.2–20.1)	0.19
HCV RNA log IU/ml	6.30 (6.12–6.78)	6.27 (6.08–6.85)	6.33 (6.17–6.71)	0.68

supplementary modeling data section.

2.7. Mathematical modeling of HCV infection and treatment in chronically HCV-infected Alb-uPA/SCID chimeric mice with humanized livers

We previously reported serum HCV RNA kinetic data at days 0, 3, 7 14, 21 and 28 in 5 HCV infected *Alb-uPA/SCID* chimeric mice treated with CCZ 50 mg daily (He et al., 2015). Since HCV viral kinetic patterns under CCZ therapy in the humanized mice were found to be monophasic or biphasic, the standard HCV model (DebRoy et al., 2016) (Supplemental Eq. S(2)) was used as described in the supplementary section.

3. Results

3.1. Baseline patient characteristics

Twenty-seven subjects were screened and enrolled. Three withdrew consent prior to dosing (Fig. 1). The demographic and baseline clinical characteristics of the subjects that were enrolled were generally balanced between the two groups (Table 1). Overall, the median age was 56 years, 54% were male and 58% were Caucasian. 46% of subjects had HCV genotype 1a infection and the overall median baseline serum HCV RNA was 6.30 log IU/ml.

3.2. Virologic responses and viral kinetic analysis in patients

At baseline, HCV RNA levels did not differ between group 1 subjects (median = 6.27 log IU/ml) and group 2 subjects (median = 6.33 log IU/ml, p = 0.68) (Table 1). By the end of treatment, no HCV RNA decline (p = 0.69) was seen in group 1 subjects (median decline = 0.18 log IU/ml) or in aggregate (median decline = 0.05 log IU/ml). However, a decline that trended towards significance, was seen in group 2 subjects that received CCZ + RBV (median decline = 0.52 log IU/ml, p = 0.07).

In examining individual HCV viral response kinetic patterns, all patients treated with CCZ monotherapy (n = 12, Supplemental Table 1) and 5 patients treated with CCZ + RBV (P20-P24 in Table 2 and Supplemental Fig. 2) were non-responders, defined as having < 3-fold change in HCV RNA during therapy. The remaining 7 patients treated with CCZ + RBV, had declines in HCV RNA with a significant average decline of 0.84 log IU/ml at EOT compared to 0.16 log IU/ml in non-responders, p = 0.03 (Fig. 2 and Table 2). Of the 7 responders, two patients (P17 and P19) demonstrated a monophasic decline after an ~14 day delay during which viral load remained at pre-treatment levels, 2 patients (P16 and P18) exhibited a biphasic partial response in which HCV virus levels declined initially and then remained at a new lower set point, and 3 patients (P13, P14 and P15) had a triphasic response pattern in which virus levels declined rapidly in the 1st phase followed by a shoulder phase before a continuation of viral decline in the 3rd phase (Fig. 2). Fitting the model (Supplemental Eq. S(1)) to the data predicted a median effectiveness of CCZ + RBV in blocking viral production (defined as ε), and/or CCZ in blocking viral entry/infection (defined as η), of ε = 59% (Interquartile range, IQR: 50%) and η = 78% (IQR: 23%), respectively. The median viral clearance rate from blood was estimated as c = 1.6/day (IQR: 0.92/day), corresponding to HCV half-life $t_{1/2}$ = 10.3 h (IQR: 9.4 h). Viral production and infected cell loss/death rate were estimated as p = 1.6 virions/cell/day (IQR:4.1) and δ = 0.31/day (IQR: 0.17), respectively (Table 3).

3.3. Biochemical responses to therapy with CCZ with or without RBV in patients

In comparing laboratory values at the end of therapy (day 28) with baseline values, subjects randomized to group 1 (CCZ) did not experience significant changes in alkaline phosphatase, ALT, or AST (Supplementary Table 2). There was a significant decrease in total bilirubin (median −0.1 mg/dL, p = 0.03), that was not clinically meaningful. In contrast, subjects randomized to group 2 (CCZ + RBV) did experience significant median declines in ALT (−26 U/L, p = 0.0009), AST (−17 U/L, p = 0.002), hemoglobin (−1.9 g/dL, p < 0.0001) and an increase in total bilirubin (0.3 mg/dL, p = 0.0003) by the end of therapy.

