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ScienceDirect
Antiviral Research 73 (2007) 78–83
Antiviral Research
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Short communication

Antiviral interactions of an HCV polymerase inhibitor with an HCV protease inhibitor or interferon *in vitro*

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Received 25 April 2006; accepted 25 July 2006

Abstract

The combinations of Abbott Hepatitis C virus (HCV) polymerase A-782759 with either Boehringer Ingelheim HCV NS3 protease inhibitor BILN-2061 or interferon (IFN) displayed additive to synergistic relationships over a range of concentrations of two-drug combination. Treatment of HCV replicon with A-782759, IFN or BILN-2061 for about 16 days resulted in dramatic reductions in HCV RNA (5.1, 3.0 and 3.9 $\log_{10}$ RNA copies, respectively). However, none of the compounds tested alone lead to replicon RNA reduction to undetectable levels. Ongoing replication in the presence of A-782759 or BILN-2061 was associated with the appearance of resistant mutations M414T in NS5B and D168V in NS3, respectively. In contrast, a combination of A-782759 with BILN-2061 resulted in greater than 7 logs RNA reduction leading to undetectable replicon RNA after 16 days of treatment. Our findings suggest that a monotherapy with either drug alone is likely to result in development of resistant mutants. However, a combination therapy with polymerase inhibitor has the potential to improve the efficacy of IFN or a protease inhibitor alone *in vivo*, due to the lower likelihood of resistance development.

Keywords: HCV replicon; Polymerase inhibitor; Protease inhibitor; Drug combination; Synergy

Hepatitis C virus (HCV) infection is one of the major health care problems in the world. The current standard of therapy consists of combination treatment with pegylated interferon (PEG-IFN) and ribavirin (RBV). PEG-IFN plus RBV therapy is now achieving sustained virologic response (SVR) rates of about 50% for genotype 1 and 80% for genotype 2 and 3. (Davis et al., 1998; McHutchison et al., 1998; Davis, 1999a,b; Manns et al., 2001; Di Bisceglie and Hoofnagle, 2002; Fried et al., 2002). Besides variable effectiveness, IFN-RBV regimens have serious side effects, which often lead to treatment discontinuation.

Inspired by the success of anti-HIV small molecule inhibitors, an industry-wide drug discovery and development effort has been ongoing for specific anti-HCV agents. Based on the reports in the scientific and patent literature, the main targets for HCV drug discovery included two essential viral enzymes: NS3 serine protease and NS5B RNA-dependent RNA polymerase (RdRp) (Dhanak et al., 2002; De Francesco et al., 2003; Pause et al., 2003; Frecer et al., 2004; Gopalsamy et al., 2004; Howe et al., 2004; Ni and Wagman, 2004; Stansfield et al., 2004; Summa et al., 2004; Perni et al., 2006). In the recent years, three protease inhibitors, BILN-2061 (Boehringer Ingelheim), SCH 503034 (Schering Plough), and VX950 (Vertex), (Lamarre et al., 2003; Hinrichsen et al., 2004; Reesink et al., 2005; Sarrazin et al., 2005; Zeuzem et al., 2005a,b) and two polymerase inhibitors, NM283 (Idenix) and HCV-796 (Wyeth/Viropharma), (Afdhal et al., 2004; O'Brien et al., 2005; ViroPharma, 2005) have been tested in human clinical trials. The first HCV protease inhibitor BILN-2061 administered in a 2-day oral dosing in HCV infected patients resulted in more than 3 log plasma HCV RNA reduction, demonstrating *in vivo* efficacy of HCV protease inhibitors for the first time. The second protease inhibitor VX950 demonstrated viral RNA decline by more than 3 $\log_{10}$ at 750 mg TID in HCV infected patients. Nucleoside polymerase inhibitor NM283, a prodrug of the active molecule NM107 demonstrated antiviral activity in HCV infected chimpanzees and humans. Nonnucleoside polymerase inhibitor HCV-796 at 1000 mg twice-daily oral administration in patients demonstrated the mean reduction in HCV RNA of 1.4 $\log_{10}$ on day 4, 1.3 $\log_{10}$ on day 7, and 0.7 $\log_{10}$

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doi:10.1016/j.antiviral.2006.07.009

on day 14. Abbott's HCV polymerase inhibitor A-782759 is highly active against the HCV replicon and has good pharmacokinetic characteristics (Pratt et al., 2004).

