Ribavirin Antagonizes the In Vitro Anti-Hepatitis C Virus Activity of 2’-C-Methylcytidine, the Active Component of Valopicitabine

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Ribavirin antagonizes the in vitro anti-hepatitis C virus (HCV) activity of the pyrimidine nucleoside analogue 2’-C-methylcytidine, the active component of the experimental anti-HCV drug valopicitabine. In contrast, the combination of ribavirin with either the purine nucleoside analogue 2’-C-methyladenosine or the HCV protease inhibitor VX-950 resulted in an additive antiviral activity. These findings may have implications when planning clinical studies with valopicitabine.

Current standard therapy for chronic hepatitis C virus (HCV) consists of the combination of pegylated interferon alpha 2a with ribavirin (RBV) (Fig. 1). This therapy is only effective in approximately 50% to 60% of patients and is associated with significant side effects (5). Several selective inhibitors of HCV replication have been reported, a number of which are being (or have been) studied in clinical trials (4, 9). Valopicitabine is the 3’-O-valine ester of 2’-C-methylcytidine (2’-C-MeCyt) (Fig. 1), a compound that, once phosphorylated intracellularly to its 5’ triphosphate metabolite, is assumed to inhibit the viral RNA-dependent RNA polymerase (3, 6, 10, 11). Valopicitabine is currently being evaluated in phase II clinical trials (1). In 1987, Vogt and colleagues reported that ribavirin antagonizes the anti-human immunodeficiency virus (HIV) activity of the pyrimidine nucleoside analogue 3’-azido-3’-deoxythymidine (AZT) (12). Ribavirin was also shown to antagonize the anti-HIV activity of several other antiretroviral pyrimidine (but not purine) nucleoside analogues (2). Because the active component of valopicitabine is a pyrimidine nucleoside analogue, and because valopicitabine might be used in combination with ribavirin for the treatment of HCV infections, we studied whether the earlier observed antagonism of ribavirin with pyrimidine nucleoside analogues against HIV also extends to the anti-HCV activity of the combination of ribavirin with 2’-C-MeCyt.

To this end, we made use of human hepatoblastoma cells (HuH6) containing a genotype 1b subgenomic HCV replicon derived from the Con1 isolate. In HuH6 cells, replicon replication is independent from ongoing cell proliferation (13). Cells were cultured as described before for Huh 9-13 cells (7). For antiviral evaluation, cells were seeded at a density of 1.5 × 10⁴ cells per well in 96-well cell culture plates, and antiviral assays were carried out as described before (7). After a 3-day incubation period at 37°C, cells were lysed in cells-to-cDNA lysis buffer (Ambion, Cambridgeshire, United Kingdom), and lysates were used to determine the amount of HCV replicon RNA by means of quantitative real-time PCR as described previously (7). The EC₅₀ were calculated as the concentration of compound that caused a 50% reduction in HCV RNA levels compared to that of the untreated control. Serial dilutions of known quantities of a plasmid containing the neomycin gene were used to set up the standard curve. The amount of viral RNA produced in treated cultures was expressed as a percentage of that in the untreated control.

To determine the cytotoxic activity of the (combination of) compounds, an assay was set up in the same way described above, but after 3 days the cell number was determined by means of the MTS/PMS method (Promega, Leiden, The Netherlands). Alternatively, cells were trypsinized and counted with a Coulter Counter (Analis, Ghent, Belgium). The CC₅₀ were

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calculated as the concentration of compound that caused a 50% reduction of the proliferation of exponentially growing replicon cells compared to that of the untreated control.

The effects of drug-drug combinations were evaluated using the method of Prichard and Shipman (8). Combination studies with ribavirin plus 2'-C-MeAdo were performed in three independent experiments: combination studies of ribavirin plus 2'-C-methyladenosine (2'-C-MeAdo) in one experiment and ribavirin plus VX-950 in two independent experiments.

Ribavirin alone inhibited HCV subgenomic replicon replication in HuH6 cells in a dose-dependent manner, with an EC\(_{50}\) of 21 \(\mu\)g/ml ± 5.4 \(\mu\)g/ml (87 \(\mu\)M ± 22 \(\mu\)M). At the highest concentration tested (33 \(\mu\)g/ml; i.e., 135 \(\mu\)M), ribavirin inhibited HCV replicon replication by 70% and did not prove cytostatic or cytotoxic, as assessed by both the MTS method and cell counting (data not shown). Also, 2'-C-MeCyt resulted in a dose-dependent inhibition of HCV replicon replication, with an EC\(_{50}\) of 0.07 \(\mu\)g/ml ± 0.01 \(\mu\)g/ml (0.27 \(\mu\)M ± 0.04 \(\mu\)M); at 6 \(\mu\)g/ml (23 \(\mu\)M) the compound achieved a maximal inhibitory activity of 97% (data not shown). The combined antiviral activity of ribavirin and 2'-C-MeCyt was next evaluated; data were analyzed for synergism, antagonism, or additive effects (8). When a combination is additive, data points form a horizontal surface that equals the zero plane. A surface that lies above the zero plane indicates a synergistic effect of the combination, and a surface below the zero plane indicates antagonism. Three independent experiments were carried out, and average data are presented (Fig. 2A). The combination of ribavirin and 2'-C-MeCyt resulted in a marked antagonistic activity across broad concentration ranges of both drugs, even at concentrations of ribavirin that were well below the EC\(_{50}\) for inhibition of HCV replication. The combination of compounds did not prove cytotoxic to the cells, thus excluding that pleiotropic effects cause the observed antagonism (data not shown).