3.4. Pharmacokinetic analysis of CCZ therapy in patients

The pharmacokinetics of CCZ in patients treated with CCZ alone is shown in Supplemental Fig. 1 and detailed in Supplemental Table 1. The concentration of CCZ after the first dose in all patients reached a peak (C_{max} median = 79.71 ng/ml, IQR = 52.89 ng/ml) at median peak time t_{max} = 3 h (IQR = 2 h) followed by a transient decrease and then a plateau from median time t_p = 10.5 days (IQR = 7 days). The median concentration of CCZ during the plateau, C_p was 229.32 ng/ml, IQR = 151.36 ng/ml (Supplemental Table 1). The pharmacokinetics of CCZ concentrations in patients treated with CCZ + RBV is shown in Supplemental Fig. 2 and detailed in Table 2. The concentration of CCZ after the first dose reached a peak (C_{max} median = 101.90 ng/ml, IQR = 47.22 ng/ml) at t_{max} = 2 h (IQR = 6 h) after initiation of therapy, which was followed by a transient decrease and then a plateau at t_p = 7 days after initiation of therapy. The median CCZ concentration during C_p was 191.66 ng/ml, IQR = 92.80 ng/ml (Table 2). There was no significant correlation between CCZ pharmacokinetic parameters

Table 2
Characteristics and HCV kinetic patterns in subjects receiving CCZ + RBV combination therapy.

Patient	HCV GT	Sex	RT	bHCV (log IU/ml)	VKP	t_0 (d)	1st phase decline slope (log/d)	final-phase decline slope (log/d)	ΔHCV EOT (log cp/ml)	C_{max} (ng/ml)	t_{max} (hr)	t_p (d)	C_p (ng/ml)
13	1b	M	R	7.26	T	0	0.13	0.05	1.12	32.42	8	7	82.56
14	1a	F	R	6.37	T	0	0.08[a]	0.04[a]	1.06	138.82	1	7	266.10
15	1a	M	R	5.61	T	0	0.33	0.04	0.92	87.43	1	2	91.57
16	3	F	R	6.70	FPR	0	0.58	0.01[a]	0.84	140.16	4	7	171.49
17	1b	F	R	6.30	M	7	0.03		0.46	101.47	2	7	197.59
18	1a	M	R	6.58	FPR	7	0.09	0.01[a]	0.45	101.90	2	14	191.63
19	1a	F	R	6.20	M	14	0.04		0.58				
R-Median (IQR)				**6.4[b] (0.50)**			**0.09 (0.29)**	**0.04 (0.04)**	**0.84[c] (0.60)**	**101.68[c] (65.48)**	**2[b] (4)**	**7[b] (3)**	**181.56[b] (125.40)**
20	1a	F	NR	6.76					0.43	116	2	7	144
21	6	M	NR	7.11					0.14	76.17	8	14	98.75
22	1a	F	NR	6.20					0.00	123.39	4	7	204.62
23	1a	M	NR	6.10					(0.16)	70.83	2	7	191.69
24	1b	M	NR	5.35		14	(0.06)		(0.92)	112.22	8	7	206.80
NR-Median (IQR)				**6.2[b] (1.21)**					**0.16[c] (0.61)**	**112.22[b] (46.20)**	**4[b] (6)**	**7[b] (3.50)**	**191.69[b] (84.34)**
All-Median (IQR)				**6.33 (0.62)**					**0.52 (0.69)**	**101.90 (47.22)**	**2 (6)**	**7 (0.00)**	**191.66 (92.80)**

bHCV, baseline (pre-treatment) HCV RNA; NR, Non-responder; R, Responder; RT, response type; VKP, viral kinetic pattern; M, monophasic; T, triphasic; FPR, flat partial response; t_0, time delay in which viral load remained in bHCV level; EOT, end of therapy (day 28); ΔHCV EOT, decline from bHCV at end of treatment, C_{max}, peak concentration of CCZ after first dose; t_{max}, time when the concentration of CCZ obtained a peak after first dose; t_p, starting time of plateau (slope not different from zero, $p \geq 0.142$); C_p, concentration of CCZ during plateau; (), defined as increase in viral load (or slope) from bHCV level; *non-responders are defined as patients who have had less than 3-fold decline from bHCV during therapy.

[a] Slope was not significant or different than 0 ($p > 0.1$); ^ Due to an increase in viral load at day 28, calculations were made up to day 21; ¶ Data is not available for concentration of CCZ.

[b] The distribution is not significantly different across the both RT (R and NR, $P \geq 0.218$).

