The cyclophilin inhibitor Debio-025 is a potent inhibitor of hepatitis C virus replication *in vitro* and has a unique resistance profile.

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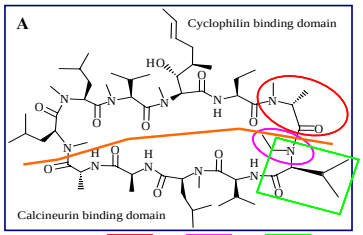
ABSTRACT

Debio-025 is a potent inhibitor of HCV replication [Hepatology 43:761-70]. In phase I clinical studies monotherapy (dose 1200 mg BID) resulted in a mean maximal decrease in viral load of 3.6 log10 [Hepatology, 44 : 4S1, 609A]. We now demonstrate that Debio-025 is equipotent against wild-type HCV, as against HCV replicons that are resistant to either HCV polymerase or protease inhibitors. Debio-025, alone at concentrations below 1 µM, was able to cure cells from their HCV replicon within 3 to 4 passages, whereas treatment with a HCV protease inhibitor, (7 passages) did not. Debio-025, at a concentration of 0.1 µM when combined with VX-950 [at a concentration that is alone not able to clear replicon cells] was able to efficiently cure the cells. Debio-025 at 0.1 or 0.5 µM was able to completely prevent the development of resistance to the protease inhibitors BILN-2061 & VX-950 as well as to nucleoside and non-nucleoside HCV polymerase inhibitors. Following long-term culture in increasing concentrations of Debio-025 or CsA, replicon resistance to both compounds was obtained. CsA and Debio-025 proved cross-resistant, but the replicons remained fully susceptible to interferon and several other anti-HCV inhibitors. Replicons resistant to Debio-025 or CsA carry one common mutation in the NS5A gene, additional mutations are detected in the Debio-025res replicons. Because of its unique mechanism of action and resistance profile, Debio-025 forms an attractive drug candidate for the treatment of HCV infections in combination with polymerase and/or protease inhibitors.

STRUCTURE



Name	Pos3	N4	Pos4
CsA	H	Me	Leu
DEBIO-025	Ala	Et	Val

Fig. 1. Structural formulae of DEBIO-025 (Panel A) and CsA (Panel B). The line delineates the cyclophilin binding domain (top part of the structures) and the calcineurin binding domain (lower part of the structures).

ANTIVIRAL ACTIVITY IN VARIOUS REPLICON CELL LINES

Aim: to compare the anti-HCV activity of Debio-025 with reference compounds in various HCV subgenomic replicon systems.

	compound	Huh mono	HuH6	Huh 9-13	Huh 5-2
Protease inhibitors	BILN-2061	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.03
	VX-950	0.96 ± 0.34	0.96 ± 0.16	1.02 ± 0.88	1.05 ± 0.71
Polymerase inhibitors	HCV 796	/	0.02 ± 0.03	0.08 ± 0.05	0.04 ± 0.01
	JT16	0.79 ± 0.35	1.21 ± 0.47	1.45 ± 0.83	0.80 ± 0.37
	2'-C-methylcytidine	0.67 ± 0.35	0.74 ± 0.29	0.43 ± 0.14	2.42 ± 1.49
	2'-C-methyladenosine	6.64 ± 8.65	0.29 ± 0.04	0.36 ± 0.24	0.41 ± 0.51
	R1479 (4'-azidocytidine)	8.22 ± 4.31	5.69 ±1.29	0.93 ±0.95	1.42 ±0.63
Cyclophilin binding molecules	CsA	0.33 ± 0.16	0.24 ± 0.06	0.46 ± 0.44	0.32 ± 0.11
	Debio-025	0.04 ± 0.02	0.008 ± 0.001	0.04 ± 0.03	0.06 ± 0.02

Data are expressed as the 50% inhibitory concentration (µM). Antiviral activity in Huh 5-2 cells was assessed by luciferase assay or the RT-qPCR assay for Huh mono, HuH6 or Huh 9-13 cells.
Data are mean values ± SD for at least 3 independent experiments.

Conclusion: Debio-025 has, when compared to other HCV inhibitors, a favourable antiviral activity. The 50% effective concentration (EC50) of Debio-025 is on average 10-fold lower then the EC50 obtained for CsA in the same cell line.

CLEARANCE

Aim: to study whether DEBIO-025 is able to clear cells from their replicon.

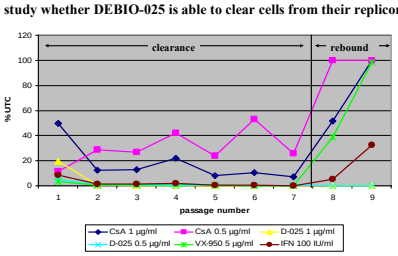
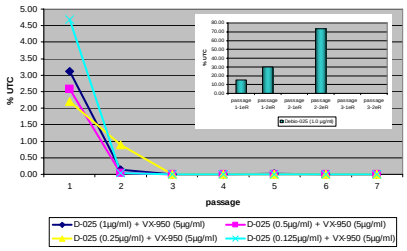


Fig. 2. Clearance and rebound experiment in Huh 9-13 cells. Cells were treated for 7 consecutive passages with the indicated concentration of compounds in the absence of G418 selective pressure. During rebound (passage 8-9) the compounds were omitted from the culture medium but cells were again cultured under the selective pressure of 1000 µg/ml of G418.