Historically, resistance development has been the Achilles heel of highly specific small molecule antivirals. Our previous studies showed that both BILN-2061 and A-782759, while possessing excellent antiviral potency, readily select highly resistant replicon variants (Lu et al., 2004; Mo et al., 2005). We have also demonstrated that the replicon variants selected with the combination of the two inhibitors occurred at a much lower frequency and were significantly less fit compared to the single resistant mutants (Mo et al., 2005).

The HCV polymerase inhibitor A-782759 and the HCV NS3 protease inhibitor, BILN-2061 (Fig. 1) have been previously characterized as potent anti-HCV agents with the 1b-N-strain replicon IC_{50} of 100 and 4 nM, respectively (Lu et al., 2004; Mo et al., 2005). Human IFN-α used in this study had the IC_{50} of 7.5 IU/ml in the 1b-N-strain replicon. To assess the potential synergistic antiviral effect of combination of A-782759 with either IFN or BILN-2061 the compounds were tested for inhibition of HCV replicon using a checkerboard titration pattern (serial two-fold dilutions). The 1b-N subgenomic replicon (Ikeda et al., 2002; Yi et al., 2002) was obtained from Dr. Stanley Lemon (UTMB, Galveston, TX, USA). Each compound was serially diluted two-fold, and six dilutions of one compound were combined with six dilutions of another compound according to the checkerboard method. Concentrations tested were chosen to ensure that the IC_{50} of the compounds were in the middle of the serial dilution range. Three experiments were performed with three replicates per experiment. Degree of HCV replication was determined by the SEAP reporter assay (Yi et al., 2002). The response was calculated as percentage in inhibition of SEAP reporter signal, and this was used for statistical analysis. Based on the inhibition curves generated by each compound individually (not shown), we determined the predicted inhibition value for the mixture of two compounds if they were to act in an additive manner, based on two mathematical models: Bliss independence and Lowe additivity (Greco et al., 1995; Thisted, 1988). If the observed inhibition value by the drug combination significantly exceeded that predicted based on additivity, the interaction was considered to be synergistic. On the other hand, if the observed inhibition level fell significantly short of the predicted value, antagonistic interaction was considered.

Using these mathematical models, we have determined that combinations of A-782759 with either IFN or BILN-2061 produced additive or synergistic effect at the majority of the concentrations tested (Fig. 2). Consistent synergy between A-782759 and BILN-2061 was observed by both models at the concentrations near their respective IC_{50}, as well as at the concentrations five times their respective IC_{50}. Synergy between A-782759 and IFN was also detected by both models at concentrations around and below these compounds' IC_{50}. This suggests that a combination therapy with A-782759 may have a potential to improve the efficacy of IFN and BILN-2061 *in vivo*. None of the compounds alone or in combination produced toxicities measured by beta-actin RNA at the concentration tested for additivity or synergistic effect.

To assess the potential advantage of a combination anti-HCV therapy, we have studied the long-term effects of A-782759, BILN-2061 or IFN alone and their combinations on the replicon in tissue culture. HCV 1b-N replicon cells were passaged in the absence or in the presence of A-782759, BILN-2061, and IFN-alpha individually or in various combinations in the absence of neomycin for 20 days. Each compound was used at concentrations 10 times (1000 nM A-782759, 40 nM BILN-2061, 75 IU/ml IFN) and 20 times (2000 nM A-782759, 80 nM BILN-2061, 150 IU/ml IFN) over their respective IC_{50}. In brief, replicon 1b-N cells (10^6 cells) were plated in a T75 flask in 15 ml of supplemented DMEM without G418. Compounds were added, and the cells were grown in a tissue culture incubator at 37 °C, 5% CO_2, to ∼95% confluency (4 days). At each passage, cells were trypsinized, 10^6 cells were frozen in 350 ml of RLT RNA lysis buffer (QIAGEN), and additional 10^6 cells were passed into another T75 flask with fresh medium and inhibitors. At the end of the assay, RNA was extracted from frozen replicon cells saved at the time of passage using QIAGEN RNeasy spin kit and was analyzed in a Real-Time RT PCR assay (Applied Biosystems).

