The Cyclophilin Inhibitor Debio-025 Shows Potent Anti–Hepatitis C Effect in Patients Coinfected with Hepatitis C and Human Immunodeficiency Virus

Robert Flisiak,[1] Andrzej Horban,[2] Philippe Gallay,[3] Michael Bobardt,[3] Suganya Selvarajah,[3] Alicja Wiercinska-Drapalo,[1] Ewa Siwak,[1] Iwona Cielniak,[2] Jozef Higersberger,[2] Jarek Kierkus,[4] Christian Aeschlimann,[5] Pierre Grosgurin,[6] Valérie Nicolas-Métral,[6] Jean-Maurice Dumont,[6] Hervé Porchet,[6] Raf Crabbé,[6] and Pietro Scalfaro[6]

Debio-025 is an oral cyclophilin (Cyp) inhibitor with potent anti–hepatitis C virus activity *in vitro*. Its effect on viral load as well as its influence on intracellular Cyp levels was investigated in a randomized, double-blind, placebo-controlled study. Mean hepatitis C viral load decreased significantly by 3.6 $\log_{10}$ after a 14-day oral treatment with 1200 mg twice daily ($P < 0.0001$) with an effect against the 3 genotypes (1, 3, and 4) represented in the study. In addition, the absence of viral rebound during treatment indicates that Debio-025 has a high barrier for the selection of resistance. In Debio-025–treated patients, cyclophilin B (CypB) levels in peripheral blood mononuclear cells decreased from 67 ± 6 (standard error) ng/mg protein (baseline) to 5 ± 1 ng/mg protein at day 15 ($P < 0.01$). *Conclusion:* Debio-025 induced a strong drop in CypB levels, coinciding with the decrease in hepatitis C viral load. These are the first preliminary human data supporting the hypothesis that CypB may play an important role in hepatitis C virus replication and that Cyp inhibition is a valid target for the development of anti–hepatitis C drugs. (HEPATOLOGY 2008; 47:817-826.)

Among human immunodeficiency virus (HIV)-infected patients, nearly one-third are coinfected with hepatitis C virus (HCV), and liver disease has emerged as a major cause of morbidity and mortality in these patients.[1,2] The efficacy of peginterferon plus ribavirin has recently been established in HIV/HCV-coinfected patients,[3] though efficacy was substantially inferior to that observed in HCV-monoinfected patients.[1]

Cyclophilins (Cyps) are abundant intracellular binding proteins that are expressed in many tissue types.[4] They catalyze the *cis-trans* interconversion of peptide bonds amino-terminal to proline residues, facilitating changes in protein conformation and protein folding, and are involved in several cellular processes such as transcriptional regulation, immune response, protein secretion, and mitochondrial function.[4,5] *In vitro*, HCV replication can be reduced by either RNA interference-mediated reduction of endogenous cyclophilin B (CypB) expression or the induced inhibition of NS5B binding to CypB,[5] indicating that CypB is a functional regulator of the NS5B-RNA–dependent RNA polymerase. In HIV-1, evidence has been found that Cyps can act as viral cofactors.[6,7] The virus recruits cyclophilin A (CypA) onto its viral capsid, potentially forming a protective shield.[7]

Debio-025 is a selective Cyp inhibitor[8] that has well-established *in vitro* antiviral properties against HCV and HIV-1. *In vitro* data have demonstrated that the 50% effective concentration for inhibition (IC_{50}) of the HCV subgenomic replicon replication in Huh 5-2 cells is

Abbreviations: AE, adverse event; AUC, area under the curve; CI, confidence interval; Cyp, cyclophilin; ELISA, enzyme-linked immunosorbent assay; GE, genome equivalents; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IgG, immunoglobulin G; MRP2, multidrug resistant associated protein; PBMC, peripheral blood mononuclear cell; PK, pharmacokinetics.

From the [1]Department of Infectious Diseases, Medical University of Bialystok, Bialystok, Poland; the [2]Hospital of Infectious Diseases, Warsaw, Poland; [3]The Scripps Research Institute, La Jolla, CA; the [4]Department of Gastroenterology, Hepatology and Immunology, The Children's Memorial Health Institute, Warsaw, Poland; the [5]Novartis Consumer Health SA, Nyon, Switzerland; and [6]Debiopharm SA, Lausanne, Switzerland.

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Address reprint requests to: Raf Crabbé, Debiopharm SA, Forum "après-demain," Chemin Messidor 5-7, CP 5911, 1002 Lausanne, Switzerland. E-mail: rcrabbe@debiopharm.com; fax: (41) 21-321-01-69.

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0.27 ± 0.03 μg/mL with a selectivity index of approximately 900.[9] This antiviral activity was confirmed *in vivo* in a study showing a synergistic effect between interferon-α and Debio-025 in chimeric mice infected with HCV genotype 1a or 1b.[8] *In vitro* studies with 42 HIV-1 isolates showed a majority of the viruses (n = 27) to be highly sensitive to Debio-025, with a mean IC_{50} of 0.097 μM ± 0.029, and that Debio-025 inhibited HIV-1 Gag/capsid–CypA interaction in a dose-dependent manner.[10] A clinical study of asymptomatic HIV-positive volunteers revealed that Debio-025 at dosages of 50, 400, and 1200 mg/day orally for 10 days had a limited anti-HIV-1 effect in individual patients, but the study failed to demonstrate a difference in viral load reduction between placebo and any of the 3 dosages tested.[11] These results prompted further exploration of the molecule's potential at higher dosages as an antiviral treatment for HIV. In addition, the significant *in vitro* anti-HCV effect displayed by Debio-025 warranted confirmation of this activity in humans. For this reason, we designed a double-blind, randomized, placebo-controlled study to investigate the antiviral potential of Debio-025 in both HIV-1–monoinfected and HIV-1/HCV–coinfected patients. Because lower dosages had been previously tested, the trial was designed as a proof-of-concept study with 1 treatment regimen close to the anticipated maximum tolerable dose. Another goal was to investigate the effect of Debio-025 on CypA and CypB levels. Cyclophilins are predominantly located intracellularly,[12] and it was expected that any clinical effect could only be correlated with inhibition of Cyps at the intracellular level. Debio-025 also easily penetrates and concentrates in blood cells after oral administration, leading to intracellular levels significantly higher than plasma levels.[13] Previous experience has demonstrated that inhibition of CypB leads to its secretion by the cells resulting in substantially reduced intracellular CypB levels. A significant reduction of intracellular CypB levels is therefore expected to give a good indication about the level of CypB inhibition. CypA behaves differently and is not secreted from the cells after inhibition.[14,15] We selected peripheral blood mononuclear cells (PBMCs) for CypA and CypB determination, because this cell fraction is relatively easy to obtain and highly relevant for HIV replication.

