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Combination Antivirals against H1N1 pandemic influenza: an *in vitro* study of potential agents that show anti-influenza activity

Introduction

One of the agents that has had, and continues to have, a great impact on human health is Influenza virus. In the U.S.A., epidemic influenza causes an average of 144,000 hospitalizations and 20,000 deaths/year[1]; World-wide deaths are estimated at 0.5 – 1M each year[2]. In addition to yearly epidemics, there have been sporadic pandemics, in which the death toll is higher. For example, the 1918 "Spanish flu" likely caused 20 – 50M deaths[3]. The H1N1 strain reemerged in 2009 with the "swine flu" pandemic. In the pandemic phase the virus is estimated to have caused over 400000 deaths[4,5]. In August 2010, WHO Director-General Dr Margaret Chan announced that the pandemic had moved into the post-pandemic period. In the post-pandemic period the virus may still cause localized outbreaks and would circulate in the population as a seasonal flu virus for years to come[6].

Taxonomically, Influenza virus belongs to the family *Orthomyxoviridae* For reviews, see. The Influenza A viruses are classified according to two proteins; the hemagglutinin (HA), of which there are currently 16 different types (H1 – H16), and the neuraminidase (NA), of which there are currently 9 known different types (N1 – 9). Virtually every possible H/N combination has been detected in water fowl, the generally-accepted reservoir, but only a few HN types (most notably the 1918 H1N1 "Spanish Flu", the 1957 H2N2 "Asian Flu" and the 1968 H3N2 "Hong Kong Flu") infect humans. A hallmark of influenza virus is the virus' genetic variability and because of this the "flu shot" needs to be reformulated each year. Therefore, it is worthwhile to consider additional strategies that may not be reliant upon specific viral genetic constitution.

One such strategy is to use anti-viral therapy. Ideally, such therapy would be effective against common aspects of the influenza replication cycle, and thus capable of inhibiting a variety of influenza strains. A large number of anti-viral compounds have been described during the past few decades[9] and many, but not all, have been tested against influenza virus. Two classes of antiviral compounds have been approved for human usage and are available for treating influenza infections. The neuraminidase inhibitors, Tamiflu® (oseltamivir phosphate) and Relenza® (zanamavir) are effective at limiting influenza complications if treatment is initiated soon after infection[10]. Oseltamivir is considered first line treatement for influenza infections in healthy adults while zanamivir is to be used as prophylaxis in at-risk populations[11,12]. However, the appearance of resistant strains of H1N1 virus in Tamiflu®-treated individuals raises significant concerns over long-term use of the drug in a pandemic[5]. The adamantine class of drugs, amantadine and rimantadine, are useful in small outbreaks of influenza but cannot be used in a pandemic, where there is rapid development of resistant viruses[13]. It is probable that the virus' capacity to rapidly mutate will allow it to develop resistance against any class of anti-viral that we can devise and that this resistance can, and will, emerge most rapidly during wide-spread use of any single anti-viral compound.

To combat this a new approach using a combination of anti-viral therapies is being

advocated[11]. These include both pharmaceutical (anti-viral drugs, vaccines) and non-pharmaceutical interventions (personal hygiene, quarantine, travel restrictions)[14]. This paper will focus on a combination approach using only anti-viral drugs. In theory, a combination approach will both prevent resistance from developing by targeting multiple sites of influenza virus infection pathway and reduce side effects by reducing the amount of individual drug needed to treat. The most promising theoretical combination would be two drugs that affect different parts of the influenza infection and replication cycle. For example, trials of oseltamivir carboxylate, a neuramindase inhibitor, with ribavarin, an inhibitor of inosine-5′-monophosphate dehydrogenase (IMPDH), in mice have shown no greater effect in combination than with ribavarin alone using a mouse-adapted A/New Caledonia/20/99 (H1N1) strain[15]. However, a combination of rimantadine and oseltamivir in a mouse model using H3N2 virus showed a synergistic effect[16]. In addition, some researchers have used up to three anti-viral drugs at once with promising results[17]. For a review of which anti-viral drugs have been tested and at what stage the testing was done see[18].