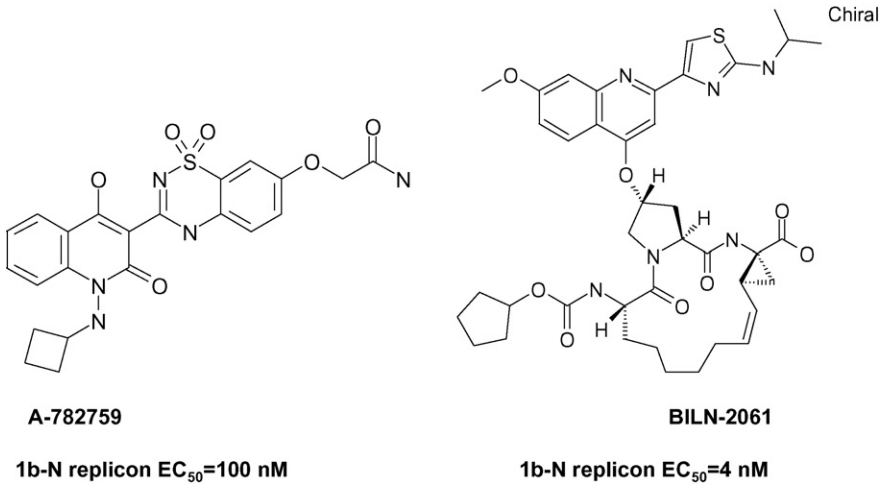


Fig. 1. Structures of the Abbott HCV polymerase inhibitor A-782759 and BILN-2061 and their activity against HCV Replicon 1b-N.