Patients and Methods

Study Design. This was a double-blind, randomized, placebo-controlled study to determine the antiviral activity, pharmacokinetics (PK), and safety of Debio-025 in asymptomatic HIV-1–monoinfected and HIV-1/HCV–coinfected patients. Treatment and assessments were identical in both groups. The study included a pharmacodynamic assessment of CypA and CypB levels in PBMCs to investigate the relationship between CypA and CypB inhibition, and antiviral effect. The sample size of 18 patients in the Debio-025 group was calculated to detect a viral load reduction of 1.0 log_{10} copies/mL with a standard deviation of 1.4 log_{10} copies/mL from pretreatment to end-of-treatment. The placebo group (4 patients) was included to ensure a blind assessment of safety during the study. Sample size was not powered to detect a difference between treatment groups. This resulted in an unbalanced randomization of patients in a 9:2 (Debio-025/placebo) ratio. One additional patient (patient 23) was included because he completed all screening procedures and was fully eligible for inclusion. Because this patient was randomized to active treatment, the study ended with 19 patients on active drug and 4 patients on placebo.

Patients were randomly assigned to Debio-025 oral solution 1200 mg or placebo twice daily for 14 consecutive days with a final last dose in the morning of day 15. The 15-day treatment was followed by a 4-week washout period. The placebo formulation was similar to that of Debio-025 except for the active compound. Visual inspection did not allow differentiation between treatments, which were packaged in a blinded fashion prior to study start. A computer-generated randomization stratified by viral infection (monoinfected or coinfected) and including a Debio-025/placebo ratio of 9:2 was issued prior to study initiation by the study biostatistician. Treatment assignment was centralized through a randomization center to be contacted by fax or e-mail by the investigator. This center was solely in charge of assigning randomization number and corresponding study drug box number to the patients enrolled based on the preestablished randomization list. A log of all randomization-related communications with the study sites was kept by the randomization center. Ethical Committees at participating centers approved the protocol. All patients provided written informed consent. The study was conducted according to the principles set forth in the 2000 Declaration of Helsinki and Good Clinical Practices guidelines.

Patients. Male and female patients were eligible for inclusion if they were between 20 and 65 years of age, infected with HIV-1 or HIV-1 and HCV, had detectable plasma HIV-1 RNA levels ≥5000 copies/mL and HCV RNA levels ≥2000 copies/mL (the latter applicable only to coinfected patients), were negative for hepatitis B surface antigen, had normal liver or compensated liver disease, and a CD4 lymphocyte cell count >250 cells/mm^3 within 28 days prior to study entry. Patients were ineligible for inclusion in case of ongoing or recent (<1 month) anti-HIV, anti-HCV, or other antiviral treatment, clini-

cally significant coexisting medical condition, major liver impairment, documented liver biopsy showing advanced inflammation, necrosis, and fibrosis (Scheuer grade 4 for necroinflammation and fibrosis, Metavir score A3F3 or superior), arterial hypertension, or significant kidney impairment defined as serum creatinine ≥2 times the upper limit of normal.

Endpoint Measures. Antiviral activity was assessed as the maximum $\log_{10}$ reduction of HCV and HIV-1 RNA copies at various time points during the study compared with day −1. Debio-025 plasma PK was assessed with the following parameters calculated according to noncompartmental analysis: C_{max} (on days 1 and 15), C_{0h} (trough levels on days 2, 4, 8, 10, 14, and 15), t_{max} (on days 1 and 15), $AUC_{0\text{-}12h}$ (area under the concentration time curve on days 1 and 15), Cl/F (oral clearance), V_z/F (apparent volume of distribution), $t_{½}$, AR_{AUC} and AR_{Cmax} (accumulation ratios) and $Flux_i$ [fluctuation index (i.e., variation between peak and trough levels at steady-state)]. Safety was assessed on changes in vital signs, 12-lead electrocardiograms, standard hematology, chemistry and urine laboratory tests, and reports of clinical adverse events (AEs).

Procedures. After prestudy evaluation, eligible patients were started on Debio-025/placebo treatment as described above. Patients were seen daily during the 15-day treatment period and on 5 occasions during the washout period. Study medication was administered under visual supervision. Blood was sampled for serum HCV and plasma HIV-1 RNA levels on day 1 at 12 hours postdose, premorning dose on days 2, 4, 8, 10, and 15, and on days 21, 35, and 42. Blood for plasma PK assessment was taken on day 1 pre-dose, and 0.5, 1, 2, 4, 8, and 12 hours postdose, premorning dose on days 2, 4, 8, 10, 14, and 15, and 0.5, 1, 2, 4, 8, 12, and 24 hours post–day 15 morning dose, and on days 21, 28, 35, and 42. Safety assessments were perfomed at regular intervals during the treatment and washout period. Blood for measurement of CypA and B levels in PBMCs was taken at screening and on days 15 and 42.

