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Development of Influenza Virus Inhibitors with a Higher Genetic Barrier To Resistance

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ABSTRACT

Influenza virus infects over 500 million people annually resulting in variable degrees of systemic symptoms, ranging from mild fatigue to respiratory failure and death. Even when the predictive strains of the trivalent annual vaccine are actually predominant in the population during the typical influenza virus season, the vaccine is only about 64% protective in immunized patients. Only four FDA-approved antivirals (AMT, RMT, OSC, ZNV) are available for treatment and they have a short window of opportunity to begin treatment following infection in order reduce the duration and severity of illness. Throughout the years, many of the seasonal influenza viruses predominant in the patient population have been resistant to AMT or OSC. The development of novel and improved anti-influenza 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 sensitivity to distinct wild type and resistant clinical strains through development of a robust cross-resistance profile. ImQuest has evaluated RBV, AMT, and OSC against a panel of wild-type and drug resistant subtype A and subtype B influenza viruses in order 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 infection; however, given the prevalence for seasonal drug-resistant virus it would be beneficial to develop therapies inhibiting multiple viral targets for broader antiviral activity and a higher genetic barrier to resistance. Using several approved influenza virus inhibitors and RBV, combination therapy was evaluated to determine potential antiviral synergy using a seasonal strain of influenza type A virus. Understanding range of anti-influenza efficacy, cross-resistance, mechanism of antiviral action and how a new inhibitor could potentially be used in combination with approved anti-influenza drugs are crucial for developing a better drug to reduce influenza infection.

METHODS

Anti-Influenza Virus Cytoprotection Assay: Inhibition of virus-induced cytopathic effects (CPE) and cell viability following influenza virus replication in MDCK cells was measured by XTT tetrazolium dye. Cells (1 x 10^4 cells per well) were seeded in 96-well flat-bottom tissue culture plates and allowed to adhere overnight at 37ºC/5% CO_2. Following incubation, media was removed from the cell monolayers and the cells were washed with DPBS. Ribavirin (RBV), Amantadine (AMT) and Oseltamivir carboxylate (OSC) purchased from Sigma Aldrich were serially diluted for six concentrations. Influenza virus strains were diluted to a pre-determined 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% CO_2 for four days, cell viability was measured by XTT staining. The optical density of the cell culture plate was determined spectrophotometrically at 450 and 650 nm using Softmax Pro 4.6 software. Percent CPE reduction of the virus-infected wells and 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 virus titer was varied to determine how the antiviral activity of Ribavirin, Amantadine and Oseltamivir carboxylate would be affected.

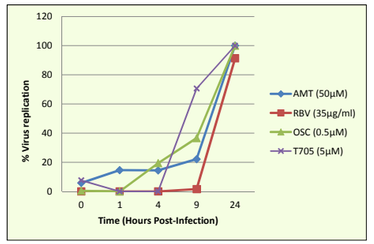
Time of Drug Addition Assay: Ribavirin, Amantadine, Oseltamivir carboxylate and T705 were added to MDCK cells at 0, 1, 4, 9 and 24 hours post-infection with wild type influenza virus strain A/PR/8/34 or Oseltamivir resistant-influenza virus strain A/NJ/15/07 containing the H274Y neuraminidase mutation to confirm mechanism of antiviral action by determining when the compound loses effectiveness.

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, generating a three dimensional dose response surface that can be evaluated statistically to define the interaction of the test compounds. Combination evaluations were performed using MDCK cells infected with influenza type A virus in a cytoprotection assay described above. The results of the assay were imported into the MacSynergy II software program and the compound interactions were calculated at the 95% and 99% confidence intervals.