Another strategy is to find novel drugs that have not previously been used against influenza but show potential by showing inhibition of other viruses. One drug that has been studied previously in the Coomb's lab is mycophenolic acid (MPA). It has been shown to inhibit reovirus which like influenza is an RNA virus[19, 20, 21, 22]. In addition, this compound has been found to inhibit replication of a wide range of viruses, including human immuodeficieny virus (HIV)[23] Herpesvirus[24], and West Nile virus[25]. There was one report from the 1960's stating MMF had anti-influenza activity *in vitro*[22].MPA is the active form of mycophenolate mofetil (MMF). MMF is a potent immunosuppressant drug and has been used for over 10 years[19]. It was first used for patients with solid organ transplants and is as of 2009 the Scientific Registry of Transplant Recipients states that over 80% of its patients use it as part of an immunosuppressive regimen[19]. MMF is an impressive immunosuppressant for its ability to suppress both B and T lymphocytes. Due to this property it is being tested as an aid for treating rheumatologic diseases[19].

The mechanism of action for MPA centers around its ability to inhibit IMPDH. IMPDH is an enzyme needed in the *de novo* synthesis of guanosine. Ultimately, if this pathway is blocked new DNA cannot be formed, thus inhibiting production of new B and T lymphocytes[22]. However, how MPA inhibits certain viral agents is as yet unclear[22]. In one study using hepatitis C virus, the inhibition was independent of guanosine depletion[26]. This is in contrast to a study in herpes and reovirus where addition of guanosine reversed the inhibition[20,27]. MPA is also a prime candidate for research as it has already been shown to been shown to enhance the activity of antiretrovirals against HIV[28, 29].

Given the above information, we hypothesize that a combinatorial anti-viral strategy will (1) synergistically attenuate influenza virus replication to a greater extent than would be achievable by single drug therapy, (2) allow lower-dose usage of each anti-viral, which, in turn will reduce cellular toxicity effects, and (3) dramatically reduce capacity of the virus to mutate to escape the anti-viral activity. Therefore, specific objectives are to use a multi-pronged approach to attempt to optimize anti-influenza drug usage. Our approach will initially involve testing the anti-influenza virus activity of a small number of compounds, some of which are known to attenuate influenza growth (oseltamavir, amantadine) and some of which do not have had well reported

use against influenza, but which are known to have anti-viral activity against other viruses (mycophenolic acid).

Methods

Tissue Culture

Madin Darby canine kidney (MDCK) and adenocarcinomic human alveolar basal epithelial cells (A549) cells were grown in separate tissue culture flasks with a media consisting of modified Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, non-essential amino acids, 0.2% weight per volume (w/v) sodium pyruvate, glucose and 2mM L-glutamine. They were passaged once every 2-3 days, using tyrpsinization, to maintain a sub-con fluency of 90-95%. MDCK are the "standard" cells that many Influenza virus strains are grown in and the cell line routinely used for influenza virus plaque assays. A549 are a human epithelial lung cell line that most influenza virus researchers consider most relevant for influenza virus research[23].

Drug Trials

Screening

The five anti-virals tested were mycophenolic acid, oseltamivir carboxylate (Oseltamivir®), ribavarin, zanamivir (Relenza®), and amantadine. All have been shown to have some activity against influenza[11]. A general set of screening concentrations was chosen to test each of the five anti-viral drugs. These were 300, 30, 3, 0.3, 0.03 and 0 μg/ml. To prevent cross contamination, MDCK cells were plated in 6 wells of a 24 well plate to be sub-confluent at 85-95%. The media used was modified DMEM with supplements with no FBS to prevent cell replication during the incubation period.

Viral samples of known titres of A/PR/8/34(H1N1) strain were prepared in a suspension using the same drug concentration as to be tested. The volume calculated to add to the cells was to give a multiplicity of infection (MOI) of 0.01 plaque forming units (PFU)/cell.

Serial dilutions of drug were created in DMEM with supplements in appropriate concentration with enough volume to both pre-treat and infect out of the same dilution tube. The culture media was removed from the cells and then they received a pre-treated of media with the appropriate concentration of drug and were incubated at 37°C for one hour. After pre-treatment plates were washed twice with phosphate-buffered saline (phosphate buffer concentration of 0.01M and a sodium chloride concentration of 0.154M) (PBS). Cells were then infected with the previously calculated amount of viral suspension and again incubated for one hour but at 35°C. Plates were rocked every 10 minutes to prevent drying out of the cells.