Determination of HCV and HIV-1 RNA Levels and HCV Genotypes. Serum HCV RNA was measured via reverse-transcription polymerase chain reaction (Abbott RealTime HCV, Abbott Laboratories, Wiesbaden, Germany; limit of detection 52 copies/mL), as was plasma HIV-1 RNA (Abbott RealTime HIV-1, Abbott Laboratories; limit of detection 40 copies/mL). HCV genotyping was determined via transcription-mediated amplification (Versant HCV Genotype Assay; Bayer Healthcare, Brussels, Belgium). HCV and HIV-1 RNA determinations and HCV genotyping were performed by the Molecular Diagnostics Laboratory, Wojewodzki Szpital Zakazny (Warsaw, Poland).

Determination of Debio-025 Concentrations. Debio-025 levels were determined in Li-heparinized plasma by RCC Ltd, Environmental Chemistry and Pharmanalytics (Itingen, Switzerland) using a validated liquid chromatography/mass spectrometry method after liquid/liquid extraction.

Determination of CypA and CypB Levels in PBMCs. PBMCs were prepared as follows: PBMCs (1 million cells) were washed twice with 10 mL sterile phosphate-buffered saline and lysed for 30 minutes on ice in 250 μL lysis buffer [10 mM NaCl, 10 mM Tris (pH 7.4), 0.5% NP40, 1x protease inhibitors]. Lysates were cleared via centrifugation at 14,000 revolutions per minute for 10 minutes in a microcentrifuge. Supernatants (200 μL) were collected and protein concentration of cell lysates measured with a Coomassie-based BioRad kit (BioRad Laboratories, Hercules, CA). Increasing protein concentrations of cell lysates (triplicates in 50 μL) were added to 96-well plates coated with anti-Cyp immunoglobulin G (IgG) and incubated for 2 hours at 4°C.

CypA and CypB levels were measured via specific enzyme-linked immunosorbent assay (ELISA) using purified antibodies specific for CypA and CypB. Ninety-six–well plates coated overnight at 4°C with rabbit anti-Cyp IgG (20 μg/mL) were washed 10 times with H_2O, blocked with 3% bovine serum albumin in phosphate-buffered saline for 1 hour at room temperature, and washed 4 times with wash buffer (phosphate-buffered saline containing 1% bovine serum albumin and 0.02% Tween). Polyclonal anti-CypA and anti-CypB IgG were obtained from New Zealand rabbits immunized with recombinant human CypA and CypB proteins. For the Cyp ELISA standard curve, recombinant CypA and CypB proteins were diluted in wash buffer, added to the ELISA plate, and incubated for 2 hours at 4°C. Wells were washed 10 times with washing buffer, incubated for 2 hours at 4°C with biotinylated rabbit anti-Cyp IgG (8 μg/mL), washed 10 times, incubated for 30 minutes at room temperature in the dark with streptavidin-conjugated horseradish peroxidase (Jackson Laboratories and Immunochemicals, West Grove, PA) (1/1000 dilution) and washed 10 times with washing buffer. The o-phenylenediamine dihydrochloride substrate (Sigma-Aldrich, St. Louis, MO) was added to the wells, and the reaction was stopped after addition of H_2SO_4 (4 N) (Fisher Scientific, Fair Lawn, NJ). Plates were read at 495 nm on a microplate reader (Molecular Devices, Sunnyvale, CA). The limit of detection was 100-200 ng/mL of recombinant Cyp or 5-10 ng/well (50 μL/well). No ELISA cross-reaction between CypA and CypB was observed using anti-CypB IgG.

Determination of CypA and CypB Levels in Huh-7 Cells. Ten micrograms of *in vitro*–transcribed genomic Con1 RNA was electroporated into Huh-7 cells. Seven days after transfection, cells were treated with or without Debio-025 (1 μM). At the indicated time points, intracellular HCV RNA was analyzed via reverse-transcription quantitative polymerase chain reaction and presented as genome equivalents (GE) per microgram total RNA as described previously.[16] The primers for reverse-transcription quantitative polymerase chain reaction were: HCV, 5′-ATGGCGTTAGTATGAGTGTC-3′ (sense) and 5′-GGCATTGAGCGGGTTGATC-3′ (antisense); glyceraldehyde 3-phosphate dehydrogenase, 5′-GAAGGTGAAGGTCGGAGTC-3′ (sense) and 5′-GAAGATGGTGATGGGATTTC-3′ (antisense). Intracellular and extracellular CypA and CypB content was quantified via ELISA as described above.

Statistical Analysis. The assumption of normality was assessed by the Shapiro-Wilk statistic on pharmacodynamic outcomes. The following parameters were analyzed separately via analysis of covariance: the maximum $\log_{10}$ change in HIV-1 and HCV RNA copies from day −1 to on-treatment or posttreatment, the $\log_{10}$ change in HIV-1 RNA and HCV-RNA copies from day −1 to day 10 and to day 15. The analysis of covariance model included terms for treatment group and day −1 baseline value as covariates. The baseline value was the respective parameter value at day −1. From the analysis of covariance were presented the adjusted treatment group means (least squares means), standard error of the mean, and associated 95% confidence intervals (CIs), as well as *P* values where applicable, within and between the Debio-025 and placebo groups.

Statistical analysis was performed with SAS version 8.2 software. The α-risk *P*-values reported were 2-sided, and the statistical significance nominal limit was set to $P < 0.05$. No adjustment for multiple comparisons was made because of the exploratory nature of the study. Pharmacokinetic parameters were estimated for each patient individually prior to the calculation of mean day 1 and day 15 parameter estimates. A noncompartmental approach was used to compute PK parameter estimates using WinNonlin Enterprise PK software version 4.1 (Pharsight Corporation, Mountain View, CA; WinNonlin model 200, extravascular dosing). The terminal elimination rate used to calculate $t_{1/2}$ was determined via log-linear regression ($r^2 \geq 0.85$) obtained on at least the 3 last quantifiable concentrations in each plasma concentration versus time profile. Areas under the curve (AUCs) were calculated according to the log-linear trapezoidal rule using actual sampling times. Other parameters were calculated assuming steady-state conditions on day 15 as follows: $C_{ss\ av}$ was calculated as $AUC_{0\text{-}12h\ (day\ 15)}/12$; Cl/F as dose/$AUC_{0\text{-}12h\ (day\ 15)}$; V_z/F as the oral clearance divided by the elimination rate; AR_{AUC} was determined by the ratio $AUC_{0\text{-}12h\ (day\ 15)}/AUC_{0\text{-}12h\ (day\ 1)}$ and AR_{Cmax} by the ratio $C_{max\ (day\ 15)}/C_{max\ (day\ 1)}$, and $Flux_i$ as $(C_{max} - C_{min})_{(day\ 15)}/C_{ss\ av}$ x100. Safety and pharmacodynamic analyses were conducted in all randomized patients who received any dose of study medication.