[c] Viral load decline from the bHCV at EOT is significantly different between R and NR, $P = 0.03$.

and the HCV RNA viral decline.

No subjects prematurely discontinued therapy due to adverse events or a serious adverse event (Supplemental Table 3). There were no significant changes from baseline on digitized electrocardiography testing performed throughout the study. The were no major distinguishing symptoms between group 1 and group 2 participants.

3.5. Viral kinetic comparison of CCZ + RBV treatment responders with historical RBV monotherapy

A viral kinetic comparison was performed between CCZ + RBV responders with our *historical* RBV monotherapy data (Pawlotsky et al., 2004) to identify potential differences in response with the addition of CCZ (Fig. 3). This analysis suggested no statistical ($P \geq 0.343$) difference in viral nadir level and viral nadir time between patients treated with RBV with or without CCZ. However, while HCV RNA viral levels increased from nadir to day 14 in RBV monotherapy subjects, the viral load continued to decline in patients treated with CCZ + RBV (Fig. 3 and Supplemental Table 4), suggesting a possible synergistic effect of CCZ with RBV.

3.6. Kinetic and modeling analyses of CCZ monotherapy in HCV-infected uPA/SCID mice with humanized livers

We previously reported that CCZ treatment of uPA/SCID mice with humanized livers reduced chronic HCV levels (He et al., 2015). Analogous to the CCZ + RBV treated responder patients described above, more than one viral inhibition kinetic patterns were identified among the 5 CCZ-treated mice. One pattern was a biphasic with a rapid 1st phase that lasted up to 3–4 days, followed by a slower 2nd phase and the other was monophasic consisting of a gradual viral load decline over the period of treatment (Supplemental Fig. 3). Modeling was performed using Supplemental Eq. S(2) to estimate viral kinetic parameters and CCZ efficacy (Supplemental Table 5). In terms of understanding CCZ mechanism of action, using this model, we previously showed that the effect of an antiviral in blocking viral infection (e.g., viral entry) has only a minimal impact on viral decline in the serum as long as the efficacy of blocking of viral production is high (Neumann et al., 1998). Consistent with this known feature of the model, the effect of CCZ in blocking entry/infection could not be defined with confidence, however, there is evidence for CCZ effectiveness in blocking viral production (median $\varepsilon = 83\%$; min-max: 36%–97%), regardless whether CCZ was blocking infection.

4. Discussion

In this first-in-humans, proof-of-concept study evaluating the feasibility of repurposing the first generation piperazine antihistamine chlorcyclizine for chronic HCV infection, we demonstrated that CCZ administered at 75 mg twice daily with and without weight-based ribavirin was safe and tolerable. Although each individual dosing of 75 mg was slightly higher than was originally approved for use in 1949, this dose administered twice daily as monotherapy against HCV did not result in significant HCV RNA declines with 28 days of therapy. However, when combined with RBV, significant declines in HCV RNA were seen in 58% of patients along with a significant improvement in ALT and AST.

The lack of HCV RNA decline seen in subjects receiving CCZ monotherapy differed from the declines seen in the *in vivo* chimeric mouse model demonstrated by He et al. (2015) At CCZ doses of 2 mg/kg/day dosed in chimeric mice infected with HCV, a ~1 log decline of HCV RNA was observed with 28 days of therapy, whereas CCZ dosing at 10 and 50 mg/kg/day resulted in substantially greater dose dependent declines approaching 2 logs (50 mg/kg/day data). In this current study, patients were dosed at 150 mg/day (equivalent to 2 mg/kg/daily for 75 kg adult), which was recommended and accepted by the FDA for this IND-exempt pilot study. Unfortunately, in contrast to the mouse studies, at this dosing, a significant antiviral response was not observed. Assessment of pharmacokinetics indicates that the plasma (S)-CCZ levels reached were in the range of 200 ng/mL. This is 2-fold over the EC_{50} of (S)-CCZ against HCV genotype 2 (100 ng/mL), however, CCZ is much more active against HCV genotype 2 than the other genotypes (EC_{50} in

