ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2008, p. 3433–3437 Vol. 52, No. 9
0066-4804/08/$08.00+0 doi:10.1128/AAC.01534-07

Comparative In Vitro Anti-Hepatitis C Virus Activities of a Selected Series of Polymerase, Protease, and Helicase Inhibitors[▽]†

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Received 28 November 2007/Returned for modification 16 January 2008/Accepted 8 July 2008

We report here a comparative study of the anti-hepatitis C virus (HCV) activities of selected (i) nucleoside polymerase, (ii) nonnucleoside polymerase, (iii) α,γ-diketo acid polymerase, (iv) NS3 protease, and (v) helicase inhibitors, as well as (vi) cyclophilin binding molecules and (vii) alpha 2b interferon in four different HCV genotype 1b replicon systems.

Worldwide, more than 170 million people are chronically infected with hepatitis C virus (HCV) and are thus at increased risk of developing serious, life-threatening liver disease. Current standard therapy for chronic hepatitis C consists of pegylated interferon in combination with ribavirin (26). Unfortunately, this therapy results in a sustained virological response in only about 50 to 60% of the patients treated and is associated with serious side effects. There is an urgent need for new therapeutic strategies (10).

Small-molecule inhibitors that target, in particular, the NS3 protease or the NS5B RNA-dependent RNA polymerase (RdRp) have been pursued as potential new therapies. BILN 2061 (culprivir), a peptidomimetic inhibitor of the HCV NS3 protease, was the first selective inhibitor of HCV to be administered to patients chronically infected with HCV (genotype 1). Administration of the compound resulted in a rapid and pronounced decline in viral replication (11, 12), but the drug was not developed further because of toxicity issues (11).

Following the pioneering studies with BILN 2061, numerous anti-HCV compounds progressed toward clinical studies; three other NS3 protease inhibitors, i.e., VX-950 (telaprevir), SCH 503034 (boceprevir), and TMC435350, entered clinical trials. VX-950 has shown good efficacy both in monotherapy (29) and in combination with the current standard therapy (8) and is currently in phase II clinical studies. Boceprevir treatment reduced the mean viral load by 1.1 to 2.7 $\log_{10}$ during a 14-day trial in HCV genotype 1-infected patients (32). Recently reported data by Schering-Plough from an ongoing phase I study reveal a high rate of early virological response when SCH 503034 was combined with pegylated interferon and ribavirin (32). TMC435350, when given as a single dose of 200 mg for 5 consecutive days, reduced the HCV viral load by 3.9 $\log_{10}$ (28).

Besides protease inhibitors, a number of nucleoside or nonnucleoside polymerase inhibitors are or have been in development. 2′-*C*-Methylcytidine (the active component of valopicitabine) inhibits, as its 5′-triphosphate metabolite, the HCV polymerase. Clinical development of the compound was stopped in phase II clinical studies due to side effects. Treatment with R1626, the prodrug of the nucleoside analog 4′-azidocytidine (R1479), resulted in an up to 3.7-$\log_{10}$ viral load reduction (30). R7128 (the prodrug of PSI-6130) resulted in a mean 2.7-$\log_{10}$ viral load decline during 14 days of monotherapy (27). Also, treatment with the nonnucleoside polymerase inhibitor HCV 796, a benzofuran, resulted in a marked viral load reduction. However, clinical development of this compound has been stopped because of adverse effects. VCH-759, a new nonnucleoside thiophene-2-carboxylic acid RdRp inhibitor, caused a 2.5-$\log_{10}$ decrease in HCV RNA after 10 days of treatment in HCV-infected patients and was generally well tolerated in early clinical studies (5). Debio-025, a nonimmunosuppressive analogue of cyclosporine A, is a potent inhibitor of HCV replication (24). During a 15-day phase 1b study of human immunodeficiency virus-HCV-coinfected patients, treatment with Debio-025 resulted in an average 3.6-$\log_{10}$ HCV viral load reduction (7). Various other inhibitors of in vitro HCV replication have been reported but were not developed further.

A direct comparative study of the in vitro anti-HCV activities of various antiviral drugs has so far not been reported. It may, however, be useful to have information available about the relative potency of molecules with reported anti-HCV activity. Therefore, we collected a variety of HCV replication inhibitors with different targets and evaluated their anti-HCV activities side by side either in Huh-7 cells carrying the genotype 1b bicistronic reporter replicon I_{389}luc-ubi-neo/NS3-3′/5.1 (Huh-5-2 cells), the bicistronic selectable replicon I_{377}/NS3-3′/wt (Huh-9-13 cells), or the monocistronic selectable replicon I_{389}/hygro-ubi-NS3-3′/5.1 (Huh-Mono cells) (1, 20, 21, 36) or

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† Supplemental material for this article may be found at http://aac.asm.org/.