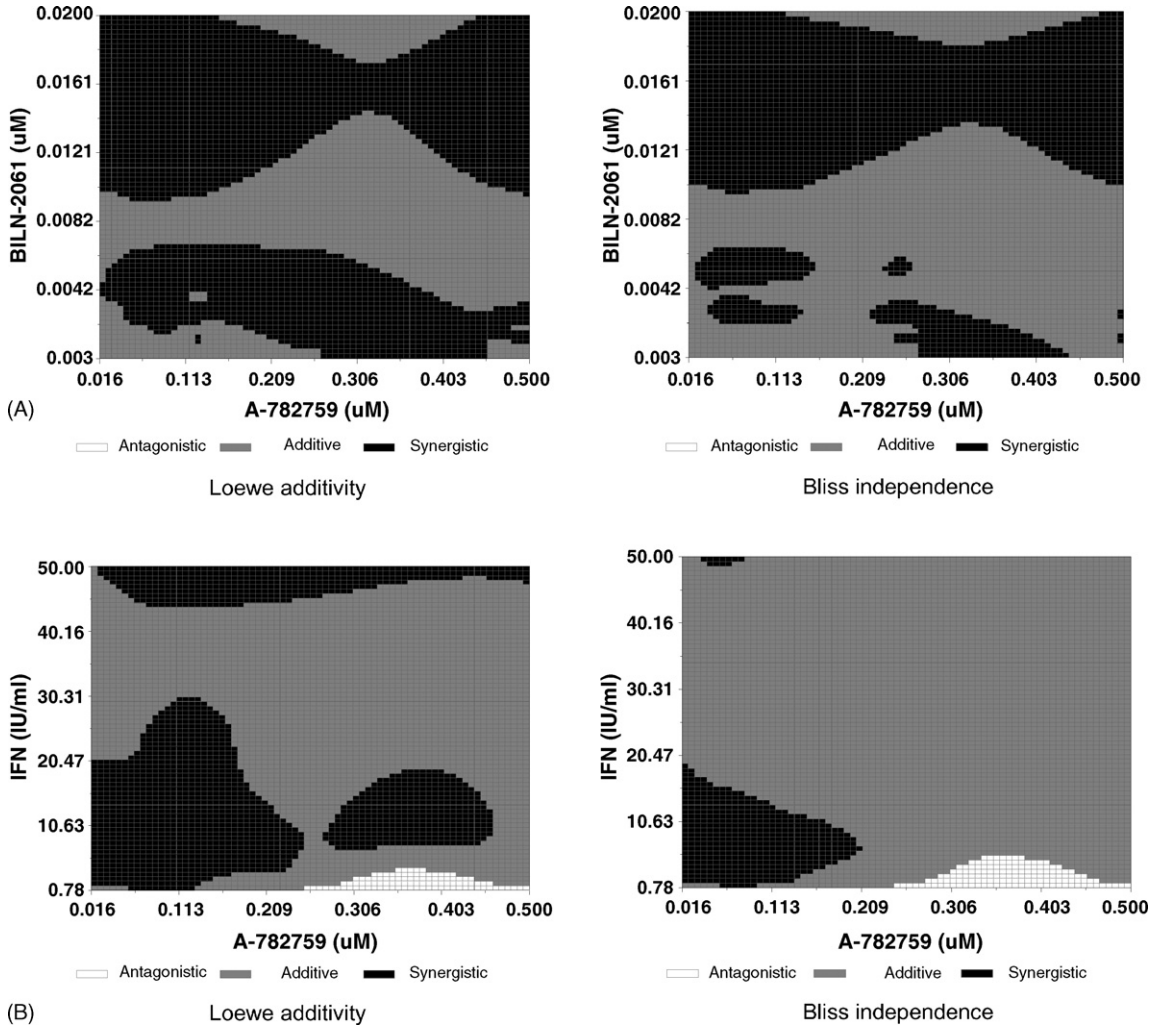


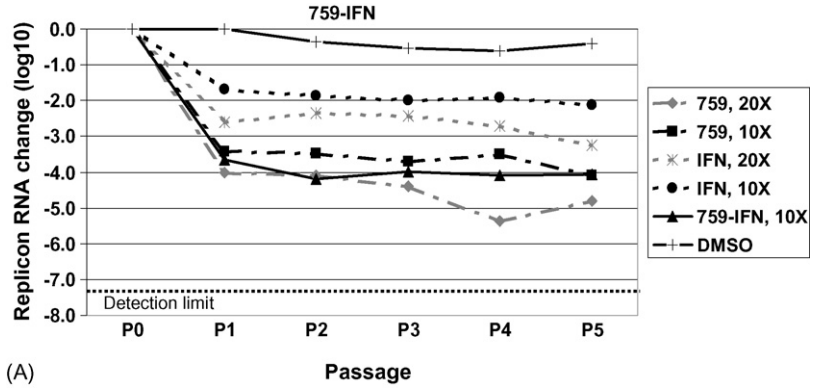
Fig. 2. Combinations of A-782759 with BILN-2061 and IFN exhibited additive and synergistic interactions in a 4-day tissue culture assay. (A) Graphic representation of the A-782759 combination with BILN-2061 at multiple concentrations of the compounds. Dark-shaded areas represent synergy, grey areas represent additivity, and white areas show antagonistic interactions. Interpolations were made at those concentration combinations that were not tested in the experiments while displayed in the contour plots. Replicon RNA inhibition data were analyzed using both Bliss independence and Loewe additivity models. (B) Graphic representation of the A-782759 combination with IFN.

In untreated control cells, the HCV RNA levels were relatively stable over the 5 passages (20 days). Although the HCV RNA levels were dramatically reduced (2–5 log) during treatment with any of the individual compounds at concentrations 10–20-fold above their respective IC_{50}, none of them completely eliminated the HCV RNA from the replicon cells (Figs. 3 and 4). To determine if the ongoing replication was associated with the appearance of resistant mutants, the NS3 and NS5B genes were amplified from the passages 2 and 3 of the replicon cells treated with BILN-2061 and A-782759, respectively (Lu et al., 2004; Mo et al., 2005). As expected, amino acid substitution D168V in NS3 gene and M414T mutation in NS5B gene were detected in the replicon cells treated with BILN-2061 or A-782759, respectively (Figs. 3 and 4A and B). M414T is more than 800 times more resistant to A-782759 than the wild-type, and D168V exhibits 144-fold resistance (Lu et al., 2004; Mo et al., 2005).

