**Influenza Antiviral Drug Development**

**Evaluation of Antiviral Drug Combinations**

**Introduction**

Only four drugs have been approved by the FDA for the prevention and treatment of seasonal influenza virus infections. The clinical usefulness of two of these drugs is limited due to the increasing incidence of resistant viruses in the population.

Combinations of antiviral treatments may offer additive or synergistic advantages over monotherapy, provide broader antiviral activity, and potentially reduce the development of drug-resistant influenza variants.

Using approved influenza virus inhibitors and ribavirin, combinations of antiviral drugs were evaluated to determine potential antiviral synergy using a seasonal strain of influenza A virus.

**Methodology**

**Anti-Influenza Virus Cytoprotection Assay:** MDCK cells (1 x 10^4 cells per well) were seeded in 96-well flat-bottom tissue culture plates and allowed to adhere overnight at 37°C, 5% CO₂. Following incubation, the medium was removed from the cell monolayers and the cells were washed with DPBS.

Six serial half-logarithmic dilutions of ribavirin (RBV), amantadine (AMT) and oseltamivir carboxylate (OSC) (purchased from Sigma Aldrich) were prepared and added to appropriate wells in the microtiter plate. Influenza virus strains were diluted to a predetermined titer to yield 85% to 95% cell killing at 4 days post-infection and were added to the plate.

Following incubation at 37°C, 5% CO₂ for four days, cell viability was measured by XTT tetrazolium dye staining. The optical density was determined spectrophotometrically at 450 and 650 nm using Softmax Pro 4.6 software.

Percent reduction of the cytopathic effects (CPE) of the virus-infected wells and the percent cell viability of uninfected drug control wells were calculated by four parameter curve fit analysis.

**Combination Therapy Assay:** The MacSynergy II combination analysis evaluates the interaction of two or three antiviral compounds in a checkerboard pattern that can be used to determine synergy, additivity, and antagonism across a wide range of concentrations of the test compounds, generating a three dimensional dose response surface to statistically define the interaction of the test compounds. Combination evaluations were performed using MDCK cells infected with influenza A virus in the cytoprotection assay described above.
The raw data from the combination assays were imported into the MacSynergy II software program and the compound interactions were calculated at the 95% confidence interval.

**Results**

The figures below depict the results of the evaluation of two-drug and three-drug combination influenza virus treatment. The AMT/OSC combination was modestly synergistic; the RBV/AMT and OSC/RBV combinations were additive. The AMT/OSC/RBV triple combination was synergistic with the peak synergy volume 5- to 8-fold greater than the synergy volume of the two compound combinations.

**Summary**

These results suggest that *in vitro* combination assays can be successfully used to identify beneficial combinations of antiviral agents and assist with the prioritization of clinical studies of combination influenza virus therapy.

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