Results

Patient Enrollment. The study population comprised 23 patients enrolled between September 2005 and March 2006. Nineteen patients were randomized to the Debio-025 group and 4 patients were randomized to the placebo group. Patient distribution is presented in Fig. 1. Patients were all Caucasian, predominantly male [18/23 (78.3%)], and the majority was HIV-1/HCV–coinfected [19/23 (83.6%)]. Patient baseline characteristics are shown in Table 1. In the Debio-025 group, 4 patients discontinued treatment because of AEs prior to day 15, and 1 patient was withdrawn during follow-up.

Response to Debio-025 Therapy. Of the 19 coinfected patients, those treated with Debio-025 (n = 16) experienced a significantly greater maximum reduction of $\log_{10}$ HCV RNA copies/mL compared with placebo-treated (n = 3) patients (difference −2.90; 95% CI, −4.76 to −1.04; $P = 0.0045$). The least squares mean of the maximum reduction of $\log_{10}$ HCV RNA copies/mL for the Debio-025 group was −3.63 (95% CI, −4.37 to

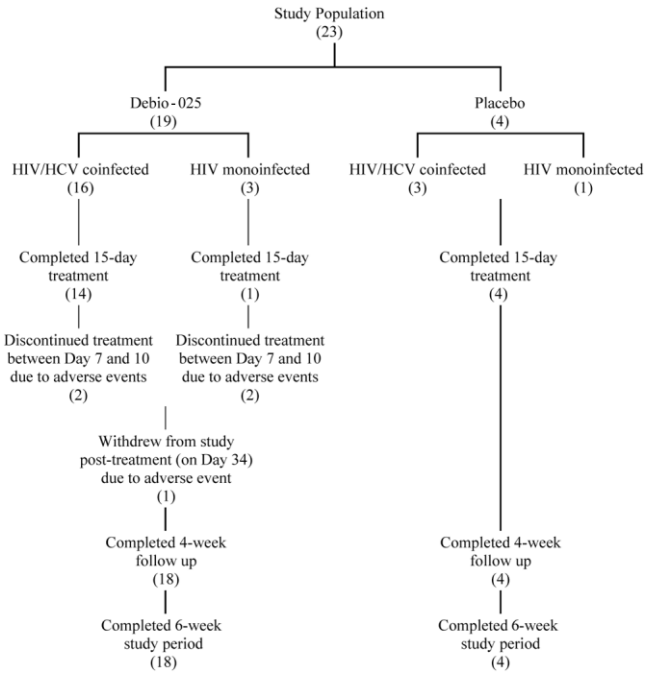

Fig. 1. Patient enrollment and outcomes.

Table 1. Baseline Characteristics of Patients

Characteristic	Placebo	Debio-025
No. of patients	4	19
Sex, n (%)		
Female	1 (25.00)	4 (21.10)
Male	3 (75.00)	15 (78.90)
Age, years (range)*	28 (26–33)	30 (19–55)
Body mass index* †	23.55 (19.10–26.60)	22.10 (16.30–27.00)
Type of viral infection, n (%)		
HIV-1/HCV–coinfected	3 (75.00)	16 (84.20)
HIV-1–monoinfected	1 (25.00)	3 (15.80)
HCV strain, n (%) |		
Genotype 1	0 (0.0)	5 (26.30)
Genotype 3	1 (25.00)	6 (31.60)
Genotype 4	2 (50.00)	5 (26.30)
HIV-1 RNA $\log_{10}$ copies/mL*	4.43 (4.10–4.80)	4.55 (3.20–5.70)
HCV RNA $\log_{10}$ copies/mL* |	7.25 (6.70–7.40)	7.12 (4.50–7.90)
Alanine aminotransferase (U/L)*	39 (35–70)	52 (20–470)‡
γ-Glutamyltransferase (U/L)*	78 (28–129)	29 (12–320)§
Total bilirubin (μmol/L)*	16.93 (10.00–24.00)	11.97 (4.96–24.00)
Creatinine (μmol/L)*	76.63 (67.00–81.00)	62.00 (44.20–100.00)
Platelet count (10^9/L)*	214 (179–255)	167 (98–294)
Neutrophil count (10^9/L)*	2.11 (1.68–5.67)	2.20 (0.48–5.18)‡
CD4 cells (cells/mm^3)*	420 (268–529)	376 (164–1195)

*Median (range).
†The body mass index is the weight in kilograms divided by the square of the height in meters.
‡n = 18.
§n = 17.
| Measured in HIV-1/HCV–coinfected subjects.

−2.90) compared with −0.73 (95% CI, −2.44 to 0.97) for the placebo group (Table 2). The difference between treatment groups was also significant for the HCV RNA reduction between day −1 and day 10 (−2.48 $\log_{10}$ RNA copies/mL; 95% CI, −4.07 to −0.88; $P = 0.0046$) as well as for the reduction between day −1 and day 15 (−2.87 $\log_{10}$ RNA copies/mL; 95% CI, −5.00 to −0.73; $P = 0.0117$). Fifteen out of 16 patients (93.8%) in the Debio-025 group experienced a reduction of HCV RNA by at least 2 $\log_{10}$ (Fig. 2A). The most important response was observed in patients with genotype 3 who achieved a least squares mean maximum reduction of $\log_{10}$ HCV RNA copies/mL of −4.46 $\log_{10}$ (95% CI, −6.06 to −2.85). In patients with genotypes 1 and 4, the maximum reduction reached −3.19 $\log_{10}$ HCV RNA copies/mL (95% CI: −4.19 to −2.18) (Fig. 2B). Viral loads decreased below detectable levels in 3 patients (1 of each genotype) at day 8 (1 patient) and at day 15 (2 patients). No patient developed a breakthrough phenomenon during treatment, and relapses occurred only after treatment cessation. A time to relapse of up to 3 weeks was observed in 3 patients, although time to relapse was highly variable overall (Fig. 2C-E).