Combination of A-782759 with IFN (Fig. 3A) displayed an additional 0.3 log RNA reduction compared to A-782759 alone (Fig. 3B), but failed to completely eliminate HCV RNA from replicon cells. However, the mechanisms of action of IFN in the treatment of HCV *in vivo* are complex and may involve both innate and adaptive immunity-mediated antiviral effects, the latter of which is not assessed in this *in vitro* study. Other small molecule inhibitors described in the literature (SCH) have also benefited from the combination with IFN (Malcolm et al., 2006; Tong et al., 2006).

The combinations of A-782759 and BILN-2061 reduced HCV RNA to undetectable levels (over 7 logs) by passage 4 (16 days, Fig. 4) at concentrations 10 and even 5 times above each respective IC_{50}. Combination of $3X + 3X$ IC_{50} (300 nM A-782759 and 12 nM BILN-2061) produced replicon RNA reduction by 6.6 log, 1.5 log greater than $20X$ IC_{50} of A-782759 (5.1 log reduction, Fig. 4B), and 2.7 log greater than $20X$ IC_{50} of BILN-2061 (3.9 log reduction, Fig. 4B).

The above experiments were conducted in the absence of G418, conditions that allow for propagation of HCV-cured cells at the rates similar to those of cells containing HCV replicon.



(A)

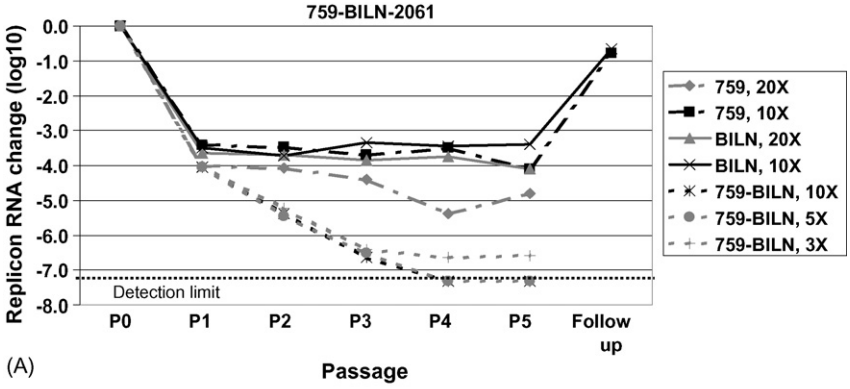
(B)

Treatment	Log RNA change	
759, 20X	-5.1	NS5B: M414T/M
759, 10X	-3.8	
IFN, 20X	-3.0	
IFN, 10X	-2.0	
759-IFN, 10X	-4.1	

Fig. 3. Long-term replicon RNA inhibition by the combination of A-782759 and IFN. 1b-N replicon cells were grown in the presence of each compound alone and in combination in the absence of the selectable marker G418. Replicon RNA levels were measured at each passage. (A) Long-term replicon RNA inhibition dynamics, passages 0 to 5. (B) Replicon RNA log change from the baseline caused by the antiviral treatments. The value shown is the average of RNA change at passages 4 and 5 (after replicon RNA decline has reached plateau). Mutation in NS5B (residue 414) was identified in the replicon population at passages 2 and 3. All experiments were performed in duplicates.

To detect any residual ongoing replication of HCV replicon in the cells following the long-term treatments with inhibitors, we plated passage five cells treated with the inhibitors in the growth medium containing G418 (400 μg/ml). Multiple colonies were selected within 2 weeks among the cells treated with single antiviral compounds and combinations of IFN with A-782759. In contrast, no colonies were selected with the cells treated with combinations of A-782759 and BILN-2061 suggesting a complete elimination of replicating RNAs by this potent combination treatment.