Conclusion: IFN (100 IU/ml), VX950 (5µg/ml) or CsA (0.5 µg/ml and 1 µg/ml) were not able to cure the cells from their replicon after 7 passages. Debio-025 could result in complete clearance. By inserting a rebound after every passage we could specify that complete clearance was obtained after 3 passages with Debio-025.
DEBIO-025 even at a concentration as low as 0.125 µg/ml when combined with a concentration of VX-950 (alone not able to clear replicon) resulted in complete clearance as detected by RT-qPCR and the lack of "rebound".

COMBINED RESISTANCE SELECTION

Aim: to study whether DEBIO-025 is able to prevent/delay resistance development against the HCV protease inhibitors VX950 and BILN-2061 and the polymerase inhibitors R1479 and JT-16.

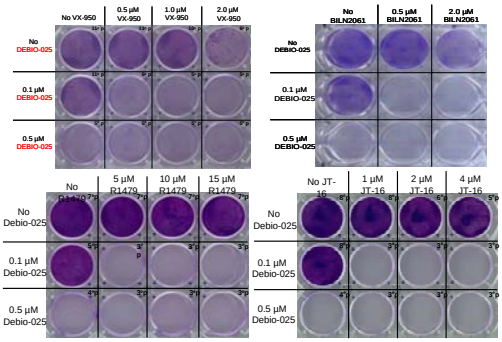


Fig. 3. Combined resistance selection DEBIO-025 with either VX-950 or with BILN-2061: Huh 9-13 cells were cultured under G418 selection in the presence of various combinations of either compound. At the time that cultures became confluent or a sufficiently large number of colonies had developed, cells were further passaged under the same experimental conditions.

Conclusion: DEBIO-025 even at concentrations, as low as 0.1 µM, prevent the emergence of VX-950, BILN-2061, R1479 and JT-16 resistant replicons.

RESISTANCE SELECTION AND PHENOTYPES

Aim: to select DEBIO-025 and CsA resistant replicons

compound	WT	Debio-025 res	CsA res
Debio-025	0.04 ± 0.03	>1.95 [>40]	0.23 ± 0.17 [>5]
CsA	0.46 ± 0.44	4.55 ± 3.98 [± 10]	3.82 ±1.00 [± 8]

Data are expressed as the 50% inhibitory concentration (µM). Antiviral activity in DEBIO-025res and CsAres was assessed by the RT-qPCR assay. Data are mean values ± SD for at least 3 independent experiments.

Conclusion: At least 28 passages of replicon containing Huh 9-13 cells in the presence of increasing concentrations of DEBIO-025 and 52 passages in the presence of CsA were required to obtain drug-resistant replicon containing cells. DEBIO-025 and CsA proved cross-resistant with each other.

ACTIVITY AGAINST VARIOUS RESISTANT REPLICONS

Aim: study whether DEBIO-025 is effective against various polymerase and protease resistant HCV replicons.

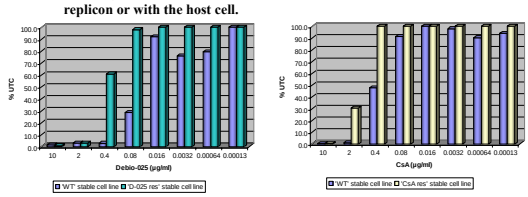
drug	WT	Debio-025 res	CsAres	2CMCres	R1479res	BILN 2061res	VX-950res
Debio-025	0.04 ± 0.03	≥1.9532	0.23 ± 0.17	0.06 ± 0.02	0.10 ± 0.04	0.08 ± 0.03	0.09 ± 0.01
CsA	0.46 ± 0.44	4.55 ± 3.98	3.82 ±1.00	0.21 ± 0.04	0.26 ± 0.05	0.17 ± 0.06	/
2CMC	0.43 ± 0.14	0.41 ± 0.53	0.41 ± 0.40	>30.15	3.16 ± 1.19	0.91 ± 1.09	1.02 ± 0.70
R1479	0.93 ± 0.95	2.76 ± 0.89	1.98 ± 0.50	1.16 ± 0.43	28.63 ± 8.68	2.16 ± 0.27	/
BILN 2061	0.02 ± 0.01	<0.004	≤0.0039	0.04 ± 0.03	0.01 ± 0.01	1.25 ± 0.47	0.79 ± 0.08
VX-950	1.02 ± 0.88	0.36 ± 0.04	0.49 ± 0.32	1.01 ± 0.32	0.65 ± 0.15	0.31 ± 0.05	13.97 ± 1.4

Data are expressed as the 50% inhibitory concentration (µM). Antiviral activity in Huh 5-2 cells was assessed by luciferase assay or the RT-qPCR assay for Huh mono, HuH 6 or Huh 9-13 cells.
Data are mean values ± SD for at least 3 independent experiments.