The maximum reduction of the $\log_{10}$ number of HIV-1 RNA copies/mL during the study was significantly different compared with baseline for patients in the Debio-025 group (n = 19) (−1.03; 95% CI, −1.28 to −0.78; $P < 0.0001$). The difference between treatment groups was not statistically significant (−0.47; 95% CI, −1.07 to 0.13; $P = 0.1150$). The least squares mean of the maximum reduction of the $\log_{10}$ number of HIV-1 RNA copies/mL was −1.03 (95% CI, −1.28 to −0.78) in the Debio-025 group compared with −0.56 (95% CI, −1.10 to −0.01) in the placebo (n = 4) group (Table 2).

Pharmacokinetics. Debio-025 plasma concentration versus time plots exhibited a clear absorption phase with peak concentrations observed between 1 and 2 hours postdosing on day 1 (Fig. 3A) and between 0.5 and 4 hours postdosing on day 15 (Fig. 3B). Visual inspection of mean predose plasma concentrations suggests that steady state was achieved by day 14 of dosing in the majority of patients (Fig. 3C). Following the last dosing on day 15, a prolonged apparent terminal $t_{1/2}$ was observed (100 hours on average), and the shape of the elimination phase indicates a multicompartmental PK. A high apparent volume of distribution was calculated (near 6000 L on average), and the plasma oral clearance ranged from 23.9 to 66.2 L/hour. There was an appreciable increase in plasma systemic exposure to Debio-025 on day 15 compared with day 1, with mean accumulation ratios of 4.9 and 10.1 for C_{max} and $AUC_{0\text{-}12h}$, respectively (Table 3).

CypA and CypB Levels in PBMCs. Among coinfected patients, PBMC mean CypB levels at baseline were similar between the Debio-025 group (n = 16) and the placebo group (n = 3) (67.31 ± 6.35 ng/mg versus 73.33 ± 22.82 ng/mg protein, respectively) (Fig. 4A). In

Table 2. Maximum Reduction in Log_{10} RNA Copies/mL

Characteristic	Placebo	Debio-025	Difference*
HCV ($\log_{10}$ RNA copies/mL)			
No. of patients	3	16	19
Least squares mean	−0.73	−3.63	−2.90
Standard error	0.80	0.35	0.88
95% CI	−2.44 to 0.97	−4.37 to −2.90	−4.76 to −1.04
P value	NA†	<0.0001‡	0.0045§
HIV-1 ($\log_{10}$ RNA copies/mL)			
No. of patients	4	19	23
Least squares mean	−0.56	−1.03	−0.47
Standard error	0.26	0.12	0.29
95% CI	−1.10 to −0.01	−1.28 to −0.78	−1.07 to 0.13
P value	NA†	<0.0001‡	0.1150§

Abbreviation: NA, not applicable.
*Represents difference (Debio-025 – placebo) between adjusted treatment means from analysis of covariance.
†Analysis was not conducted.
‡*P* value associated with test of Debio-025 least squares mean change equal to zero.
§*P* value associated with test for difference between adjusted treatment means.

