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Synergy of a Hepatitis C Virus (HCV) NS4A Antagonist in Combination with HCV Protease and Polymerase Inhibitors[▽]

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Rapid emergence of resistance to monotherapy with virus-specific inhibitors necessitates combination therapy. ACH-806 is a hepatitis C virus NS4A inhibitor with a novel mechanism of action and resistance pathway. This compound was synergistic with NS3 protease inhibitors and NS5B nucleoside and nonnucleoside polymerase inhibitors.

Significant progress has been made in the discovery and testing of novel inhibitors of hepatitis C virus (HCV) replication (4). The majority of the compounds evaluated in vitro and in early clinical trials have belonged to one of three classes of HCV inhibitors: NS3 protease inhibitors (PI) (11, 17, 29), NS5B nucleoside polymerase inhibitors (21, 22, 24), and NS5B nonnucleoside (NNI) polymerase inhibitors (2, 7, 8, 12). Importantly, resistance to each of these compound classes has been described with some resistance mutations conveying cross-resistance to several inhibitors within a given class (e.g., A156T in HCV protease) (15, 18, 20, 28). Analogous to the experience with human immunodeficiency virus type 1 therapy, combinations of several classes of viral inhibitors with unique mechanisms of action and resistance pathways will be integral to the success of small-molecule-based antiviral therapy for chronic HCV infection.

ACH-806 [1-(4-pentyloxy-3-trifluoromethylphenyl)-3-(pyridine-3-carbonyl)thiourea] is a novel acylthiourea compound with a 50% effective concentration (EC_{50}) of 14 nM in the genotype 1b replicon system and 30 nM in a genotype 1a replicon system (13). A phase 1b proof-of-concept study showed significant antiviral activity at the lowest dose tested (23). ACH-806 possesses a unique mechanism of action. It selectively binds to the NS4A protein, resulting in altered protein composition and inactivation of the replicase complex (13). Given its unique mechanism of action, we sought to evaluate ACH-806 in combination with other small-molecule inhibitors of HCV replication, as well as alpha interferon, in a genotype 1b luciferase reporter replicon system.

(A portion of the results described here was presented as an abstract at the XVI International HIV Drug Resistance Workshop, Hilton Barbados, Bridgetown, Barbados, 12 to 16 June 2007.)

Replicon constructs. The BM4-5 replicon is a subgenomic HCV genotype 1b replicon which contains a deletion of a serine in NS5A (10). The firefly luciferase gene was inserted into the BM4-5 replicon, in a manner we and others have previously described (26, 27), to generate the BM4-5 FEO replicon. The sequence of the replicon was verified by DNA sequencing.

Cell culture and luciferase compound assays. Cell culture and luciferase compound assays were performed as previously described in detail (9, 27). Briefly, 10,000 BM4-5 FEO cells/well were seeded into 96-well plates and incubated for 4 h. Medium was then aspirated and replaced with 100 μl of complete medium containing a single compound or combinations at the desired concentration(s). Plates with compounds were incubated for 48 h and then assayed for luciferase expression (Bright-Glo; Promega). All conditions were run in triplicate, and the number of relative light units for each condition was reported as the mean ± the standard error of the mean of the three wells.

Compounds tested. The Achillion NS4A antagonist ACH-806 (Fig. 1) (John Pottage, Achillion Pharmaceuticals, New Haven, CT) was dissolved in dimethyl sulfoxide to a concentration of 2 mM; further serial 10-fold dilutions were made in complete medium. The additional compounds tested included two peptidomimetic HCV PI, BILN 2061 (14) and a Vertex PI which is a close structural analog of VX-950 (16) (Vicki Sato, Vertex Pharmaceuticals, Cambridge, MA); a trans-lactam GSK-PI active-site mimic (compound 4d in reference 1) (Karen Romines, GlaxoSmithKline, Research Triangle Park, NC); one nucleoside analog HCV RNA-dependent RNA polymerase inhibitor, 2′-C-methyladenosine (6) (William Lee, Gilead Sciences, Foster City, CA); one GSK-NNI benzothiadiazine RNA polymerase inhibitor (compound 4 in reference 5) (Karen Romines, GlaxoSmithKline); and human recombinant alpha interferon A/D (Sigma-Aldrich I4401).