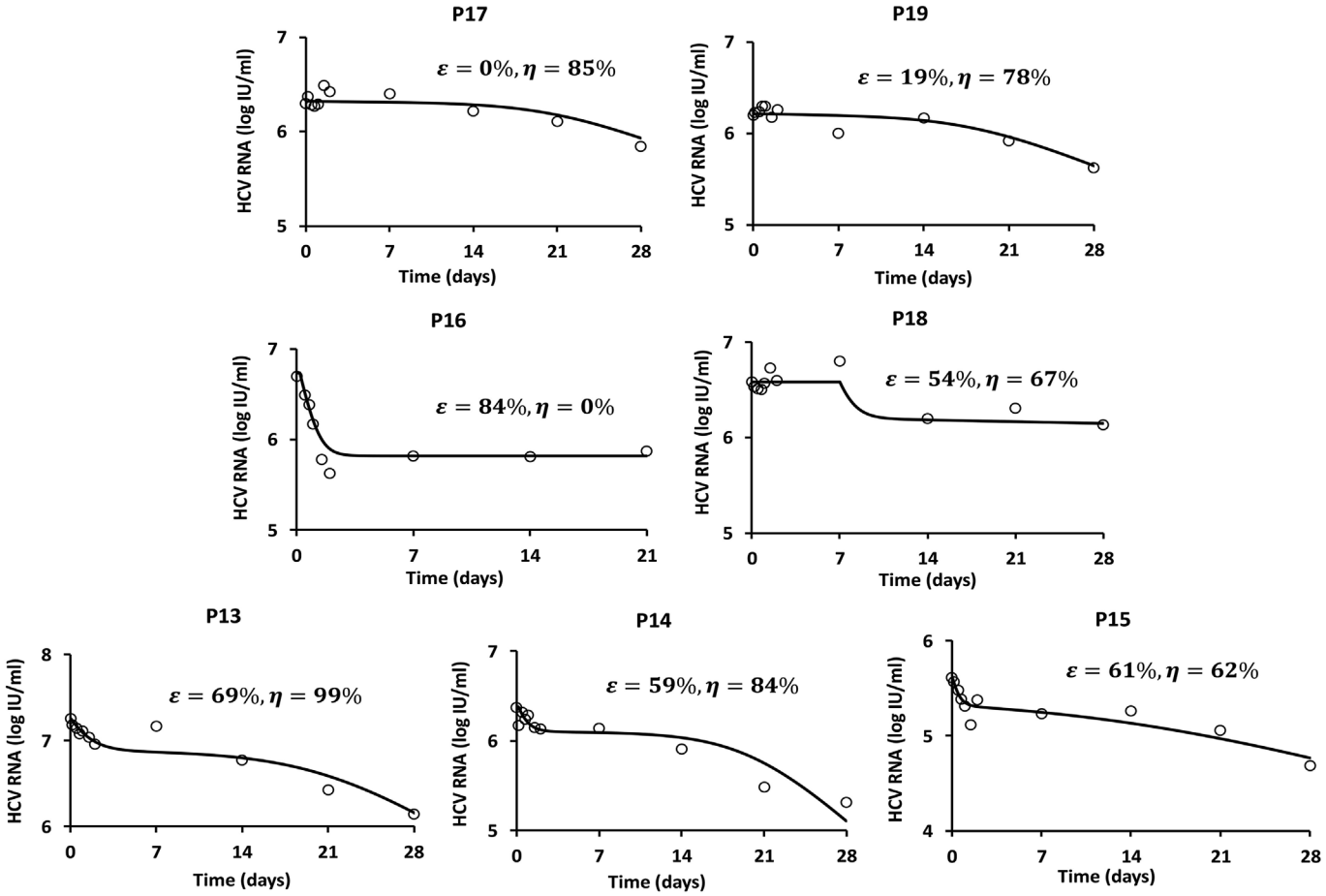

Fig. 2. Viral kinetic patterns and modeling of response in patients who responded to 28 days of CCZ + RBV therapy. Serum HCV RNA levels are demonstrated (circles) in 7 patients during therapy. Modeling (Supplemental Eq. S(1)) of response (solid lines) demonstrates three types of responses: monophasic (P17 and P19), biphasic (P16 and P18), or triphasic (P13, P14 and P15). The model is in agreement with HCV RNA data and the viral kinetic modeling parameter values are demonstrated in Table 3; ε and η indicate estimated efficacy of CCZ + RBV in blocking viral production and infection, respectively.

the range of 1–5 μg/mL) (Rolt et al., 2018; Huang et al., 2011). Because only one patient treated in this study was infected with genotype 2, these low plasma levels may account for sub-therapeutic effects of CCZ in these patients. Thus, increased dosing beyond what was explored in this study to achieve greater plasma drug levels may demonstrate a more profound response; however this could be at the cost of increased side effects. Alternatively, the lack of response seen in this study could also be attributable to human factors related to different drug metabolism, bioavailability or distribution, which perhaps cannot be overcome simply by increasing the dosing.