After incubation as much liquid as possible was removed from the wells. The cells were the covered in media like that used in the pre-treatment but supplemented to contain

100 U/mL of penicillin, 100 μg/mL of streptomycin sulfate, 0.000125% trypsin and 1 μg/mL of amphotericin B. Amphoteracin-B and penicillin/streptomycin are used to prevent contamination in the experiment, of fungus and bacteria respectively. Trypsin is a digestive enzyme that will cleave the hemagglutinin molecules of the viral particles[30]. If these proteins are not cleaved they will not be able to undergo a fusion to create an active protein. In the host, cellular enzymes complete this task but need to be replaced in culture[30]. Plates are incubated at 35ºC for set time points dependent upon cell line used. MDCK cells are incubated for 72 hours and A549 cells are incubated for 48 hours.

After incubation the liquid from each well was harvested and centrifuged at 2500rpm for three minutes. The supernatant was extracted and combined with 10% glycerol volume by volume (v/v), which acted as a preservative. Samples were kept at -80ºC until needed.

Antiviral Combination

After reviewing the results of the screening trials it was decided that the combination of MPA and oseltamivir carboxylate would be tested. As a starting point, the inhibitory concentrations (IC) at 50 and 90 were calculated. The IC50 is the concentration of drug at which the percent yield of virus is 50. The design was based partly on another experiment using multiple chemotherapy against influenza virus, where the concentration of one drug remained constant with serial dilutions of the other[31]. For the combination experiment, set concentrations of 5, 10, 25 and 100 μg/ml were used with the concentrations of 10, 1, 0.1, 1E-2, 1E-3 and 1E4 μg/ml oseltamivir carboxylate. The methods used were as above, except in addition the strain A/NY/55, a model H3N2 virus, was tested.

Plaque Assay

Viral titre was measured using plaque assay (plaque reduction). The samples collected during the drug trials were used to make serial dilutions up to 10^{-6}, as influenza titires *in vitro* generally do not rise greater than 2E8. The suspensions were prepared in gel saline (137 mM NaCl, 0.2 mM CaCl2, 0.8 mM MgCl2, 19 mM HBO3, 0.1 mM Na1B407, (0.3% [w/v] gelatin) and kept on ice until needed.

Cells were set up to be 90-95% sub-confluent in 6-well plates. Cell culture media was removed and the cell monolayers were washed twice with PBS. The plates were infected with a set amount of virus suspension from each dilution tube. Plates were washed in sets of 6-12 to ensure they could be washed and infected within 20 minutes to prevent drying out of the cells.

Infected plates were placed in the 35ºC incubator and rocked every 10 minutes for one hour to allow for adsorption to occur. As much liquid as possible was removed from each well after the sixty minutes. To keep the cells stable for the incubation period an overlay media was added. The media contained equal parts 1.2% agarose (at about 37ºC) and modified DMEM with, 100 U/mL of penicillin, 100 μg/mL of streptomycin sulfate, 0.000125% trypsin and1 μg/mL of amphotericin B.

At 66-70 hours post infection (hpi) plates were checked for plaques. If they could be seen the plates were stained using either neutral red or crystal violet staining methods. Which method chosen was based upon timing, ease of use, and reagents available in the lab. For neutral red staining, 0.8ml neutral red reagent was used for every 100ml 1.2% agarose. The overlay was added to the wells and left for 14-18 hours before counting. The crystal violet staining involved fixing the cells with formaldehyde before adding the crystal violet stain. After the crystal violet stain had been removed and the plates washed to remove excess stain the plaques were counted.

Viral production was compared by determining the titre of the virus with drug and comparing it to the titire of virus without drug. This was plotted on a graph to visualize the percent yield. A best fit line was applied and the IC50 and IC90 calculated from the equation.

WST-1 Cell Viability Assay

The protocol for the assay was adapted from one used previously in the lab and from ScienCell© Research laboratories[32]. MDCK cells were set up in a 96-well plate to be sub-confluent the next day. The media used was a mix of the culturing media with set concentrations of anti-virals that had been tested during the drug trials. Wells for negative controls (cells with no treatment), positive controls (cells treated with 0.05% sodium azide) and blank wells (media only) were also set up on the 96-well plate. See diagram below for plate template. After incubating for 24 hours in the 37º incubator, reconstituted WST reagent was added. The amount of reagent added was equivalent to 10% of the volume already in each well. The plates were placed in the incubator for 3 hours. They were then rocked for one minute to ensure proper mixing of reagent and placed in the ELISA plate reader. Readings were taken at 610nm and 440nm. The 610nm readings were subtracted from/ the 440nm readings. The average of the media blank samples was calculated and subtracted from the calculated readings. A graph was set up using the average of the negative controls (100% viability) and the positive controls (0% viability) as the scale for the y-axis. The drug concentrations were placed as the x-axis and the values from the calculations as the y-axis.

