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Synergy of Small Molecular Inhibitors of Hepatitis C Virus Replication Directed at Multiple Viral Targets▽

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Chronic hepatitis C virus (HCV) infection is a significant worldwide health problem with limited therapeutic options. A number of novel, small molecular inhibitors of HCV replication are now entering early clinical trials in humans. Resistance to small molecular inhibitors is likely to be a significant hurdle to their use in patients. A systematic assessment of combinations of interferon and/or novel anti-hepatitis C virus agents from several different mechanistic classes was performed in vitro. Combinations of inhibitors with different mechanisms of action consistently demonstrated more synergy than did compounds with similar mechanisms of action. These results suggest that combinations of inhibitors with different mechanisms of action should be prioritized for assessment in clinical trials for chronic hepatitis C virus infection.

Chronic hepatitis C virus (HCV) infection is a major worldwide health problem; in the United States, an estimated 3 million persons are chronically infected (4). Estimates of the health care burden of chronic HCV infection predict a drastic increase in hospitalizations and medical costs related to complications such as cirrhosis and hepatocellular carcinoma over the next 1 to 2 decades (3). Effective and better-tolerated therapy for HCV could effectively stem this tide (7).

Current interferon-based therapy for chronic HCV infection results in sustained responses in roughly 55% of patients and is accompanied by significant toxicity. Genotype 1 HCV, the most prevalent genotype in the United States, responds less well to therapy with pegylated interferon plus ribavirin, with response rates of 42 to 46% (11, 23). These limitations have spurred an intense drug discovery effort, resulting in a number of promising compounds (8).

Hepatitis C virus replication takes place in the cytoplasm, with the replication complex being tightly associated with lipid membranes (1). Key components of the replication complex include several promising antiviral targets, including the NS3/4A protease and the NS5B RNA-dependent RNA polymerase. A number of candidate protease inhibitors (PIs) which have excellent potency in vitro have been developed (2, 17, 20); several of these compounds have also been evaluated in phase I/II trials, with encouraging results (15, 16, 29, 36). Resistance to this class of inhibitors has been described, with some mutations conferring cross-resistance to several compounds (17, 18, 21, 34, 35).

The NS5B RNA polymerase is also essential for viral replication, and a number of nucleoside inhibitors and nonnucleoside inhibitors (NNIs) of the HCV polymerase with potent activity in vitro and in early clinical trials have been described (5, 12, 13, 27, 30). Resistance to both nucleoside and nonnucleoside inhibitors in vitro has been described (22, 24, 26). We have assessed a number of combinations of HCV inhibitors with several molecular targets currently in development, using an HCV genotype 1 replicon-based luciferase reporter system.

Replicon constructs. The BM4-5 replicon is a subgenomic HCV genotype 1b replicon which contains a deletion of a serine in NS5A and has been previously described (14). The firefly luciferase gene was inserted in the BM4-5 replicon, in a manner previously described (33), to create a luciferase/neomycin phosphotransferase fusion protein (FEO) and the replicon (BM4-5 FEO). Briefly, the *Photinus pyralis* luciferase gene was amplified using primers coding for the AscI restriction site. Following amplification, both the BM4-5 plasmid and luciferase PCR product were restriction digested with AscI. Ligation was then carried out to insert the luciferase gene in phase with the neomycin phosphotransferase gene, creating the desired BM4-5 FEO replicon. The sequence of the replicon was verified by DNA sequencing.

Cell culture. Human hepatoma Huh-7.5.1 cells (a kind gift from Francis Chisari, Scripps Research Institute, La Jolla, CA) and BM4-5 FEO cells stably expressing the BM4-5 FEO replicon were grown at 37°C and 5% CO_2 in Dulbecco's modification of Eagle's medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. BM4-5 FEO cells were additionally grown in the presence of 500 μg/ml of G-418.

Transfection and clone selection. The BM4-5 FEO plasmid was linearized with ScaI. In vitro transcription (Megascript; Ambion) was carried out according to the manufacturer's instruction to yield BM4-5 FEO RNA. Transfection was performed as previously described (32). Four hundred microliters of a Huh-7.5.1 cell suspension (10^7 cells/ml) was placed in a 0.4-cm cuvette with 10 μg of BM4-5 FEO RNA. The mixture was electroporated (Bio-Rad Gene Pulser) at 270 V and 975 μF and transferred to a 10-cm tissue culture dish. G-418 at 500 μg/ml was added at 24 h, and the medium was changed every 3 to 4 days. Individual G-418-resistant colonies were visible within 2 to 3 weeks. Individual colonies were harvested and expanded for characterization of luciferase expression.

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Luciferase compound assay. BM4-5 FEO cells were seeded into 96-well plates at a density of 10,000 cells per well in 100 μl medium. After allowing 4 h for attachment, compounds were added to wells at the specified concentrations. All conditions were run in triplicate. Cells and compounds were incubated for 48 h. The luciferase assay (Bright-Glo; Promega) was carried out according to the manufacturer's instructions. Luciferase activity was determined using a microplate luminometer (Veritas microplate luminometer; Turner Biosystems). The relative light units (RLU) for each condition were reported as the mean ± the standard error of the mean for the three wells.

