IN VITRO COMBINATION STUDIES OF ACH-1625 (HCV NS3 Protease Inhibitor) & ACH-2928 (HCV NS5A Inhibitor) IN PRESENCE AND ABSENCE OF RIBAVIRIN

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ABSTRACT
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Background

Due to high replication rate and poor fidelity of RNA polymerase, preexisting variants of hepatitis C virus resistant to any single direct-acting anti-HCV agent are deemed inevitable. Therefore, therapy with direct antiviral agents (DAAs) will require combination treatment to achieve sustained virologic response (SVR). In light of the profound effect of ribavirin (RBV) on sustained viral response (SVR) when telaprevir was combined with Peg-interferon-α [1], inclusion of RBV in development of DAA regimens may help prevent virologic breakthrough. In this in vitro study, the combinatory effects of two direct-acting antiviral agents, ACH-1625 and ACH-2928, were investigated for potency and for prevention of emergence of resistance in presence or absence of RBV.

ACH-1625 is a potent, linear, noncovalent HCV NS3 protease inhibitor. In a phase I study, ACH-1625 was safe and well-tolerated at doses up to 2000 mg / day for 4.5 days. Administration of ACH-1625 in hepatitis C patients at doses of 200, 500 and 600 mg BID for 4.5 days or 600 mg QD for 5 days resulted in 3.63, 4.25, 3.94, and 3.81 $\log_{10}$ mean maximum drop of plasma HCV RNA, respectively. Currently, ACH-1625 is being evaluated in a phase 2 study in combination with Peg-interferon-α and ribarvirin.

ACH-2928 is an NS5A inhibitor that displays low picomolar potency against genotypes 1-6 HCV replicons. Preclinical pharmacokinetic characteristics of ACH-2928 support once-daily dosing. The potency and safety profile of ACH-2928 support its evaluation for treatment of HCV infected patients.

Methods

Potency Assay

- **Cells and replicons:** The Huh-Luc/neo cell line harboring a genotype 1b/strain Con-1 HCV subgenomic replicon was used in the study. Transiently replicating subgenomic replicons were also used. These transiently replicating replicons were derived as follows: 1) Sequences encoding either the NS3 protease domain or the entire NS5A were PCR amplified from HCV RNA isolated from patient plasmas and were cloned into a shuttle vector which encodes a transiently replicating Con-1 subgenomic replicon missing either NS3 protease domain or NS5A domain. 2) Pooled plasmid clones (NS3 protease) or a plasmid clone (NS5A) were amplified. 3) Replicon RNAs were synthesized in vitro from the aforementioned plasmid preparations. 4) Replicon RNAs were transfected into Huh-Lunet cells.
- **Evaluation of antiviral activity:** Cells were seeded in 96-well plates and inhibitors were added on the second day in 6 half-log serial dilutions. Three days after addition of inhibitors, the level of HCV RNA replication was quantified with luciferase reporter assay.

Short Term Combination Assay

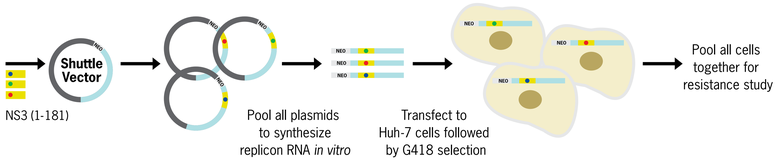
Huh-luc/neo cells were seeded in 96-well plates. On the second day, inhibitors were serially diluted 1:2 and added in a matrix format to the 96-well plates, achieving a defined set of varying drug concentrations and ratios. Cells were incubated for 3 days, after which the level of HCV RNA replication was quantified with luciferase reporter assay. The data were analyzed with a three-dimensional analytical method developed by Prichard and Shipman [2], yielding volumes, in positive (above the plane in a three-dimensional graph) or negative (below the plane in a three-dimensional graph). A positive volume indicates synergy whereas a negative volume indicates antagonism.

Colony Formation Assay

Replicon cells: In addition to Huh-luc/neo cells which harbor a laboratory strain, cells that harbor a population of replicons carrying natural variations in NS3 protease from a patient sample (ID 2034) were also used (Figure 1).

Evaluation of effect on emergence of Resistant Variants: Cells seeded into 6-well dish at a density of 5 X 10^4 were treated with inhibitors either alone or together at several concentrations in the presence of G418 till the cells formed visible colonies.