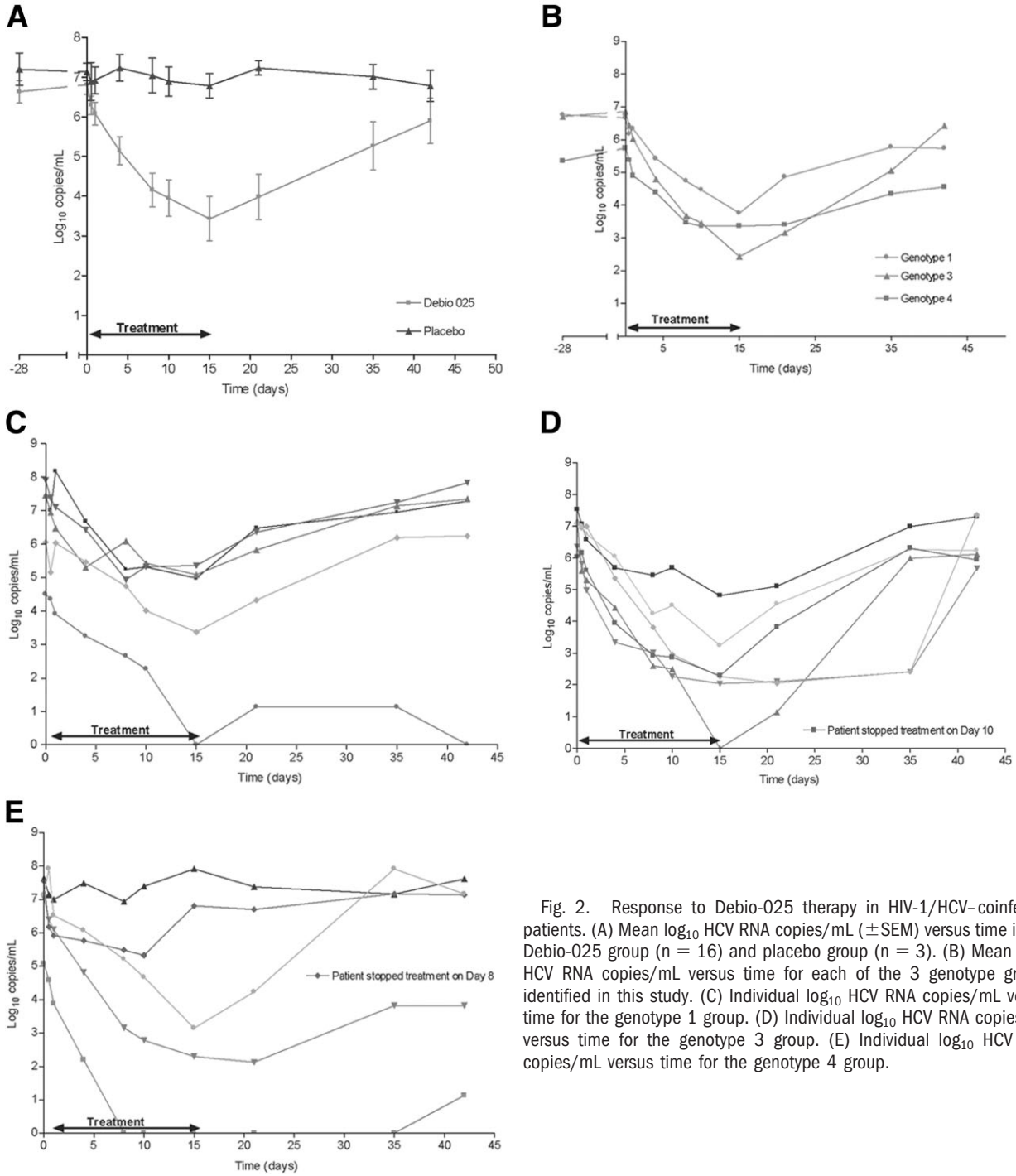

Fig. 2. Response to Debio-025 therapy in HIV-1/HCV–coinfected patients. (A) Mean $\log_{10}$ HCV RNA copies/mL (±SEM) versus time in the Debio-025 group (n = 16) and placebo group (n = 3). (B) Mean $\log_{10}$ HCV RNA copies/mL versus time for each of the 3 genotype groups identified in this study. (C) Individual $\log_{10}$ HCV RNA copies/mL versus time for the genotype 1 group. (D) Individual $\log_{10}$ HCV RNA copies/mL versus time for the genotype 3 group. (E) Individual $\log_{10}$ HCV RNA copies/mL versus time for the genotype 4 group.

the Debio-025 group, the mean level decreased to 5.30 ± 0.57 ng/mg protein on day 15 ($P < 0.001$). On day 42, the mean level (19.76 ± 3.03 ng/mg protein) was higher than on day 15, but remained inferior to the baseline level. In the placebo group, the mean CypB level remained unchanged during the study (76.00 ± 25.42 ng/mg protein on day 15 versus 77.33 ± 24.97 ng/mg on day 42) (Fig. 4A). No statistically significant correlation was found between HCV viral load decrease and CypB change from baseline to day 15 levels ($P = 0.37$). PBMC mean CypA levels (±SEM) at baseline (day −28) were similar in the placebo group (0.54 ± 0.20 μg/mg protein) and in the Debio-025 group (0.54 ± 0.04 μg/mg protein) (Fig. 4B). These mean levels remained unchanged during the study in both groups. Western blot analysis confirmed that Debio-025 drastically reduced CypB levels but not CypA levels at day 15 (end of treatment) (Fig. 4C).

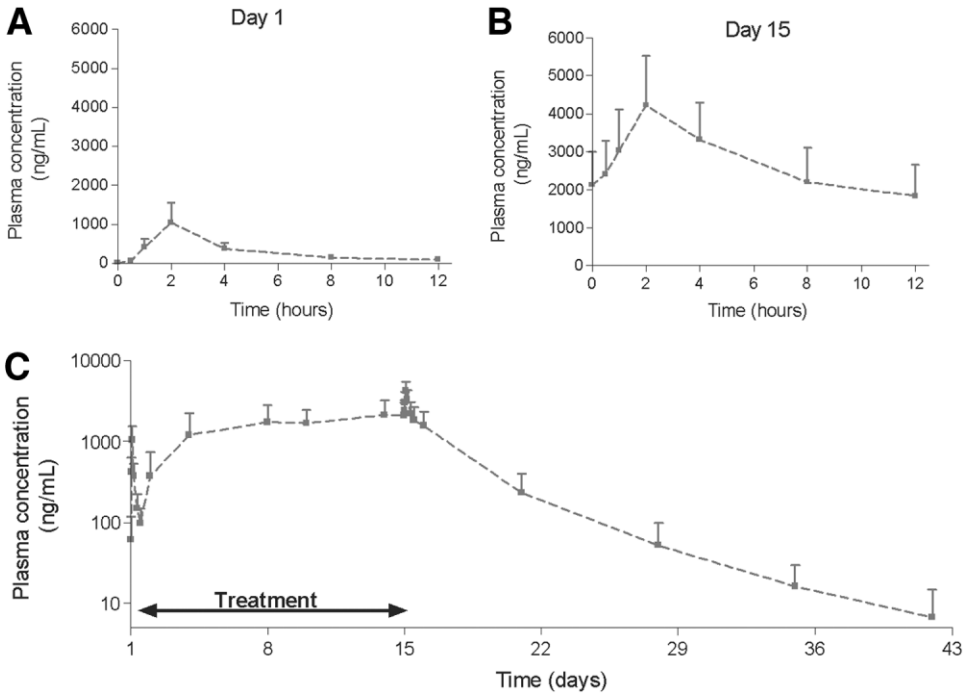

Fig. 3. Debio-025 plasma concentrations over time. (A) Mean Debio-025 plasma concentration (ng/mL) (± SD) versus time at day 1 — linear scale. (B) Mean Debio-025 plasma concentration (ng/mL) (± SD) versus time at day 15 — linear scale. (C) Mean Debio-025 plasma concentrations (ng/mL) (± SD) versus time over the entire study period — log/linear scale.

