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Combination of TMC435 with two novel NS5B inhibitors increases anti-HCV activity and results in a higher genetic barrier *in vitro*

O. Lenz, J.M. Berke, H. de Kock, P. Van Remoortere, O. Nyanguile, S. Vendeville, T. Jonckers, P. Raboisson, K. Simmen, G. Fanning, T. Lin

Tibotec BVBA, Belgium

Corresponding author:
Oliver Lenz
Tibotec BVBA
Generaal De Wittelaan L11 B3
B-2800, Mechelen
Belgium
+3215461624
e-mail olenz@its.jnj.com

Introduction

- **TMC435 is a macrocyclic NS3/4A protease inhibitor currently in Phase IIb clinical development for the treatment of hepatitis C virus (HCV) infection.**
- **It is a potent and selective inhibitor of NS3/4A *in vitro*, with a 50% effective concentration (EC_{50}) of 8 nM in a genotype-1b replicon cell line.[1]**
- **Findings from Phase I and IIa studies have demonstrated that TMC435 is well tolerated, has a pharmacokinetic profile that supports a once-daily (QD) dosing regimen, and demonstrates potent antiviral activity in both treatment-naive and -experienced genotype-1-infected patients.[2-5]**
- **Since combinations of specifically targeted antiviral therapies for HCV (STAT-Cs) with different mechanisms of action may provide more efficacious HCV treatment, we performed *in vitro* replicon studies to assess the potential of combining TMC435 with one or two novel HCV NS5B polymerase inhibitors (a non-nucleoside inhibitor [Tib-NNI] and a nucleoside inhibitor [Tib-NI]); here we report these findings.**

Methods

- The effect of combining TMC435 with Tib-NNI and/or Tib-NI on *in vitro* anti-HCV activity, genetic barrier to resistance and replicon clearance was assessed using three different assays.

Anti-HCV activity

- This was a 3-day antiviral assay conducted using HCV-genotype-1b-replicon-containing cells with luciferase (Luc) readout, followed by combination analysis.
- Huh7-Luc replicon-containing cells were seeded at a density of 2,500 cells/well in a 384-well plate in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal calf serum (FCS) and incubated in the absence of G418 with a range of serially diluted combinations of TMC435, Tib-NNI and Tib-NI, according to the checkerboard method.
- After 72 hours of incubation, the Luc signal was measured with a ViewLux reader (PerkinElmer), and the effect of each combination was assessed using the Bliss independence model based on the algorithm developed by Prichard and Shipman,[6] using the MacSynergy™ II software.

Colony formation

- Colony formation was determined using HCV-genotype-1b-replicon-containing cells in the presence of TMC435, Tib-NNI and/or Tib-NI.
- Huh7-Luc replicon cells (20,000) were seeded in a 10 cm dish containing DMEM plus 10% FCS and treated with different concentrations of a single inhibitor or with two inhibitors combined, in the presence of 1,000 µg/mL G418.
- Cells were incubated, and inhibitor and media were refreshed twice weekly.
- When significant cell death had occurred (approximately 2–3 weeks), the remaining colonies were stained with neutral red and counted.

Replicon clearance-rebound assay

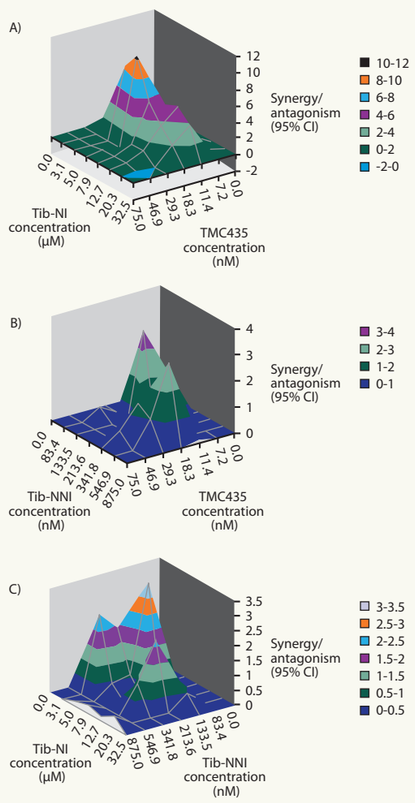
- HCV-replicon ribonucleic acid (RNA) levels during clearance-rebound were assessed using HCV-genotype-1b-replicon-containing cells.
- Huh7-Luc replicon cells (300,000) were seeded in a 10 cm dish containing DMEM plus 10% FCS and cultured in the presence of one or more of the inhibitors in the absence of G418 (clearance phase).
- Cells were passaged as needed (typically twice weekly) and HCV RNA was extracted.
- After 14 days, inhibitors were removed and cells were incubated for 21 days in the presence of 250 µg/mL G418 (rebound phase).
- HCV replicon RNA and cellular RPL13A transcript levels were quantified using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR), and HCV replicon RNA levels were normalised to RPL13A transcript levels.
- The number of cell colonies observed at the end of the experiment was counted.