Compounds tested. Compounds tested included two peptidomimetic HCV PIs, BILN 2061 (16) and a Vertex PI (19) (Vicki Sato, Vertex Pharmaceuticals, Cambridge, MA); a GlaxoSmithKline *trans*-lactam PI active-site mimic (2) (Karen Romines, GlaxoSmithKline, Research Triangle Park, NC); one nucleoside analog HCV RNA-dependent RNA polymerase inhibitor (RdRpI), 2′-C-methyladenosine (10) (William Lee, Gilead Sciences, Foster City, CA); one nonnucleoside GSK benzo-thiadiazine RNA polymerase inhibitor directed at the "thumb" region of the polymerase (Karen Romines, GlaxoSmithKline) (9); and alpha interferon (Interferon-αA; Sigma-Aldrich).

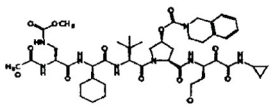
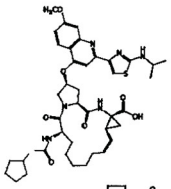
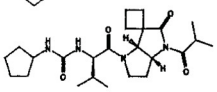
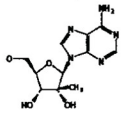
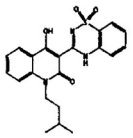
The 50% inhibitory concentration (IC_{50}) of each compound was determined independently and used to set the range of concentrations used for the synergy experiments. Each compound was tested singly and in combination at two twofold serial dilutions above and below the IC_{50}. The ratio of the two compounds tested remained fixed across the dosing range. Potential cytotoxicity of individual compounds and all combinations was assessed using a luminescent ATP-based cell viability assay (Cell Titer-Glo; Promega).

Data analysis. Determinations of compound interactions were based on the median-effect principle and the multiple drug effect equation as described by Chou and Talalay (6). Combination indices (CIs) were determined using Calcusyn (Biosoft) for each experiment at the IC_{50}, IC_{70}, and IC_{90} levels. In total, 15 combinations were evaluated with from three to five replicates per condition; this yielded a total of 61 data points per CI level analyzed. A CI of <0.9 was considered synergistic, a CI of ≥ 0.9 or ≤ 1.1 was considered additive, and a CI of >1.1 was deemed antagonistic.

Statistical analysis. At each of the three inhibitory concentrations evaluated (IC_{50}, IC_{70}, and IC_{90}) the CIs in the three synergy groups were compared using a linear mixed-effects model allowing for different means in the three synergy groups and random effects for the individual drug combinations. The random effects were not significant (likelihood ratio test), indicating no statistical difference in CI values between the antiviral compound combinations in the same synergy group. The CI replicates were further compared between synergy groups by using the Wilcoxon rank test.

Synergy of small molecular inhibitors. Transfection of Huh-7.5.1 cells with BM4-5 FEO RNA yielded numerous (>50) G-418-resistant clones. Individual clones were expanded and assessed using the luciferase assay to determine the individual clones with highest RLU per cell. Four clones yielded from 30,000 to 50,000 RLU per 10,000 cells at 48 h (data not shown); these clones were expanded and used for all subsequent studies.

TABLE 1. Activities of different small molecular inhibitors in the BM4-5 replicon

Compound	Structure	HCV BM4-5 replicon IC_{50} (nM)[a]
IFN-alpha		3.59 IU/ml ± 0.41
Vertex PI (19)		293.10 ± 53.92
BILN 2061 (16)		9.44 ± 0.88
GSK PI (2)		308.6 ± 28.9
2'-C-methyladenosine (10)		427.2 ± 51.2
GSK NNI (9)		3826 ± 460.2

[a] The IC_{50} is the average ± standard error of mean of the results from at least three independent experiments.

The IC_{50} for each of the individual compounds is listed in Table 1. CI_{50}, CI_{70}, and CI_{90} refer to the combination index at the IC_{50}, IC_{70}, and IC_{90}, respectively, of each drug. All compounds tested were additive (CI_{50} and CI_{70}) or mildly synergistic (CI_{90}) with alpha interferon. Antagonism was not demonstrated for any combination of small molecular inhibitors, including compounds targeting the same viral protein. Significantly more synergy was demonstrated between compounds in the group combining two small molecular inhibitors targeting the same viral enzyme (in this case NS3 protease) than between the group of compounds combined with alpha interferon at both the CI_{70} ($P = 0.043$) and CI_{90} ($P = 0.017$) levels. There was no significant difference between the groups at the CI_{50} level ($P = 0.108$) (Fig. 1). Similarly, the group consisting of two inhibitors with different viral targets showed significantly lower combination indices than either of the other two groups, i.e., compounds with interferon ($P < 0.001$ at all levels) or compounds with same mechanism of action ($P = 0.038$ and 0.037 at the CI_{50} and CI_{70} levels, respectively). The comparison of CI_{90}s between small molecular inhibitors with the same and different viral targets showed a trend toward a lower combination index in the group with two compounds with different viral targets ($P = 0.056$ at the CI_{90} level) (Fig. 1). None of the compounds or combinations showed cytotoxicity at the concentrations tested in the activity and synergy studies (data not shown).