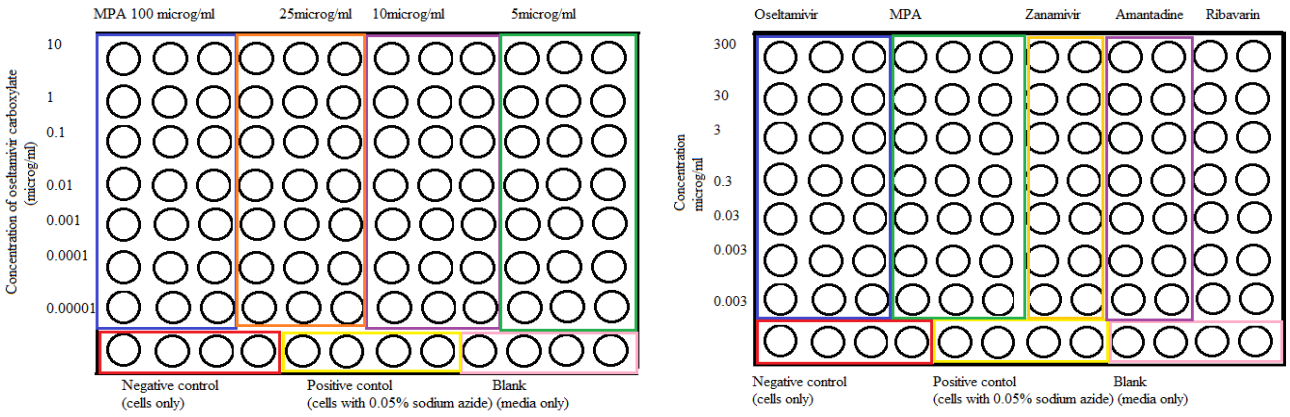


Figure 1: 96-well plate template used for WST-1 cell viability assay showing inclusion of positive and negative controls, blank, and individual and combination anti-viral dosing.

Results

Screening

While plaque assays were initially performed using both MDCK and A549 cell lines it was found that the A549 cell line is not able to produce a viral titre high enough to be counted. Thus only the results of assays done with MDCK cells are presented below. As the first line treatments, one would expect zanamivir and oseltamivir to show the greatest activity. Amantadine, a second line drug, shows the least. This is displayed in the figures below.

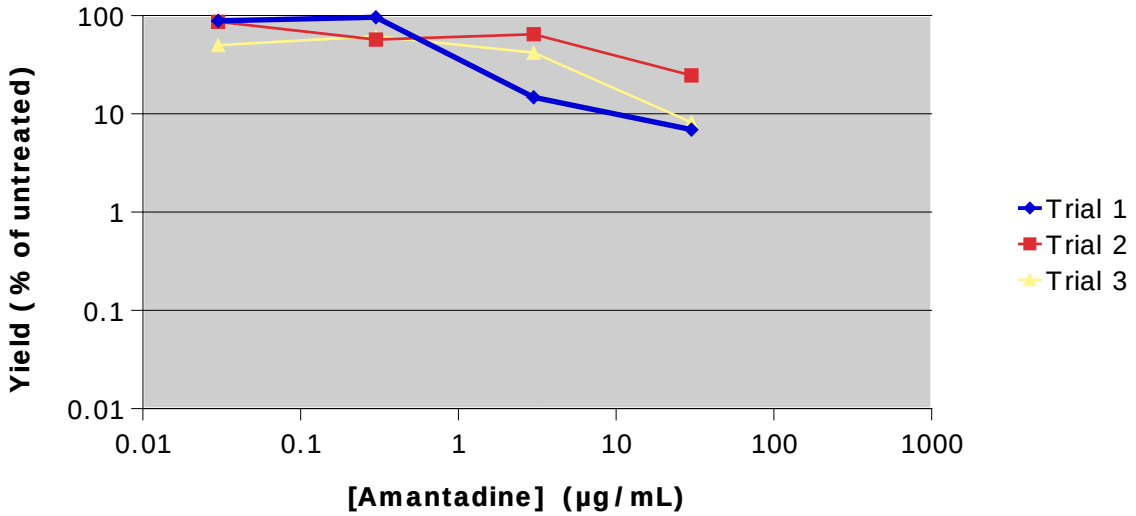


Figure 2*:* Yield of Influenza A/PR/8/34(H1N1) when in presence of various concentrations of Amantadine HCl and grown in MDCK cells after 72 hours at 35°C.