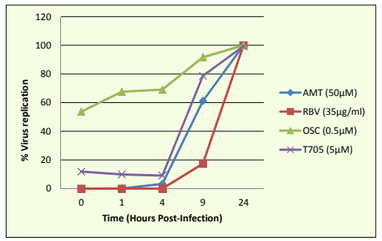
ANTI-INFLUENZA VIRUS CYTOPROTECTION EVALUATIONS

Influenza Strain	Ribivirin (µg/mL)			Amantadine (µM)			Oseltamivir (µg/mL)		
	EC_{50}	TC_{50}	TI	EC_{50}	TC_{50}	TI	EC_{50}	TC_{50}	TI
A/Denver/1/57 (H1N1)	1.58	>100	>63.3	0.17	>10	>58.8	>1	>1	---
A/NWS/33 (H1N1)	1.68	>100	>59.5	>10	>10	---	0.2	>1	>5
A/WS/33 (H1N1)	2.05	>100	>48.8	>10	>10	---	0.02	>1	>50
A/HK/8/68 (H3N2)	0.56	>100	>179	1.78	>10	>5.62	0.008	>1	>125
A/PR/8/34 (H1N1)	1.87	>100	>53.5	7.33	>10	>1.36	0.37	>1	>2.7
A/Fort Monmouth/1/47 (H1N1)	2.1	>100	>47.6	0.09	>10	>111	>1	>1	---
A/Aichi/2/68 (H3N2)	0.81	>100	>123	0.51	>10	>19.6	0.006	>1	>167
A/CA/27/07 (H1N1)	1.41	>100	>70.9	0.99	>10	>10.1	0.23	>1	>4.35
A/NY/18/09 (H1N1)	2.11	>100	>47.4	>10	>10	---	0.02	>1	>50
A/CA/04/09 (H1N1)	1.48	>100	>67.6	0.71	>10	>14.1	0.02	>1	>50
A/CA/05/09 (H1N1)	1.65	>100	>60.6	>10	>10	---	0.007	>1	>143
A/New Caledonia/20/99 (H1N1)	3.20	>100	>31.3	0.43	>10		0.17	>1	>5.88
A/Wisconsin/06/1994 (H3N2) A30V	16.7	>100	>5.99	>10	>10	---	0.00001	>0.1	>10,000
A/Venezuela/6971/2005 (H3N2) V27A	21.3	>100	>4.69	>10	>10	---	0.02	>1	>50
A/Virginia/01/2006 (H1N1) S31N	4.45	>100	>22.5	>10	>10	---	0.07	>1	>14.3
A/Wisconsin/25/2007 (H1N1) L26F	7.68	>100	>13	>10	>10	---	2.45	>10	>4.08
A/HK/2652/2006 (H1N1) S31N	8.92	>100	>11.2	>10	>10	---	0.09	>1	>11.1
A/Brisbane/10/2007 (H3N2) S31N	0.85	>100	>118	>10	>10	---	>1	>1	---
A/Taiwan/760/2007 (H3N2) S31N	23.1	>100	>4.33	>10	>10	---	0.05	>0.1	>2
A/New Jersey/15/2007 (H1N1) H274Y	2.45	>100	>40.8	0.77	>10	>13	>1	>1	---
A/Wuhan/395/95-like (H3N2)	1.23	>100	>81.3	0.94	>10	>10.6	0.67	>1	>1.49
A/HK/2369/2009 (H1N1) H274Y	1.66	>100	>60.2	>10	>10	---	>1	>1	---
B/Taiwan/2/62	0.99	>100	>101	>10	>10	---	0.02	>1	>50
B/Mass/3/66	1.81	>100	>55.2	>10	>10	---	0.001	>0.1	>100
B/MD/1/59	0.86	>100	>116	>10	>10	---	0.52	>1	>1.92
B/Allen/45	0.87	>100	>115	>10	>10	---	0.27	>1	>3.7
B/Bridgit	2.73	>100	>36.6	>10	>10	---	>1	>1	---
B/Great Lakes/1739/1954	0.63	>100	>159	>10	>10	---	0.07	>1	>14.3
B/Lee/40	0.78	>100	>128	>10	>10	---	0.02	>1	>50
B/Memphis/20/96	0.91	>100	>110	>10	>10	---	>1	>1	---
B/Memphis/20/96 R152K	0.57	>100	>175	>10	>10	---	0.11	>1	>9.09