The colonies selected with G418 after treatments with A-782759 or BILN-2061 alone were pooled to form A-782759 or BILN-2061-resistant cell lines. HCV RNA analysis showed that both resistant pools had 3600 and 4700 copies per cells in the A-782759 and BILN-206-resistant pools, respectively (Fig. 4A: follow-up) slightly lower than the RNA level in the



(A)

(B)

Treatment	Log RNA change	
759, 20X	-5.1	NS5B: M414T/M
759, 10X	-3.8	
BILN, 20X	-3.9	NS3: D168V/D
BILN, 10X	-3.4	
759-BILN, 10X	-7.3	
759-BILN, 5X	-7.3	
759-BILN, 3X	-6.6	

Fig. 4. Long-term replicon RNA inhibition by the combination of A-782759 and BILN-2061. (A) Long-term RNA dynamics, passages 0 to 5 (see legend for Fig. 3). After passage 5, cell populations treated with 10*X* EC_{50} of either A-782759 or BILN-2061 were grown for 3 weeks in the absence of the antiviral compounds in the media containing 400 μg/ml G418. The follow-up time point represents replicon RNA levels in the resistant cell population. (B) Replicon RNA log change from the baseline caused by the antiviral treatments (see legend for Fig. 3). Mutations in NS3 (residue 168) and NS5B (residue 414) were identified in the replicon population at passages 2 and 3. All experiments were performed in duplicates.

wild-type 1b-N replicon, ~10,000 copies per cell. This suggests that the drug-resistant variants developed in this study had an only slightly reduced level of fitness compared to that of the wild-type replicon.

Based on what is known about HCV and our experience with HIV, patient heterogeneity and rapid resistance development are anticipated. Indeed, *in vitro* selection experiments using HCV replicon have resulted in identification of resistant mutants to the majority of currently known HCV polymerase and protease inhibitors (Nguyen et al., 2003; Lin et al., 2004, 2005; Lu et al., 2004; Olsen et al., 2004; Kukolj et al., 2005; Mo et al., 2005; Yi et al., 2005; Malcolm et al., 2006; Tong et al., 2006). Results from the clinical trial of VX950 indicated emergence of resistant HCV variants in response to the drug monotherapy (Sarrazin et al., 2005). NS3 protease mutations at residues 36, 54, 155, and 156 confer various levels of resistance *in vitro*. Some of these mutations were observed in patients treated with VX950 as monotherapy (Sarrazin et al., 2005). Therefore, combinations of multiple antiviral agents acting against different targets are likely to be required for consistent achievement of antiviral response in patients.

Our results showed additive and synergistic interactions between A-782759 and IFN and between A-782759 and BILN-2061, respectively, in the 4-day replicon assay. In the long-term assay, the A-782759 plus BILN-2061 combination showed strong combined effect, which resulted in clearing HCV replicon RNA. We find it useful to complement short-term synergy assays with long-term replicon clearance assays because a long-term treatment puts drug synergy in the context of resistance mutants. In a short-term replicon assay (2–4 days), the maximum RNA inhibition usually does not exceed 99.9%, which equals to only a 3 log RNA drop. This fraction mostly represents the wild-type virus population highly susceptible to the potent antiviral compounds. The remaining 0.1% of viral RNA and its quasispecies composition is key to determining the long-term outcome of the treatment. These quasispecies are by chance likely to contain pre-existing mutations that confer resistance to the compound. Therefore, it is useful to determine whether a synergistic pair of drugs can actually clear the virus in a long-term treatment and not select dual-resistant variants.

In conclusion, we have demonstrated that the Abbott HCV RNA polymerase inhibitor A-782759 exhibits additive to synergistic interactions with IFN and BILN-2061. Furthermore, the combination of A-782759 and BILN-2061 is capable of clearing HCV RNA in a long-term replicon assay, which would predict an excellent clinical performance of A-782759 (or a structurally similar compound) in combination with a peptidomimetic protease inhibitor such as BILN-2061.

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