Interestingly, in patients receiving CCZ + RBV therapy, 7 of 12 (58%) of subjects demonstrated a > 3-fold change in HCV RNA during therapy. We previously demonstrated (Pawlotsky et al., 2004) that RBV alone had an early but transient effect on viral levels in 4 of 7 treated patients. In the current study, a similar early viral decline was observed in 4 of the 7 responders treated with CCZ + RBV; however, in contrast to viral rebound observed under RBV alone, viral load continued to decline in CCZ + RBV treated patients, consistent with a synergistic anti-HCV effect of CCZ and RBV we previously observed *in vitro* (He et al., 2015).

In patients receiving CCZ + RBV therapy who had a significant antiviral response, three distinct viral kinetic response patterns were observed; monophasic response, biphasic partial response, and triphasic response (Fig. 2). In the era of HCV direct-acting antiviral therapies, the

Table 3
Viral kinetics parameters of responder patients.

Patient	VKP	δ (d^{-1})	p (virions $cell^{-1}$ d^{-1})	c (d^{-1})	HCV $t_{1/2}$ (d)	ε	η
13	T	0.15	5.7	0.8	0.38	0.69	0.99
14	T	0.32	1.6	0.90	0.77	0.59	0.84
15	T	0.125	0.37	2.42	0.29	0.61	0.62
16	FPR	0.32	5.4	1.60	0.43	0.84	≃0 (3e-6)
17	M	0.31	1.3	0.90	0.77	≃0 (1e-6)	0.85
18	FPR	0.32	4.8	1.60	0.43	0.54*	0.67
19	M	0.30	1	0.90	0.77	0.19	0.78
Median(IQR)		**0.31**	**1.6**	**1.6**	**0.43**	**0.59**	**0.78**
		(0.17)	**(4.05)**	**(0.92)**	**(0.39)**	**(0.50)**	**(0.23)**

VKP, viral kinetic pattern; M, monophasic; FPR, flat partial response; T, triphasic; $t_{1/2}$, half-life of HCV in blood; **δ,** death/loss rate of infected cells; **p**, production rate of virions; **c**, HCV clearance rate in blood; ε, blocking of viral production; η, blocking of viral entry/infection. * Initiated 7 days after the initiation of treatment.

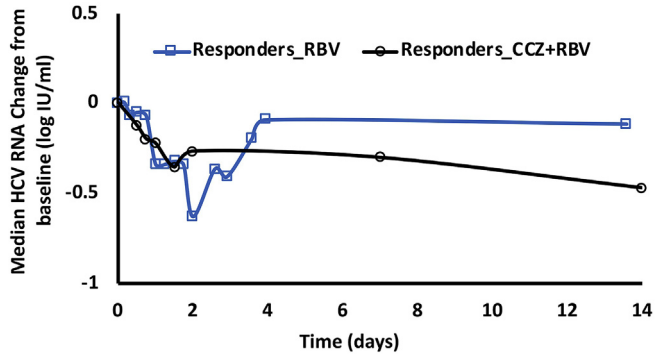

Fig. 3. Comparison of median HCV RNA change from baseline in patients who responded to CCZ + RBV (black circles) versus *historical* responders to RBV monotherapy (blue rectangles) during 14 days of treatment. The initial declines in serum HCV RNA during the first few days of therapy appear to be similar between the two groups, however HCV RNA levels return to near baseline levels around day 4 in the RBV monotherapy group whereas a continued decline is seen in those treated with CCZ + RBV. Lines are used to emphasize the kinetic patterns of each treatment group.

typical viral inhibition kinetics is biphasic (Canini et al., 2017; Dahari et al., 2016), while the multiple complex patterns observed in this study are more reminiscent of the viral kinetics observed under (pegylated) interferon-α (IFN) with or without RBV therapy (Dahari et al., 2009). Since IFN, RBV and CCZ may have diverse effects and result in slower viral decline, perhaps these variable kinetic patterns reflect, in part, different immune responses among treated subjects. This may be why only two of the patterns (i.e., monophasic and biphasic) were observed in the chimeric mice which lack any adaptive immune response.