FIGURE 1. Establishment of Cells Stably Harboring Replicons Carrying Natural Variations in NS3 Protease



Results

1 ANTI-REPLICON ACTIVITY IN VITRO

TABLE 1. Potency against Cell Line Harboring Lab-Strain Replicon[1]

Compound	DMEM + 10% FBS				DMEM + 40% Human Serum	
	EC_{50} ± SD	EC_{90} ± SD	CC_{50}	SI[2]	EC_{50}	FI[3]
ACH-1625	11 ± 4.8 nM	31 ± 9.0 nM	>31.6 μM	>2873	37 ± 13 nM	3.2
ACH-2928	2.7 ± 1.3 pM	6.8 ± 2.4 pM	>10 μM	>3712454	18 ± 5.9 pM	6.6

1. The study was performed by using Huh-luc/neo cell line which harbors a GT-1b and strian-Con-1 subgenomic replicon and the anti-replicon activity was determined by measuring the reporter luciferase activity.
2. SI (Selective Index) = CC_{50}/EC_{50}
3. FI (Fold Increase) = EC_{50} in the presence of 40% Human Serum/EC_{50} in the presence of 10% FBS.

TABLE 2. ACH-1625 Potency vs Chimeric Replicons with NS3 Protease from Clinical Strains

Compound	EC_{50} against GT-1a (nM)						EC_{50} against GT-1b (nM)						EC_{50} (nM)		
	H77	P1	P2	P3	P4	P5	Con-1	P6	P7	P8	P9	P10	Range	Mean	Median
ACH-1625	15	3.9	3.2	2.8	7.9	30	4.6	3.0	20	6.7	30	1.9	1.9–30	13	7.3
Telaprevir	213	133	63	190	246	63	301	355	7225	82	300	400	63–7225	851	230

TABLE 3. ACH-2928 Potency vs Chimeric Replicons with NS5A from Clinical Strains

Compound	EC_{50} against GT-1a (pM)						EC_{50} against GT-1b (pM)						EC_{50} (pM)		
	H77	P1	P2	P3	P4	P5	Con-1	P6	P7	P8	P9	P10	Range	Mean	Median
ACH-2928	13	13	17	13	12	9.6	1.4	0.5	1.5	0.5	1.1	6.8	0.5–17	7.5	8.2

ACH-1625

- Displays potent activity against both genotype 1a and 1b replicons including ones harboring the pooled NS3 protease domain from clinical isolates.
- The EC_{50} mean, median and range of ACH-1625 against genotype-1 strains is 13, 7.3 and 1.9 – 30 nM, respectively.
- The EC_{50} mean, median and range of Telaprevir against all tested genotype-1 strains is 851, 230 and 63 – 7225 nM, respectively.

ACH-2928

- Displays pM potency against both genotype 1a and 1b replicons including ones harboring the NS5A protein from clinical isolates.
- The EC_{50} mean, median and range of ACH-2928 against genotype-1 strains is 7.5, 8.2 and 0.5 – 17 pM, respectively.

2 SHORT TERM COMBINATORY ANTI-REPLICON ACTIVITY

TABLE 4. Combination of ACH-1625 and ACH-2928 in Presence and Absence of RBV for Three Days

	VOLUME (Mean + SD)(95% Confidence Intervals)			
	ACH-1625 and RBV	ACH-2928 and RBV	ACH-1625 and ACH-2928	ACH-1625 and ACH-2928 and RBV[1]
Synergy	47 ± 28	54 ± 3.5	115 ± 0.6	239 ± 36
Antagonism	1.5 ± 2.1	17 ± 23	1 ± 1.6	0 ± 0.57
Overall	Additive to Synergistic	Additive to Synergistic	Synergistic	Highly Synergistic

1. RBV (ribavirin) concentration used is 10 μM, a concentration approximately equal to the plasma trough concentration of RBV in clinical setting [3]. At this concentration, RBV shows no anti-replicon activity nor toxicity in our assay system.

REFERENCES
1. Hezode, C., et al., Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. N Engl J Med, 2009. 360(18): p. 1839-50.
2. Prichard, M.N., L.E. Prichard, and C. Shipman, Jr., Strategic design and three-dimensional analysis of antiviral drug combinations. Antimicrob Agents Chemother, 1993. 37(3): p. 540-5.
3. Maynard M, Pradat P, Gagnieu MC, Souvignet C, Trepo C.Prediction of sustained virological response by ribavirin plasma concentration at week 4 of therapy in hepatitis C virus genotype 1 patients.Antivir Ther. 2008;13(4):607-11.