The EC_{50} of each compound was determined independently and used to determine the range of concentrations used for the synergy experiments. ACH-806 was tested singly and in combination with each of the compounds listed above at two twofold serial dilutions above and below the EC_{50}. The ratio of the two compounds, based on the compound's EC_{50}, remained fixed across the dosing range. The potential cytotoxicity of individual compounds and all combinations was assessed with a luminescent ATP-based cell viability assay (Cell Titer-Glo; Promega). All compounds were assessed for cytotoxicity at the highest concentration used both singly and in combination.

Data analysis. Compound interactions were quantified by the approach described by Chou and Talalay (3), relying on the

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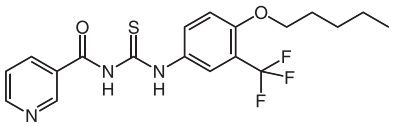

FIG. 1. Chemical structure of ACH-806 [1-(4-pentyloxy-3-trifluoromethylphenyl)-3-(pyridine-3-carbonyl)thiourea].

median effect principle and the multiple-drug effect equation. Isobolograms were generated for each combination tested and were used to determine the additivity, synergism, or antagonism of inhibitor combinations. Combination indices (CI) were determined with Calcusyn (Biosoft) for each experiment at the EC_{50}, EC_{70}, and EC_{90} of the combination. In total, six combinations were evaluated with three to eight experiment replicates per condition. By convention, 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.

The EC_{50} ($\pm$ the standard error of the mean) of ACH-806 in the BM4-5 FEO replicon system was 116.8 $\pm$ 5.4 nM. The EC_{50}s of the other compounds used in this study were as follows: interferon, 4.45 $\pm$ 0.6 IU/ml; Vertex PI, 310.3 $\pm$ 48.4 nM; BILN-2061, 9.33 $\pm$ 0.7 nM; GSK-PI, 301 $\pm$ 23.9 nM; 2′-*C*-methyladenosine, 446.8 $\pm$ 46.2 nM; GSK-NNI, 3.5 $\pm$ 0.4 μM. ACH-806 was additive with alpha interferon at the CI_{50} and CI_{70}; at the CI_{90}, the CI was 0.83 with the 95% confidence interval crossing 0.9 (additivity). Combinations of ACH-806 and either NS3 PI or NS5B polymerase inhibitors (nucleoside and NNI) showed consistent synergy (Fig. 2). No individual compounds or compound combinations showed cytotoxicity at the highest concentrations used in the activity and synergy studies (data not shown).

We have shown that an HCV NS4A antagonist, ACH-806, is synergistic with other small-molecule inhibitors of HCV replication in an HCV genotype 1b replicon system. In vitro, ACH-806 binds directly to NS4A and inhibits HCV replicon replication by altering the composition of the replication complex, resulting in nonfunctional complexes (13). ACH-806-resistant mutants contain mutations in the portion of NS3 which interacts with NS4A; importantly, no cross-resistance has been shown in vitro between ACH-806 and NS3 PI such as VX-950.

The various inhibitors tested in combination with ACH-806 are representative of the major classes of HCV therapeutics currently being developed. Given its error-prone RNA polymerase and high rate of viral turnover (19) and the early appearance of resistant mutants seen both in vitro (15, 18, 20) and in vivo (25) during monotherapy, we believe that combination therapy with several inhibitors will be needed to avoid selection of preexisting viral mutants and obtain durable virus inhibition. To that end, inhibitors which possess complementary actions in vitro (i.e., show synergy) and have divergent resistance pathways should be prioritized for study in clinical