Results

Effect of inhibitor combinations in an antiviral assay

- The effect of combining TMC435, Tib-NNI and Tib-NI on anti-HCV activity is shown in Figure 1 and Table 1.

Figure 1. Effect of combining (A) TMC435 and Tib-NI, (B) TMC435 and a Tib-NNI, and (C) Tib-NI and Tib-NNI on antiviral activity.



Three-dimensional synergy plots at the 95% confidence interval, as produced by the MacSynergy™ II software for representative experiments are shown
CI, confidence interval

Table 1. Antiviral activity of different combinations of TMC435, Tib-NNI and Tib-NI.

Combinations	Synergy volumes at 95% CI (µM²%)	Antagonism volumes at 95% CI (µM²%)	Combination effect
TMC435 + Tib-NNI	5.67	-0.43	Additive (insignificant synergism)
TMC435 + Tib-NI	37.89	0.11	Synergistic
Tib-NNI + Tib-NI	16.91	-0.61	Additive (insignificant synergism)

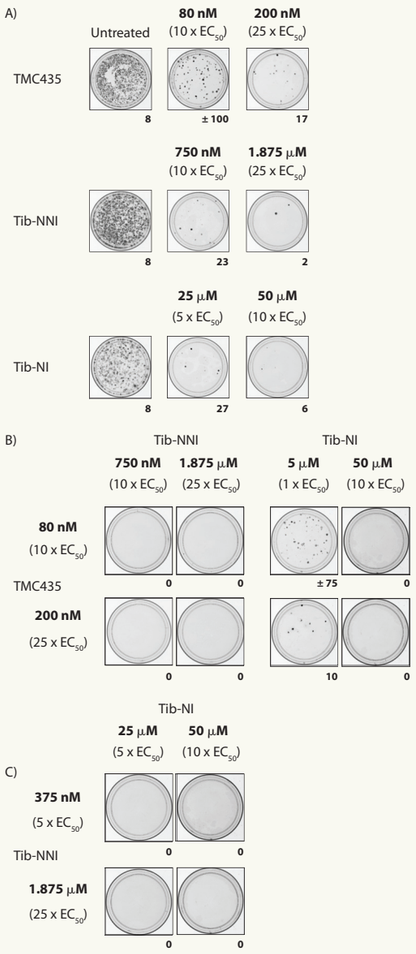
Synergy and antagonism volumes at the 95% confidence interval (CI), as produced by the MacSynergy™ II software. Synergy volumes of <25, 25–50, 50–100 and >100 indicate insignificant synergism, slight synergism, moderate synergism and strong synergism, respectively. Results shown are averages from two or more experiments

- Treatment of the cells with TMC435 in combination with Tib-NNI or Tib-NI resulted in additive or synergistic anti-HCV activity, respectively.
- Treatment with Tib-NNI in combination with Tib-NI resulted in additive anti-HCV activity.
- No cytotoxicity was observed with any of the combinations tested.

Effect of inhibitor combinations on colony formation

- Cell colony formation, in the presence of TMC435, Tib-NNI or Tib-NI alone and in combination, is shown in Figure 2.

Figure 2. Cell colony formation in the presence of TMC435, Tib-NI or Tib-NNI alone (A), or in combination (B and C).



