Influenza virus infects over 500 million people annually resulting in variable degrees of systemic symptoms, ranging from mild to respiratory failure and death. Even when the predictive strains of the annual vaccine are actually predominant in the population during the typical influenza season, the vaccine is only about 40% protective in infected patients. Only four FDA-approved antivirals (AMT, RBV, OSC, ZM) are available for treatment and they have a short window of opportunity to begin treatment and reduce viral replication and illness. Throughout the years, many of the seasonal influenza viruses predominant to the population have become resistant to AMT or OSC. The development of novel and improved antiviral drugs is still an international need. Therefore, it is important to have well-characterized influenza virus strains for screening potential inhibitors. For drug candidates with varying MOA, it is important to understand the compound selectivity to determine the most appropriate assay to use to better understand the resistance profile. ImQuest has evaluated RBV, AMT, and OSC against a panel of viruses and drug resistant subtypes and antiviral drugs in vitro to better understand the replication kinetics and phenotype of each strain. Time of drug addition experiments were performed using wild-type and drug-resistant influenza viruses to confirm mechanism of antiviral action. Combination therapy is not yet FDA-approved for the treatment of influenza; however, the use of combination therapy would be beneficial in developing therapies inhibiting multiple viral targets for influenza antiviral therapy and a higher genetic barrier to resistance. Using several approved influenza virus inhibitors and RBV, combination therapy was evaluated to determine potential synergistic enhancement using a panel of strains of influenza type A virus. Understanding range of antiviral efficacy, cross-resistance mechanism of action and how a new antiviral could potentially be used in combination with approved antivirals is crucial for developing a better drug to reduce influenza infection.

**METHODS**

**Anti-Influenza Virus Cytoprotection Assay**

Inhibition of viral replication in MDCK cells was measured by XTT tetrazolium dye. Cells (1 x 10^5 cells/well) were seeded in 96-well tissue culture plates, and allowed to adhere overnight at 37°C/5% CO₂. Following inoculation, cells were treated with test compounds for 24 hours. The 50% cell kill was measured by XTT staining. The optical density of the cell culture plate was determined spectrophotometrically at 450 and 690 nm. The percent cell viability of uninfected drug control wells were calculated by four parameter curve fit analysis.

**Variable MOI Assay**

Using the cytoprotection assay described above, the time window was determined how the antiviral activity of Ribavirin, Amantadine and Oseltamivir carboxylate would be affected.

**Time of Drug Addition Assay**

Ribavirin, Amantadine, Oseltamivir carboxylate and T705 were added to MDCK cells at 0, 1, 4, 9 and 24 hours post-infection with wild-type influenza virus and assessed for the antiviral activity of the drug. Concentrations of each drug were varied to yield 25% cell killing at 4, 9 and 24 hours post-infection and were added at the time described above.

**Combination Therapy Assay**

The MacSynergy II combination analysis evaluates the interaction of two or three antiviral compounds in a checkerboard pattern that allows the evaluation of synergy, additivity, and antagonism across a wide range of concentrations of the test compounds. The MacSynergy II combination analysis determines the percentage cell kill that each drug combination is able to achieve.