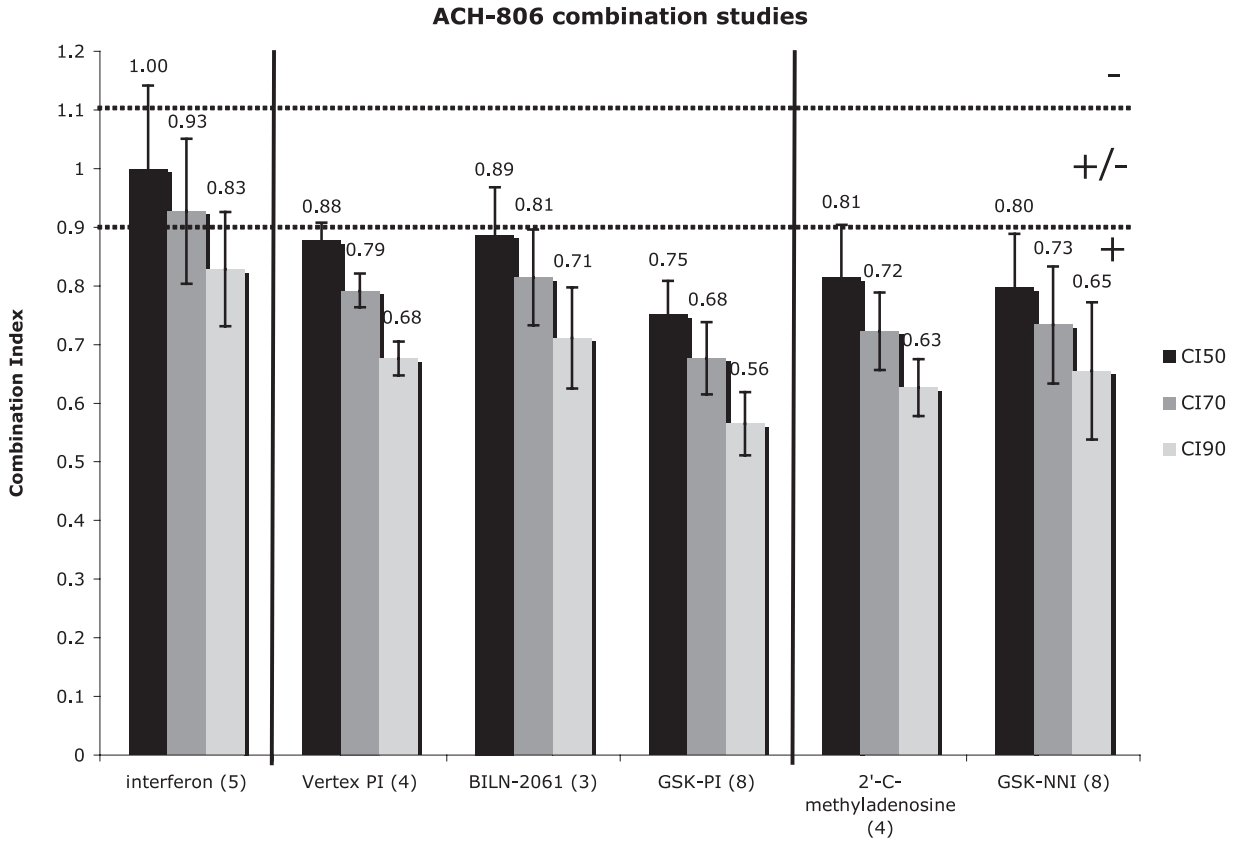

FIG. 2. CI of ACH-806 in combination with various anti-HCV compounds. Numerical values above the bars are mean CI. Error bars represent the standard error of the mean of the CI calculated from the experimental replicates (in parentheses). The dotted lines at 0.9 and 1.1 represent the bounds of an additive interaction. +, synergy; $\pm$, additivity; $-$, antagonism.

trials of combination therapy for HCV infection. NS4A antagonists, such as ACH-806, are attractive compounds to potentially combine with both protease and polymerase inhibitors.

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