▽ Published ahead of print on 14 July 2008.

in HuH6 cells carrying a replicon (HuH6*) similar to that carried by Huh-5-2 cells (38). For detailed descriptions of the genetic makeup of the different replicons and methods used, see the supplemental material.

BILN 2061 proved to be significantly more potent than the other two protease inhibitors studied ($P < 0.05$ [Mann-Whitney U test] for all data set pairs in each replicon-containing cell line) (Table 1). BILN-2061 is about 15- to 250-fold more potent than VX-950 and 13- to 200-fold more potent than SCH 503034. Comparable differences in potency between BILN 2061 and VX-950 were reported earlier (17). The in vitro anti-HCV activity of BILN 2061 reported here is comparable to the activity reported by Lin and colleagues (17), whereas VX-950 proved about threefold less potent in our study. Lin et al. generated their data by using a replicon that is very comparable to the Huh-9-13 system; the difference observed may be the result of a variety of factors, such as the higher number of cells seeded at the start of the assay (10,000 cells/well versus 5,000 cells/well in our assay), the lower amount of serum in the culture medium (2% versus 10% in our assay), or a shorter assay duration (48 h versus 72 h in our assay) (17, 18). The activity of SCH 503034 in Huh-5-2 cells was comparable to the activity reported by Malcolm et al. (0.2 μM) (22); only in the Huh-Mono replicon system did this molecule prove about sevenfold less effective than the published data (22). Again, comparable replicon systems were used in a slightly altered assay format (4,000 cells/well versus 5,000 cells/well in our assay and daily refreshing of the inhibitor). These slight alterations might explain the difference in 50% effective concentrations (EC_{50}s) for Huh-Mono cells; however, they do not explain why these parameters did not affect the data obtained with other replicon constructs.

Overall, most of the nucleoside inhibitors have an anti-HCV activity that is comparable to that of SCH 503034 or VX-950. Our data are consistent with those reported in the literature for genotype 1b replicons (3, 12, 14, 33, 34). 4′-Azidocytidine proved to be about six- to eightfold less active than the 2′-*C*-methyl analogues in HuH6* cells. A possible explanation for this difference can be a different nucleoside/nucleotide metabolism in HuH6 cells compared to Huh-7 cells affecting phosphorylation of the compound(s).

The other RdRp inhibitors studied had activities largely comparable to those of the protease inhibitors VX-950 and SCH 503034, except for HCV 796 benzofuran, which proved significantly more potent than VX-950 and SCH 503034 ($P < 0.05$, except for SCH 503034 versus HCV 796 in Huh-Mono cells [$P = 0.057$]). The benzofuran HCV 796 proved to be the most potent nonnucleoside inhibitor in all of the replicon-containing cell lines studied (for all data pairs of HCV 796 with other nonnucleoside inhibitors in different cell lines, $P < 0.01$, except for HCV 796 versus GSK-4 or versus JT16 in Huh-Mono or HuH6* cells and HCV 796 versus thiophene carboxylic acid in Huh-9-13 cells [$P > 0.05$]), with a potency comparable to that of the protease inhibitor BILN 2061 (for all HCV 796 versus BILN 2061 pairs with both data sets obtained in the same cell line, $P > 0.05$, except for HCV 796 versus BILN 2061 in HuH6* cells [$P < 0.01$]). The thiophene carboxylic acid was significantly more active in Huh-Mono cells than both protease inhibitors VX-950 ($P = 0.01$) and SCH 503034 ($P = 0.001$) in the same cell line. The thiophene carboxylic acid proved also to be more potent in HuH6* cells than VX-950 ($P = 0.002$). Overall, the thiophene carboxylic acid inhibitor had comparable activities in different replicon systems and was slightly less active than reported in the literature (15). Factors that may explain this variation include differences in the fetal bovine serum concentration or the detection method used (*Renilla reniformis* luciferase instead of firefly luciferase or quantitative reverse transcription-PCR). All other parameters are essentially the same in our study and in the previously published report. The benzothiadiazine RdRp inhibitor proved, overall, to be as potent as VX-950. However, the benzothiadiazine was about 5- to 20-fold less active in HuH6* cells and roughly 3-fold less potent in Huh-5-2 or Huh-Mono cells compared to the activity reported by Dhanak et al. ($EC_{50} = 0.5 \pm 0.1$ μM) (6). A possible explanation for the differences observed might be the different cell lines used and/or the difference in the experimental setups (number of cells per well, normalization procedure, or assay duration). The activity of the nonnucleoside RdRp inhibitor JT-16 proved comparable to that of either VX-950 or SCH 503034. However, published data report the compound to be about threefold more potent than in the present study (9).

