Title: Synergy of an HCV NS4A antagonist in combination with HCV protease and polymerase inhibitors.

Running title: synergy of an HCV NS4A antagonist

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Abstract: Rapid emergence of resistance to monotherapy with virus specific inhibitors necessitates combination therapy. ACH-806 is an HCV NS4A inhibitor with a novel mechanism of action and resistance pathway. This compound was synergistic with NS3 protease inhibitors, NS5B nucleoside and non-nucleoside polymerase inhibitors.
Significant progress has been made in the discovery and testing of novel inhibitors of HCV replication (4). The majority of compounds evaluated \textit{in vitro} and in early clinical trials have belonged to one of three classes of HCV inhibitors: NS3 protease inhibitors (11,17,29), NS5B nucleoside inhibitors (21,22,24), and NS5B non-nucleoside 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 HIV-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 hepatitis C infection.

ACH-806 $[1\-(4\text{-}pentyloxy\text{-}3\text{-}trifluoromethylphenyl)\text{-}3\text{-}(pyridine\text{-}3\text{-}carbonyl)]$ is a novel acylthiourea compound with an EC$_{50}$ of 14nM in the 1b replicon system and 30nM in a genotype 1a replicon system (13). A phase 1b proof of concept study showed significant anti-viral 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 molecular inhibitors of HCV replication as well as interferon alpha in a genotype 1b luciferase reporter replicon system.

\textit{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 in 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 assay: Cell culture and compound assays were performed in a manner identical to those previously described in detail (9,27). Briefly, 10,000 BM4-5 FEO cells/well were seeded into 96 well plates and incubated for 4 hours. Media was then aspirated and replaced with 100 μL of complete media containing a single compound or combinations at the desired concentration(s). Plates with compounds were incubated for 48 hours and then assayed for luciferase expression (Bright-Glo, Promega). All conditions were run in triplicate and 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: The Achillion NS4A antagonist ACH-806 (figure 1) (John Pottage, Achillion Pharmaceuticals, New Haven, CT) was dissolved in DMSO to a concentration of 2mM; further serial 10-fold dilutions were made in complete media. Additional compounds tested included: 2 peptidomimetic HCV protease inhibitors (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 GSK trans-lactam PI active site mimic (compound 4d in the cited reference) (1) (Karen Romines, GlaxoSmithKline, Research Triangle Park, NC); one nucleoside analog HCV RNA dependent RNA polymerase inhibitor (RdRpi), 2’-C-methyladenosine (6) (William Lee, Gilead Sciences, Foster City, CA); one non-nucleoside (NNI) GSK benzo-thiadiazine RNA polymerase inhibitor (compound 4 in the cited reference) (5) (Karen Romines, GlaxoSmithKline); and human recombinant Interferon-αA/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 2-fold serial dilutions above and below the EC$_{50}$. The ratio of the two compounds, based on the compound EC$_{50}$, 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). All compound cytotoxicity was assessed at the highest concentration used both singly and in combination.

**Data analysis:** Compound interactions were quantified using the approach described by Chou and Talalay (3) relying on the 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 using CalcuSyn (Biosoft) for each experiment at the EC$_{50}$, EC$_{70}$, and EC$_{90}$ levels for the combination. In total six combinations were evaluated with 3-8 experiment replicates per condition. By convention a CI of < 0.9 was considered synergistic, a CI of $\geq 0.9$ or $\leq 1.1$ was considered additive, and a CI of $> 1.1$ was deemed antagonistic.

The EC$_{50}$ (±SEM) for ACH-806 in the BM4-5 FEO replicon system was 116.8 nM (±5.4). The EC$_{50}$ values for the other compounds used in the studies were: IFN 4.45 IU/mL (±0.6), Vertex PI 310.3 nM (±48.4), BILN-2061 9.33 nM (±0.7), GSK-PI 301 nM (±23.9), 2’-C-methyladenosine 446.8 nM (±46.2), and GSK-NNI 3.5 µM (±0.4). ACH-806 was additive with interferon-α at the CI$_{50}$ and CI$_{70}$ levels; at the CI$_{90}$ level the CI was 0.83 with the 95% confidence interval crossing 0.9 (additivity). Combinations of ACH-
806 and either NS3 protease inhibitors or NS5B polymerase inhibitors (nucleoside and non-nucleoside) showed consistent synergy (figure 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 molecular 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 replication complex resulting in non-functional 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 protease inhibitors 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, 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 pre-existing viral mutants and obtain durable virus inhibition. To that end, inhibitors which posses complementary actions in vitro (i.e. show synergy) and have divergent resistance pathways should be prioritized for study in clinical trials of combination therapy for HCV. NS4A antagonists, such as ACH-806, are attractive compounds to potentially combine with both protease and polymerase inhibitors.
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Figure 1: Chemical structure of ACH-806 [1-(4-pentyloxy-3-trifluoromethylphenyl)-3-(pyridine-3-carbonyl)thiourea].
Figure 2: Combination indices for ACH-806 in combination with various anti-HCV compounds. Numerical values above the bars indicate the mean CI value. Error bars represent the standard error of the mean for the CI value calculated from the experimental replicates (indicated in parentheses). The dotted lines at 0.9 and 1.1 represent the bounds of an additive interaction. +, synergy; +/-, additivity; -, antagonism.