To study whether the antagonistic effect is specific for the combination of ribavirin with a pyrimidine nucleoside analogue such as 2'-C-MeCyt, we next studied the effect of the combination of ribavirin with either (i) 2'-C-MeAdo (Fig. 2B), a purine nucleoside analogue with a mechanism of antiviral activity similar to that of 2'-C-MeCyt, or (ii) the HCV protease inhibitor VX-950 (Fig. 2C). 2'-C-MeAdo alone inhibited HCV subgenomic replicon replication in HuH6 cells in a dose-dependent manner, with an EC\(_{50}\) of 0.17 \(\mu\)g/ml ± 0.02 \(\mu\)g/ml (0.60 \(\mu\)M ± 0.07 \(\mu\)M). At the highest concentration tested (3.7 \(\mu\)g/ml; i.e., 13 \(\mu\)M), 2'-C-MeAdo inhibited HCV replicon replication by 98% and was not cytotoxic (data not shown). Also, VX-950 resulted in a dose-dependent inhibition of HCV replicon replication, with an EC\(_{50}\) of 1.2 \(\mu\)g/ml ± 0.07 \(\mu\)g/ml (1.7 \(\mu\)M ± 0.10 \(\mu\)M); at 6.0 \(\mu\)g/ml (8.5 \(\mu\)M) it achieved a maximal inhibitory activity of 95% (data not shown). The combinations of ribavirin with either compound resulted in an additive activity (a slight, reproducible, but not significant synergism was observed at low concentrations of ribavirin and 2'-C-MeAdo). An additive effect may be expected for two drugs that have a different mode of action and that do not interfere with each other’s metabolism. None of the combinations containing 2'-C-MeAdo or VX-950 resulted in any detectable cytotoxic activities at the concentrations used. Comparing the averages of the theoretical additive surfaces with those of the experimental surfaces by means of a Student’s \(t\) test, of all three combinations only the combination of RBV with 2'-C-MeCyt resulted in a significant difference (\(P = 0.05\)) (concentrations ranged from 0.22 \(\mu\)g/ml to 0.07 \(\mu\)g/ml for 2'-C-MeCyt and 11 \(\mu\)g/ml to 0.41 \(\mu\)g/ml for RBV).
Our findings that ribavirin antagonizes the antiviral activity of 2′-C-MeCyt are in agreement with earlier observations of the anti-HIV activity of the combination of ribavirin with pyrimidine nucleoside analogues (2, 12). The negative effect of ribavirin on the anti-HIV activity of AZT was shown to result from an inhibition of the intracellular phosphorylation (activation) of AZT (12). It remains to be studied whether (i) ribavirin also inhibits the phosphorylation of 2′-C-MeCyt and (ii) whether the present observation also extends to other pyrimidine analogues with anti-HCV activity. The concentrations at which ribavirin and 2′-C-MeCyt resulted in an antagonistic effect against HCV are within the same range as those observed in human plasma. Following oral administration of 800 to 1,200 mg/day of ribavirin (depending on body weight and HCV genotype), average plasma concentrations of 1.1 to 2.2 μg/ml were reached (M. Nunez, P. Barreiro, and A. Ocampo, 15th Int. AIDS Conf., abstr. MoPeB3277, 2004). Doses of 500 mg/kg of body weight of valopicitabine result in average plasma concentrations of 2′-C-MeCyt of 4.3 μg/ml ± 0.7 μg/ml (X. J. Zhou, N. Afdhal, and E. Godofsky, 40th Annu. Meet. EASL, abstr. 626, 2005).

Since ribavirin is extensively used for the treatment of infections with HCV, and since the oral produrg form of 2′-C-MeCyt (valopicitabine) is being evaluated in clinical studies, a combined therapy of both drugs might be envisaged. However, our present findings argue against a combination therapy of ribavirin and valopicitabine. Moreover, our data also suggest that a combined treatment of patients with ribavirin and HCV protease inhibitors or purine nucleoside analogues may result in an additive antiviral activity.

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REFERENCES