Although the diketo acid (compound 30) was reported to be a nanomolar (i.e., 45 nM) in vitro inhibitor of the HCV RdRp (35), we show here that the molecule exhibits very little activity in cell culture. 4,5,6,7-Tetrabromobenzotriazole (TBBT) and 5,6-dichloro-1-(β-D-ribofuranosyl)benzotriazole (DRBT) were reported to inhibit HCV helicase in an in vitro assay (50% inhibitory concentration of 20 to 60 μM for TBBT [2] and 0.1 to 1.5 μM for DRBT [2], depending on the nature of the template). TBBT resulted in some inhibitory effect in the replicon system which was comparable to the inhibition in the enzymatic assay. DRBT, however, proved much less effective in the HCV subgenomic replicon system than anticipated from the activity in the enzymatic system.

The anti-HCV activity of cyclosporine A was comparable to published data ($EC_{50} = 0.25$ μM) (23, 24, 37). The nonimmunosuppressive cyclophilin binding molecule Debio-025 inhibited HCV replication in all of the assay systems tested, with EC_{50}s ranging from 8 to 60 nM. The compound proved equipotent to BILN 2061 in both Huh-5-2 and Huh-9-13 cells; however, in Huh-Mono ($P = 0.023$) and HuH6* ($P = 0.005$) cells, Debio-025 proved less (~4- to 12-fold) potent than BILN 2061.

Alpha 2b interferon proved to be a very potent inhibitor of HCV replicon replication in all of the systems used. In genotype 1-infected patients, however, host factors (for example, age, sex, race, body weight, insulin resistance, etc.) and viral factors (for example, HCV genotype and initial HCV RNA level) decrease the efficacy of interferon therapy to a mere 50 to 60% sustained virological response rate (4).

Overall, no significant differences were observed between the EC_{50}s of all of the compounds studied in the different replicon systems ($H = 0.131$, df = 3, $P = 0.988$ [Kruskal-Wallis test]). The interreplicon variation of the average EC_{50} appears to be random and not inherent to a specific replicon construct, as shown by the location of the median EC_{50} in the box plots and the similar distribution of the data sets for each cell line (see Fig. S6 in the supplemental material). Employing a single HCV 1b replicon construct should thus suffice to detect the

TABLE 1. Effects of selected compounds on HCV replicon replication[a]

Compound	EC_{50} (μM)				CC_{50} (μM)			
	Huh-Mono cells	HCV replicon-containing HuH6 cells	Huh-9-13 cells	Huh-5-2 cells	Huh-Mono cells	HCV replicon-containing HuH6 cells	Huh-9-13 cells	Huh-5-2 cells
Protease inhibitors								
BILN 2061	0.010 ± 0.005	0.004 ± 0.002	0.015 ± 0.009	0.04 ± 0.03	>3	>3	>3	>3
SCH 503034	2.0 ± 0.1	0.35 ± 0.07	0.13 ± 0.04	1.4 ± 0.8	>33	>33	>33	>33
VX-950	1.0 ± 0.3	1.0 ± 0.2	0.6 ± 0.1	1.0 ± 0.7	>33	25 ± 7	24 ± 4	>33
Polymerase inhibitors								
Nucleoside inhibitors								
2′-*C*-Methylcytidine	0.7 ± 0.3	0.7 ± 0.3	0.4 ± 0.1	2 ± 1	>33	>33	25 ± 10	>33
2′-*C*-Methyladenosine	1.7 ± 0.5	0.29 ± 0.04	0.4 ± 0.2	0.12 ± 0.03	>33	>33	21 ± 6	>33
4′-Azidocytidine	2.2 ± 0.8	6 ± 2	1.6 ± 0.8	1.4 ± 0.6	>33	>33	>33	>33
β-D-2′-Deoxy-2′-*F*-2′-*C*-methylcytidine	5 ± 2	1.1 ± 0.7	1.7 ± 0.6	2.1 ± 0.4	>33	>33	>33	>33
Nonnucleoside inhibitors								
GSK-4 benzothiadiazine	1.8 ± 0.5	11.3 ± 0.8	0.6 ± 0.1	1.8 ± 0.4	>33	>33	>33	>33
Thiophene carboxylic acid	0.6 ± 0.2	0.29 ± 0.04	0.08 ± 0.07	0.3 ± 0.1	>33	>33	>33	>33
JT16 benzimidazole	0.8 ± 0.3	1.2 ± 0.5	1.5 ± 0.8	1.1 ± 0.8	24 ± 2	21 ± 2	24 ± 5	21.5 ± 0.7
HCV 796 benzofuran	0.004 ± 0.001	0.02 ± 0.01	0.03 ± 0.02	0.019 ± 0.005	>33	>33	>33	>33
Diketo acid inhibitor compound 30	23 ± 8	>33	>33	23 ± 8	28 ± 4	>33	>33	>33
Helicase inhibitors								
TBBT	61 ± 2	43 ± 19	65 ± 6	40 ± 23	>100	>100	>100	>100
DRBT	53 ± 13	12 ± 10	34 ± 16	10 ± 4	>100	>100	>100	>100
Cyclophilin binding compounds								
Cyclosporine A	0.3 ± 0.2	0.24 ± 0.06	0.5 ± 0.4	0.3 ± 0.1	24 ± 2	7 ± 4	7 ± 3	6 ± 3
Debio-025	0.04 ± 0.02	0.05 ± 0.04	0.04 ± 0.03	0.06 ± 0.02	>3	>3	>3	>3
Interferon								
Intron A	0.020 ± 0.006[b]	0.00012 ± 0.00007[b]	0.031 ± 0.016[b]	0.005 ± 0.003[b]	>0.002[b]	>0.002[b]	>0.002[b]	>0.002[b]
Intron A	30 ± 27[c]	38 ± 37[c]	20 ± 9[c]	7 ± 5[c]	>1,200[c]	>1,200[c]	>1,200[c]	>1,200[c]