Conclusion: DEBIO-025 was equipotent against WT HCV replicon as against the resistant replicon cell lines. Also note that the other compounds stayed active against the D025 resistant replicon.

RESISTANCE IS CELL OR GENOME ASSOCIATED?

Aim: to study whether DEBIO-025 or CsA resistance is associated with the replicon or with the host cell.

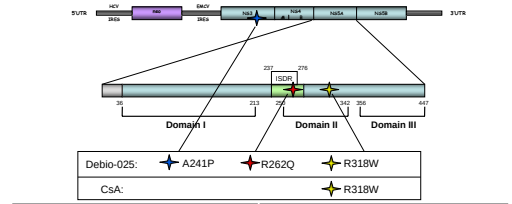


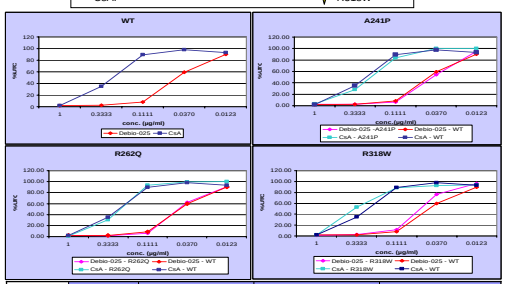
	WT	Debio-025 res	CsA res
Debio-025	0.05 ± 0.01	0.54 ± 0.08 [± 10]	0.69 ± 0.62 [>10]
CsA	0.37 ± 0.06	4.60 ± 0.66 [>10]	1.30 ± 0.15 [± 3.5]

Fig. 4. Antiviral effect of DEBIO-025 or CsA on Huh7-Lunet cells transfected with DEBIO-025res or CsAres replicons: Total RNA was isolated from DEBIO-25res or CsAres replicon containing cells. The RNA was transfected in Huh-7-Lunet cells using DMRIE-C reagents. Transfected cells were subjected to G418 selection until a stable cell line was obtained. Next, 5000 cells were seeded in each well of a 96 well plate and different dilutions of the tested compound were added. EC_{50} values were determined using RT-qPCR.

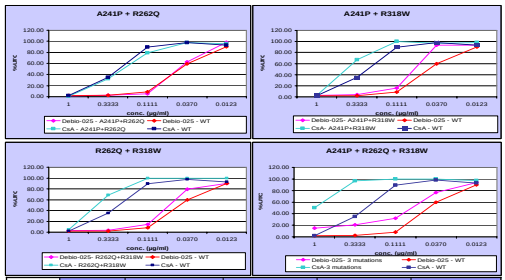
Conclusion: The observed resistance in the original cell lines is not fully restored, indicating that resistance is in part replicon-associated. Furthermore, cross-resistance of Debio-025 and CsA was confirmed using these cell lines. In conclusion the results indicate that resistance is in part replicon-associated.

RESISTANCE ASSOCIATED MUTATIONS





	WT	A241P	R262Q	R318W
Debio-025	0.05 ± 0.01	0.04 ± 0.01 [0.91]	0.05 ± 0.02 [0.98]	0.06 ± 0.01 [1.28]
CsA	0.25 ± 0.01	0.22 ± 0.01 [0.87]	0.24 ± 0.04 [0.96]	0.35 ± 0.01 [1.43]



	WT	A241P + R262Q	A241P + R318W	R262Q + R318W	3 mutations
Debio-025	0.05 ± 0.01	0.05 ± 0.02 [1.02]	0.07 ± 0.00 [1.58]	0.06 ± 0.01 [1.37]	0.08 ± 0.01 [1.72]
CsA	0.25 ± 0.01	0.22 ± 0.03 [0.89]	0.45 ± 0.01 [1.81]	0.46 ± 0.03 [1.84]	0.56 [2.35]

Fig. 5. Identified mutations in resistant cell lines. The genotype of resistant HCV subgenomic replicon cell lines was determined by sequencing. The observed mutations were reintroduced into the replicon by means of site-directed mutagenesis, after which RNA transcript were transiently transfected into Lunet cells and an antiviral assay was set up to determine the antiviral activity of Debio-025 and CsA.

Conclusion: Only the R318W mutation resulted in a small shift of the EC50 value whereas no shift was noted for the other mutations. With the double or triple mutations a more pronounced shift was observed provided that R318W was present.

CONCLUSIONS

DEBIO-025, a potent cyclophilin inhibitor, which is devoid of the immunosuppressive action of CsA has/is:

- **Excellent anti-HCV properties *in vitro*.**
- **Resistance occurs but is a slow process *in vitro*.**
- **Can delay onset of resistance against other compounds *in vitro*.**
- **Remains active against resistant replicons (protease and polymerase inhibitors)**
- **Unique mechanism of action and resistance profile**
- **Currently in phase 2 clinical trial**

REFERENCES

Paeshuyse *et al.*, (2006) Hepatology 43: 761-770