Interestingly, within the cohort of patients receiving CCZ + RBV therapy, 5 of 12 (42%) subjects did not demonstrate significant antiviral responses during therapy despite having similar pharmacokinetic profiles to those with response. Likewise, there was no difference in HCV genotype between responders and nonresponders. Finally, although viral mutation analysis was not performed in this study, previous *in vivo* studies in Alb-uPA/Scid mice on HCV genotype 1b and 2a treated for 4 and 6 weeks with CCZ have not demonstrated evidence of drug resistance (He et al., 2015). Future human studies with CCZ or its derivatives could further explore the development of viral resistant variants and/or investigate other host factors that might correlate with these two different response patterns.

Previous *in vitro* analysis of CCZ anti-HCV mechanism of action indicated a block of late stage HCV entry (He et al., 2015). Interestingly, our modeling of HCV RNA kinetics during CCZ mono-therapy in the humanized chimeric mice as well as CCZ + RBV treated humans suggest that these CCZ treatments may not only inhibit HCV entry into cells, but also inhibit viral production (Fig. 2 and Supplemental Fig. 3). Because the model lacks molecular detail (i.e. decreased viral production could result from many different specific MOA) and cannot distinguish direct from indirect effects, there are multiple possible explanations that the model may be predicting including a second MOA that has not been experimentally characterized *in vitro*. In particular, it is possible that CCZ may have additional effects on the systemic innate immune response in chimeric mice and both innate and adaptive immune responses in humans which may not be recapitulated in cell culture. Regardless of mechanism, the model clearly suggests that the continuous decline in viral load seen in some patients and mice cannot be maintained solely by the blocking of HCV entry/infection alone. As such, further *in vivo* experiments and theoretical efforts are warranted to define what additional mechanisms of HCV inhibition might be induced by CCZ therapy.

In this study, there were no premature discontinuations or serious adverse events during 28 days of CCZ therapy. The known anticholinergic effects of CCZ (dry mouth/dry eyes, drowsiness, and nervousness) were the most common and not seen at frequencies higher than expected as has been described in the literature despite the higher doses employed (Brown and Fox, 1950). Interestingly, the development of fatigue was clearly more prevalent in the group receiving RBV, which is also a known side effect of RBV and anemia (Rebetol, 2017). Future exploration of CCZ based therapies, potentially at higher doses, may result in increasing frequency and severity of these anticholinergic side effects which could result in poor long term tolerability.

Our study has several limitations. First, the model (Supplemental Eq. S(1)) was not designed to distinguish between the effects of CCZ and RBV, i.e. their MOAs or individual inhibition efficiency. In terms of mechanism, we show that CCZ treatment alone in HCV-infected humanized mice can block viral production (Supplemental Fig. 3 and Supplemental Table 5), implicating this as at least one MOA of CCZ. Historical data (Pawlotsky et al., 2004) indicates that RBV treatment alone can lead to a transient HCV decline in patients (Fig. 3) and in particular is thought to reduce progeny HCV infectivity (Dahari et al., 2007; Dixit et al., 2004). Hence, in the model we assumed that RBV reduces HCV infectivity (see function $\rho(t)$ in Supplemental Eq. S(1)). However, the model cannot rule out that both CCZ and RBV may contribute to the mean estimate of $\varepsilon = 59\%$ in blocking viral production or the mean estimated efficacy of $\eta = 78\%$ in blocking HCV infection. A related limitation is the inability to couple in the model the measured CCZ PK (Supplemental Fig. 2) with viral response due not only to the inability to distinguish the effects of the two drugs, but also the lack of RBV PK data and similar CCZ PK among responders and nonresponders.

In conclusion, despite the small numbers, this pilot-feasibility study demonstrates that CCZ may have some anti-HCV effects in humans, but the study was limited by the CCZ dosing that may be sub-therapeutic in humans. This study provides patient level data and an early framework for the development of new drugs with similarities to CCZ. Our recent structure-activity relationship campaign has generated several CCZ derivatives that are more potent and have more optimal pharmacokinetic features (Rolt et al., 2018). These improved compounds may be more suitable for further human testing and therapeutic development.

Conflicts of interest

None of the authors has financial interests or conflicts of interest related to this research.

Writing assistance

None.

Authors contributions

Study concept and design: CK, TH, TJL.
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Analysis and interpretation of data: CK, MAT, PS, PD, PJW, HMG, NB, NS, SJC, OE, TH, HD, TJL.
Drafting of manuscript: CK, PD, SLU, SJC, HD, TJL.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.01.017.

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