CypA and CypB Levels in Huh-7 Cells. Treatment with Debio-025 induced a strong reduction of HCV RNA (Fig. 4D), which was concomitant with an intracellular CypB depletion and a corresponding CypB release within the extracellular medium (Fig. 4E). HCV RNA in Debio-025–treated cells decreased from 6.6 ± 0.4 $\log_{10}$ GE/μg at baseline to 1.1 ± 0.4 $\log_{10}$ GE/μg at day 8 versus 6.5 ± 0.4 and 9.6 ± 0.4 $\log_{10}$ GE/μg, respectively, in controls (Fig. 4D). Intracellular CypB levels in Debio-025–treated cells declined from 32 ± 5 μg/mL at baseline to 2 ± 0.5 μg/mL at day 8, whereas extracellular levels increased from 0.1 ± 0.1 μg/mL to 19.3 ± 2.7 μg/mL between baseline and day 8 (Fig. 4E). No change in CypB levels was observed in controls.

Safety. The incidence of AEs was similar between patients in the Debio-025 group and in the placebo group [15/19 (78.9%) versus 3/4 (75%), respectively]. The majority of patients [12/19 (63.2%)] in the Debio-025 group experienced AEs considered to be related to study medication. Jaundice, abdominal pain, feeling hot, vomiting, fatigue, and pyrexia were the most commonly reported AEs in the Debio-025 group. All were mild or moderate in severity, and most were resolved by the end of the study. Fifteen (78.9%) patients in the Debio-025 group experienced elevated total

Table 3. Summary of Debio-025 Plasma Pharmacokinetic Parameters

Characteristic	Day	Mean	Standard Deviation	Range
C_{max} (ng/mL)	Day 1*	1052	± 503	489–2298
C_{max} (ng/mL)	Day 15†	4250	± 1213	2569–6262
t_{max} (hours)	Day 1*	2.0‡	NA	1.0–2.0
t_{max} (hours)	Day 15†	2.0‡	NA	0.5–4.0
$AUC_{0\text{-}12h}$ (hours · ng/mL)	Day 1*	3858	± 1586	1983–7627
$AUC_{0\text{-}12h}$ (hours · ng/mL)	Day 15†	32,898	± 10,526	18,136–50,109
$C_{ss\ av}$ (ng/mL)	Day 15†	2742	± 877	1511–4176
$t_{1/2}$ (hours)	Day 15†	100	± 18	70–138
Cl/F (L/hours)	Day 15†	40.1	± 12.8	23.9–66.2
V_z/F (L)	Day 15†	5593	± 1399	3122–7871
AR_{Cmax}	Day 15†	4.9	± 2.0	1.9–9.4
AR_{AUC}	Day 15†	10.1	± 4.7	4.1–21.5
$Flux_i$ (%)	Day 15†	88.5	± 40.6	11.0–180.5

Values of the pharmacokinetic parameters have been rounded to the nearest whole number or significant digit.
Abbreviation: NA, not applicable.
*n = 18.
†n = 14.
‡Median.

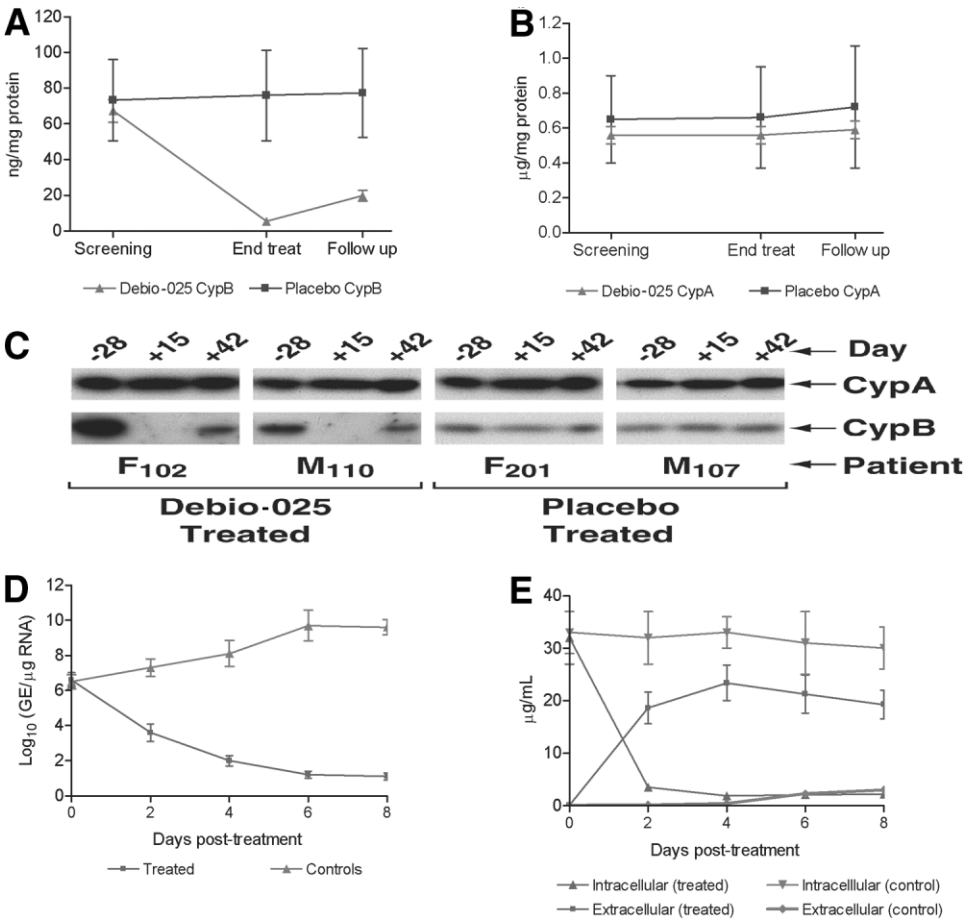

Fig. 4. Cyp levels in PBMCs and Huh-7 cells. (A) Mean CypB ng/mg protein (± SEM) in PBMCs versus time (screening at day −28, end of treatment at day 15, and follow-up at day 42). (B) Mean CypA µg/mg protein (± SEM) in PBMCs versus time (screening at day −28, end of treatment at day 15, and follow-up at day 42). (C) Western blot analysis of the PBMC lysates of 2 Debio-025–treated versus 2 placebo-treated patients at screening (day −28), end of treatment (day 15), and follow-up (day 42). (D) Mean log HCV RNA genome equivalents/µg (± SD) versus time in Debio-025–treated Huh-7 cells and controls. (E) Mean CypB µg/mL (± SD) intracellular and extracellular levels versus time in Debio-025–treated Huh-7 cells and controls.