[a] The EC_{50} is the concentration of compound required to reduce the replicon content by 50% as measured by either the luciferase (Huh-5-2 cells) or the quantitative reverse transcription-PCR assay (Huh-Mono, Huh-9-13, and HuH6 cells). The CC_{50} is the concentration of compound required to inhibit the exponential proliferation of the cells by 50% as determined by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt] assay. Data are mean values ± standard deviations for 2 to 10 independent experiments.

[b] Nanomolar concentration.

[c] Value is in international units.

anti-HCV activity of a particular compound. However, parallel analysis with a number of replicon constructs and cell lines might be of use in excluding a potential inhibitory effect on heterologous elements (for example, luciferase, encephalomyocarditis virus internal ribosome entry site, etc.) required for optimal function of the replicon.

Most, if not all, of the inhibitors included in this study were discovered following lead optimization with (due to technical limitations) a genotype 1b subgenomic replicon system. However, more than six genotypes and 50 subtypes of HCV have been identified (19). Furthermore, within one patient, the virus (a specific subtype of a specific genotype) exists as a quasispecies. This genetic diversity can also result in amino acid diversity in drug binding pockets. Therefore, molecules optimized for genotype 1b are not necessarily (equally) active against other genotypes (13, 16, 25, 31). Furthermore, variants resistant to selective HCV drugs may readily emerge. For example, HCV 796-resistant variants were readily selected during the dosing period of the phase I clinical trial in which patients received this drug as monotherapy. VX-950-resistant viruses were also readily selected with suboptimal dosing of VX-950 (31). Thus, a drug should (i) be effective against as many genotypes as possible and (ii) have a high barrier to resistance selection (16) to be used in a specifically targeted antiviral therapy regimen for HCV. Moreover, inhibitors that comprise a successful specifically targeted antiviral therapy regimen for HCV should not have an overlapping resistance profile.

In conclusion, we report on a side-by-side comparison of the anti-HCV activities of a selection of anti-HCV compounds that have various molecular targets. Overall, no significant differences in an inhibitor between different replicon-containing cell lines were observed. If any variation of the average EC_{50}s was observed, it appeared to be random and not inherent to a specific replicon construct. To rapidly exclude the possibility that newly identified inhibitors exert their activity via an effect on heterologous elements, parallel analysis with cell lines containing different HCV subgenomic replicon constructs may be warranted. Our data show that activity in enzymatic assays (as was shown for the helicase inhibitors DRBT and diketo acid compound 30) do not necessarily guarantee activity in cell culture-based assays. The data presented here may serve as a reference panel to estimate the potency of novel HCV inhibitors that may be discovered in the future, as well as for other available molecules that were not included in this study.

We acknowledge Katrien Geerts for excellent technical assistance and Dominique Brabants for dedicated editorial help.

This work was supported by a postdoctoral position in part from the Fonds voor Wetenschappelijk Onderzoek, Vlaanderen, and from the Onderzoeksfonds of the Katholieke Universiteit Leuven to Jan Paeshuyse and by VIRGIL, the European Network of Excellence on Antiviral Drug Resistance (grant LSHM-CT-2004-503359 from the Priority 1 Life Sciences, Genomics and Biotechnology).

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