FIGURE 2. ACH-1625 + RBV

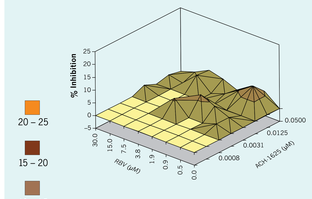


FIGURE 3. ACH-2928 + RBV

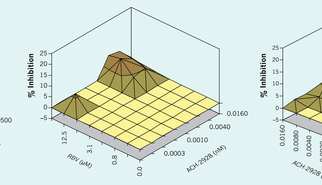
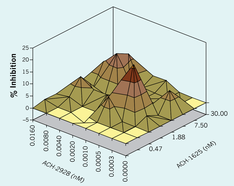
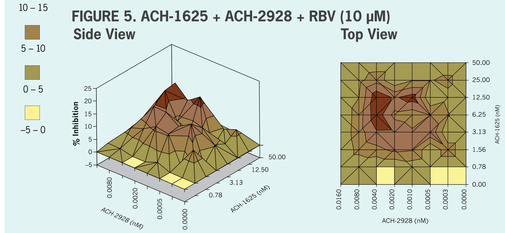


FIGURE 4. ACH-1625 + ACH-2928



20 – 25
15 – 20
10 – 15
5 – 10
0 – 5
–5 – 0

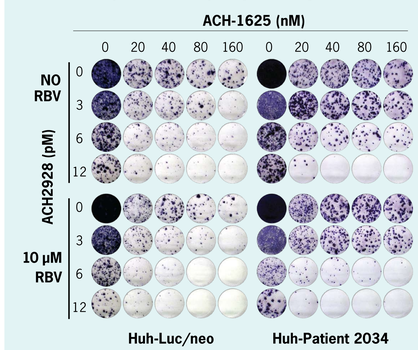
FIGURE 5. ACH-1625 + ACH-2928 + RBV (10 μM)



- ACH-1625 and ACH-2928 in combination with RBV respectively yielded no antagonistic effect.
- Combination of ACH-1625 and ACH-2928 in the absence of RBV yielded high synergistic effect.
- In presence of 10 μM RBV, concentration approximately equal to the plasma trough concentration of RBV in clinical setting, the synergistic effect is enhanced.

3 COMBINATORY EFFECT ON EMERGENCE OF RESISTANCE COLONIES

FIGURE 6. Comparison of Frequency of Colony Formation



Note: The treated wells underwent massive cell death followed by formation of colonies. The media were replaced twice a week in treated wells whereas the cells became confluent shortly after plating in the untreated wells so the media had to be replenished more frequently in the untreated wells to avoid cell death due to lack of nutrients.

TABLE 5. Comparison of Susceptibility and Amino Acid Sequences in NS3 Protease Domain

Sample ID	Amino Acid Position in NS3						EC_{50} (nM)	
	7	13	26	48	54	56	ACH-1625	Telaprevir
Con-1	S	L	R	Y	T	Y	3.9	261
2034[1]	A	V	K	I	S	F	9.8	2298

1. PCR products from HCV RNA isolated from a patient plasma were subjected directly for sequencing as well as cloned into a transient shutter vector. Replicon RNA were made from pooled plasmids and were tested for susceptibility after transfecting into Huh-Lunet cells.

- The frequency of colony formation, a consequence of development of resistant to the inhibitor(s), is higher in cells harboring a population of replicons with natural variation in NS3 protease domain under ACH-1625 selection alone.
- Combination of ACH-1625 and ACH-2928 reduced the frequency of resistant colonies significantly in both cell types.
- In presence of RBV, the frequency of resistant colonies was further reduced in most wells.

Conclusions

- Combination of ACH-1625 and ACH-2928 results in a synergistic antiviral effect.
- Combination of ACH-1625 and ACH-2928 blocks the emergence of the resistant colonies.
- In presence of RBV, the synergistic antiviral activity as well as the effect on preventing the emergence of the resistant colonies is enhanced.
- These data support clinical evaluation of combination of NS3 protease inhibitor ACH-1625, NS5A inhibitor ACH-2928 and RBV.

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