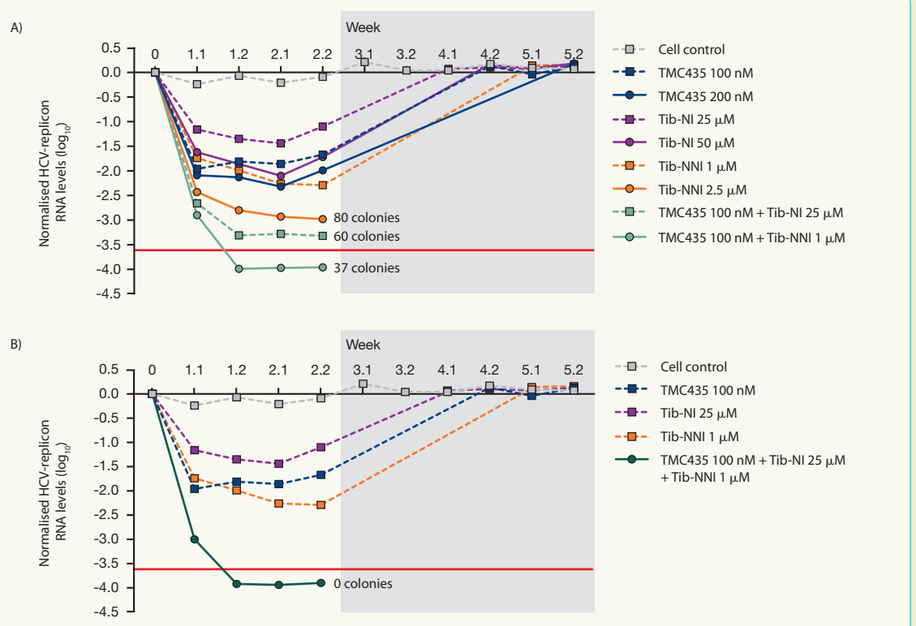
The number of surviving cell colonies is indicated on the right lower corner for each cell culture dish
EC_{50}, 50% effective concentration

- Increasing concentrations of each inhibitor alone resulted in a dose-dependent reduction in colony formation but did not completely prevent resistant replicon colony formation.
- Treatment with TMC435 in combination with Tib-NNI or Tib-NI prevented the formation of resistant replicon colonies.
- Treatment with Tib-NNI in combination with Tib-NI prevented the formation of resistant replicon colonies at the lowest concentration tested.

Replicon clearance-rebound assay

- Clearance of HCV RNA from replicon-containing cells in the presence (clearance phase) and absence (rebound phase) of TMC435, Tib-NNI or Tib-NI alone and in combination are shown in Figure 3.
- All three inhibitors reduced replicon HCV RNA levels during the 2-week clearance phase, but did not lead to total clearance of replicon HCV RNA from cells.
- Treatment with TMC435 in combination with Tib-NNI or Tib-NI increased the initial HCV RNA reduction, although a few replicon colonies were observed in the rebound phase, suggesting incomplete replicon HCV RNA clearance for some combinations.
- Treatment with a combination of all three inhibitors at the lowest concentrations tested resulted in a pronounced reduction in replicon HCV RNA and the most efficient replicon clearance.

Figure 3. Clearance of HCV RNA from replicon-containing cells in the presence of TMC435, Tib-NI and Tib-NNI alone and in combination.



The rebound phase is shaded in grey, and the RT-PCR cut-off is shown as a red line. The number of surviving replicon cell colonies is indicated.
HCV, hepatitis C virus; RNA, ribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction

Conclusions

- **The *in vitro* replicon studies reported here show that combined treatment with an HCV NS3/4A protease inhibitor (TMC435) and an HCV NS5B polymerase NNI or NI:**
  - **is additive or synergistic, with no antagonism observed**
  - **increases anti-HCV activity and raises the genetic barrier to resistance**
  - **results in improved clearance of replicon HCV RNA.**
- ***In vitro* treatment with a combination of all three inhibitors at low concentration further increases replicon HCV RNA clearance.**
- **These *in vitro* virology findings support the further evaluation of TMC435 in combination with HCV NS5B inhibitors.**

References

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