Small molecular inhibitors of the HCV protease and poly-

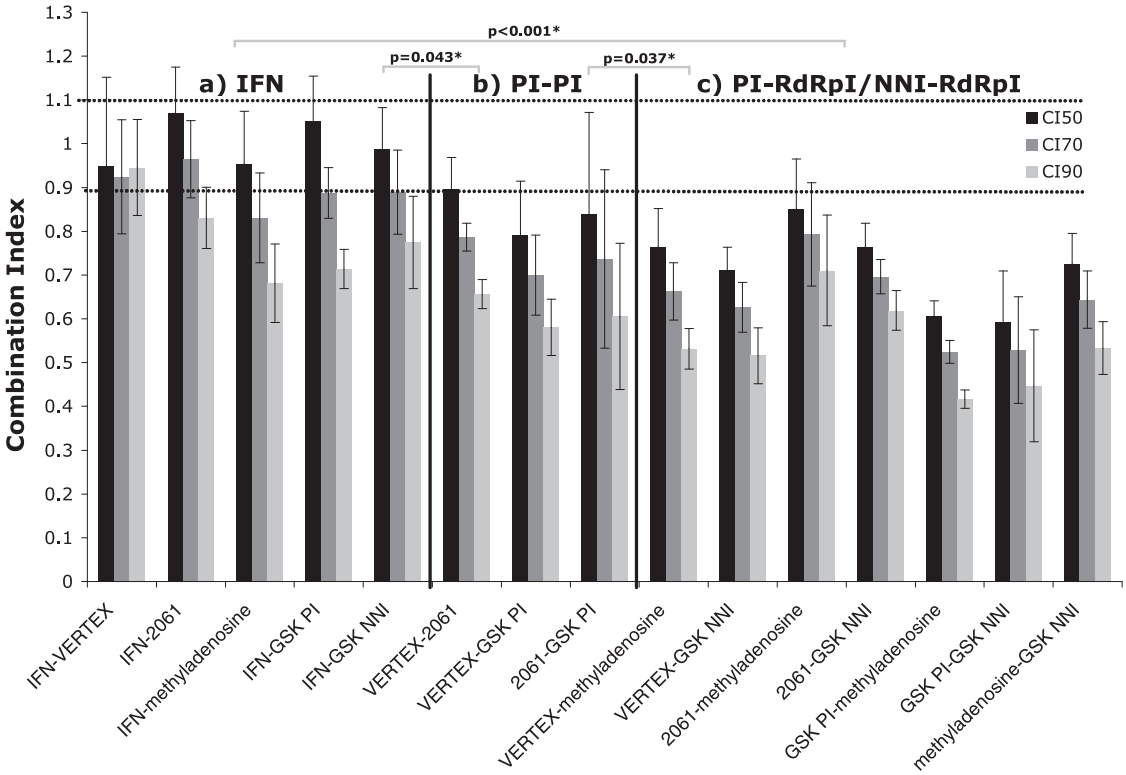


FIG. 1. CI_{50}s, CI_{70}s, and CI_{90}s for the compound combinations evaluated. Dotted lines at combination index values of 0.9 and 1.1 indicate the boundaries of an additive interaction. The *P* values displayed (*) are for analyses at the CI_{70} level.

merase show antiviral activity in our genotype 1 replicon system. Most importantly, no combination of small molecular inhibitors of HCV replication demonstrated antagonism in our system, including those with the same mechanism of action or viral target. Combinations of inhibitors targeting different viral proteins (PI-RdRpI or PI-NNI) or with different mechanisms of inhibiting the same viral protein (RdRpI-NNI) were strongly synergistic and had significantly lower combination indices than the other two groups. Combinations targeting the same site within a viral protein showed lesser degrees of synergy or were additive, but they still possessed significantly lower combination indices than the group composed of the same compounds with alpha interferon. It is important to remember that the definition of synergy as a CI of less than 0.9 is an arbitrary distinction (along a continuum) and thus does not preclude two inhibitors which occupy the same site from being "synergistic" according to a CI of <0.9. Additionally, metabolic interactions between compounds or the impact of divergent resistance pathways on different compounds may also affect the appearance of drug-drug interactions as assessed by the combination index.

HCV, like human immunodeficiency virus type 1, possesses an error-prone RNA polymerase, and it replicates to levels 10- to 100-fold higher than those of human immunodeficiency virus type 1 in chronically infected individuals (25, 28). These characteristics suggest that selection of drug-resistant viral variants will be a challenge to the use of small molecular inhibitors. In fact, resistance to these compounds both in vitro and in vivo has already been described (17, 24, 26, 31, 34).

Synergistic combinations of HCV inhibitors may produce greater viral load decreases in vivo and could potentially delay the appearance of multiply drug-resistant virus. This system provides a useful approach for the in vitro testing of antiviral combinations in anticipation of rationally designed clinical studies of combination chemotherapy directed at HCV. Our results support the evaluation of combinations of small molecular inhibitors in human clinical trials and further suggest that combinations with different mechanisms of action may be particularly attractive.

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