bilirubin levels. Median values increased from 12 µmol/L (range, 5-24) at baseline to 33.4 µmol/L (range, 7.4-108.1) at day 15 (normal, 3-22 µmol/L). In 10 (52.6%) of these patients, the increase was associated with onset of icterus and 4 (17.4%) patients discontinued the study medication for this reason. There were no associated increases in aspartate aminotransferase, alanine aminotransferase, alkaline phosphatases, or γ-glutamyltransferase and no changes in laboratory results indicative of increased haemolysis. There was, however, a clear increase in bile acid plasma levels, indicating that inhibition of biliary canalicular transporters such as biliary salt excretion protein or multidrug resistance associated protein (MRP2) may be involved. Bilirubin rapidly returned to baseline levels after treatment cessation. Decreases in platelet count in 2 patients to 62 and 67 10^9/L at the most were reported as AEs. These decreases were not associated with any clinical signs of bleeding, and platelet levels returned to near baseline by the end of the study on day 42.

Discussion

This study provides the first evidence that the Cyp inhibitor Debio-025 has a significant antiviral effect against HCV in human patients. The least squares mean maximum drop of −3.63 $\log_{10}$ in the Debio-025–treated group is important and compares favorably with results reported with other compounds such as interferon-α, polymerase, and protease inhibitors.[17,18] The data also indicate that the drug is at least active against the 3 HCV genotypes that were represented in the study (genotypes 1, 3, and 4). The number of patients within each genotype was, however, too small to draw a conclusion about differences in efficacy against different genotypes. Larger studies are needed to make quantitative comparisons between the antiviral effect in different genotypes, including genotype 2. Rapid selection of resistant strains, resulting in an HCV RNA breakthrough during treatment is a phenomenon that has been regularly reported in early short-term trials with polymerase and protease inhibitor monotherapy.[17] No single patient in this study developed a breakthrough phenomenon, suggesting that Cyp inhibitors represent a higher barrier to resistance than some other compounds in development. One out of 16 patients treated with Debio-025 was a true null responder (<1 $\log_{10}$ decrease in HCV RNA during treatment). The reason for this primary insensitivity is

not clear, but because this patient had a clear reduction in CypB levels, viral factors are more likely to be the cause of this nonresponse. This also indicates that intracellular reduction of CypB levels alone cannot completely explain the anti-HCV effect and that further investigation into the exact mode of action of Debio-025 is necessary. The reduction of HIV viral load was much less pronounced than the reduction of HCV RNA, (i.e., only 1 $\log_{10}$). We do not believe that the 1 log drop in HIV RNA is associated with this specific subpopulation, because the observed effect-size was anticipated based on PK/PD modeling performed up front on results of a double-blind, placebo-controlled study in HIV monoinfected patients treated with Debio-025.[11] One possible explanation is that the Cyps involved in HIV and in HCV replication are different. CypA is present in much larger quantities in cells than CypB (about eightfold), which is consistent with previously published data.[19] Because CypA is not secreted after inhibition, the unchanged intracellular CypA levels do not allow us to reach any conclusion on the level of CypA inhibition. It is possible that the Debio-025 plasma levels achieved in this study were insufficient to induce a substantial inhibition of CypA and therefore only a small reduction in HIV viral load. In contrast, PBMC CypB levels were substantially reduced in patients treated with Debio-025, and the *in vitro* replicon data clearly show that treatment with Debio-025 significantly reduces intracellular CypB levels over time. This reduction is associated with a clear decrease in HCV RNA in the model. CypB is considered an important cofactor for RNA-dependent RNA polymerase, and the inhibition and subsequent reduction of intracellular CypB levels therefore supports the proposed mechanism of action of Debio-025 in HCV.[5,20] The *in vitro* data in the replicon model suggest that the same phenomenon may very well occur *in vivo* at the hepatocyte level.

Debio-025 was in general well tolerated. Most reported AEs were aspecific apart from isolated and reversible hyperbilirubinemia. An isolated increase of total bilirubin and bile acids suggests a saturation or inhibition of biliary canalicular transporters such as biliary salt excretion protein and MRP2. This phenomenon was not observed in previous studies with Debio-025 at oral dosages up to 1200 mg/day for 10 days.[11] Consistent with the accumulation of Debio-025 in plasma after 2 weeks at 2400 mg/day, these findings suggest that there is a dose-dependent and/or concentration-dependent saturation of 1 or more biliary canalicular transporters that results in a reversible decrease of bilirubin excretion. The high incidence of hyperbilirubinemia (in 10 of 19 patients) leading to treatment discontinuation in 4 patients indicates that the dosage of 1200 mg twice daily used in this exploratory study is probably too high, and future studies will have to establish significant anti-HCV effects at lower doses without induction of clinically relevant hyperbilirubinemia. Although the decrease in thrombocytes in 2 patients remained within acceptable limits and was fully reversible, this phenomenon may, if confirmed, limit the use of this drug in cirrhotic patients with thrombocytopenia.

In conclusion, these results demonstrate that the Cyp inhibitor Debio-025 at a dosage of 1200 mg twice daily has a significant antiviral effect against different genotypes of the HCV virus. Signs of emerging resistance such as a rebound of HCV viral load during treatment were not observed. Together with the concurrent decrease of CypB levels in PBMCs, these data suggest that targeting the HCV host–viral interaction by inhibiting CypB with Debio-025 may represent a new approach in anti-HCV treatment. These results warrant further investigations to confirm the long-term anti-HCV effects of different dosages of Debio-025 in combination with peginterferon and/or ribavirin as well as studies to elucidate the exact role of Cyps in HCV replication.

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