ANTI-INFLUENZA VIRUS A/PR/8/34 TIME OF DRUG ADDITION ASSAY



ANTI-INFLUENZA VIRUS A/NJ/15/07 (H274Y) TIME OF DRUG ADDITION ASSAY

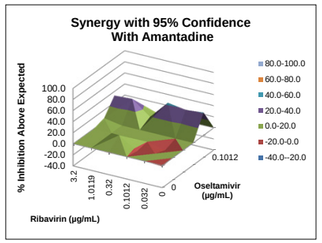


VARIABLE MOI ANTI-INFLUENZA VIRUS CYTOPROTECTION EVALUTIONS

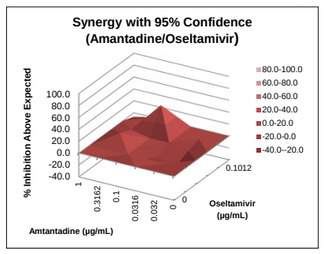
MDCK/ Influenza$_{A/HK/8/68}$ Titer	Ribavirin				Amantadine				Oseltamivir carboxylate			
	EC_{50} (µg/mL)		TI		EC_{50} (µg/mL)		TI		EC_{50} (µg/mL)		TI	
	Day 3	Day 5	Day 3	Day 5	Day 3	Day 5	Day 3	Day 5	Day 3	Day 5	Day 3	Day 5
4x 95% cell kill	>100.0	>100.0	---	---	>100.0	>100.0	---	---	>100.0	>100	---	---
2x 95% cell kill	69.1	>100.0	>1.5	---	>100.0	>100.0	---	---	>100.0	>100	---	---
95% cell kill	49.4	21.6	>2.0	4.2	0.06	1.6	1163.8	25.7	93.9	33.2	>1.1	>3.0
90% cell kill	28.2	20.8	>4.8	>10.4	0.02	0.08	3185.3	255.2	27.3	15.1	>3.7	>6.6
½ x 90% cell kill	17.4	9.6	>5.8	>10.4	0.018	0.3	3808.1	78.9	1.7	0.1	>58.1	>990.1
¼ x 90% cell kill*	0.01	5.4	>10000	>18.6	0.01	0.2	7252.2	163.3	0.002	0.05	>50000	>2222.

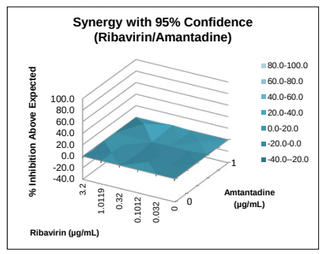
*Triplicate values inconsistent due to low MOI.

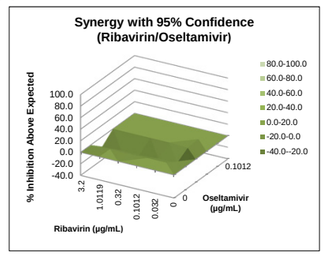
THREE-DRUG COMBINATION INFLUENZA VIRUS THERAPY



TWO-DRUG COMBINATION INFLUENZA VIRUS THERAPY







COMBINATION THERAPY EVALUTIONS

Drug Combination	MacSynergy II Synergy Volume (mcg/mL%)	Drug Interaction
AMT+OSC	155	Synergistic
AMT+RBV	104	Synergistic
RBV+OSC	-81	Additive
RBV+OSC+AMT	410	Synergistic

CONCLUSIONS

- **Many seasonal influenza strains are resistant to AMT, OSC or both. Characterization of each influenza virus strain used to evaluate the range of antiviral activity of new/novel test compounds is important in understanding their mechanism of action. Identifying the drug-sensitivity phenotype of each virus is important in understanding how certain classes of antiviral compounds will perform.**
- **Variation in MOI greatly affected the antiviral activity of RBV, AMT and OSC, but AMT was less sensitive. This is likely due to the mechanism of action of AMT or its pharmacodynamic properties.**
- **Time of drug addition assays are useful in determining the mechanism of antiviral action. The nucleoside polymerase inhibitor T705 remained active when added to influenza virus infected MDCK cells up to 4 hours post-infection. RBV, M2 ion channel inhibitor AMT, and neuraminidase inhibitor OSC remained active when added up to 9 hours post-infection against wild type influenza virus.**
- **Two drug combinations of AMT/OSC and AMT/RBV were modestly synergistic while the combination of OSC/RBV was additive. The triple combination of AMT/OSC/RBV was highly synergistic with the peak synergy volume 5- to 8-fold greater than the synergy volume of the double combinations.**