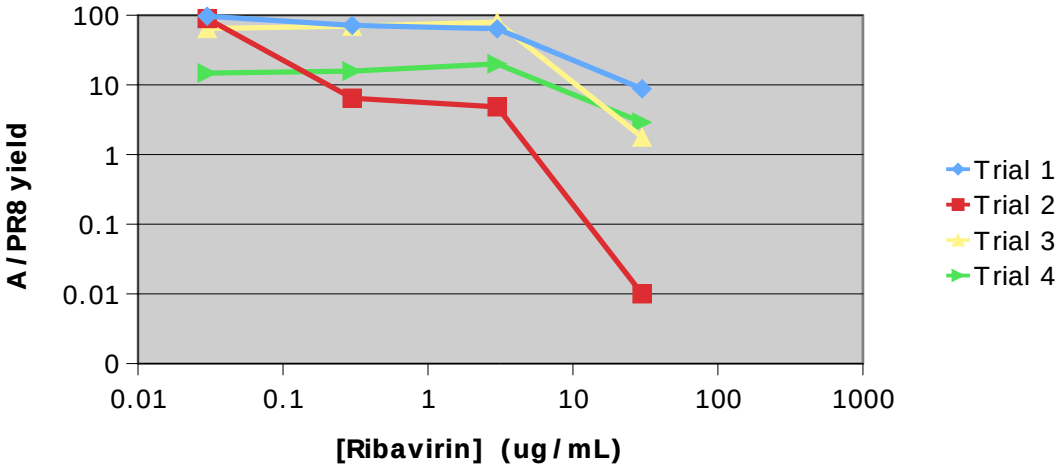


Figure 3: Yield of Influenza A/PR/8/34(H1N1) when in presence of various concentrations of ribavirin and grown in MDCK cells after 72 hours at 35°C.

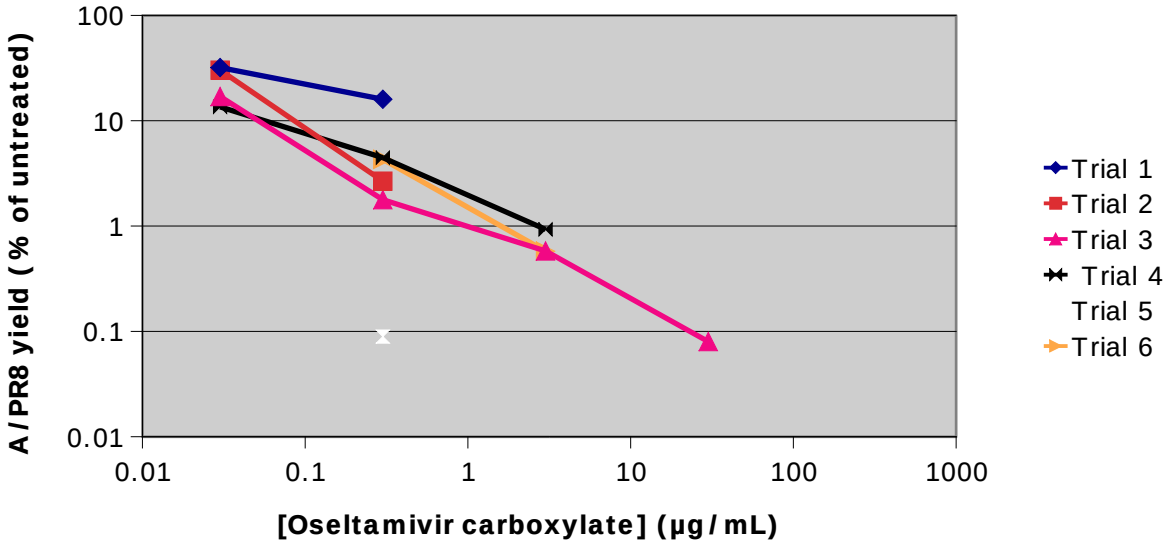


Figure 4: Yield of Influenza A/PR/8/34(H1N1) when in presence of various concentrations of Oseltamivir carboxylate and grown in MDCK cells after 72 hours at 35°C.

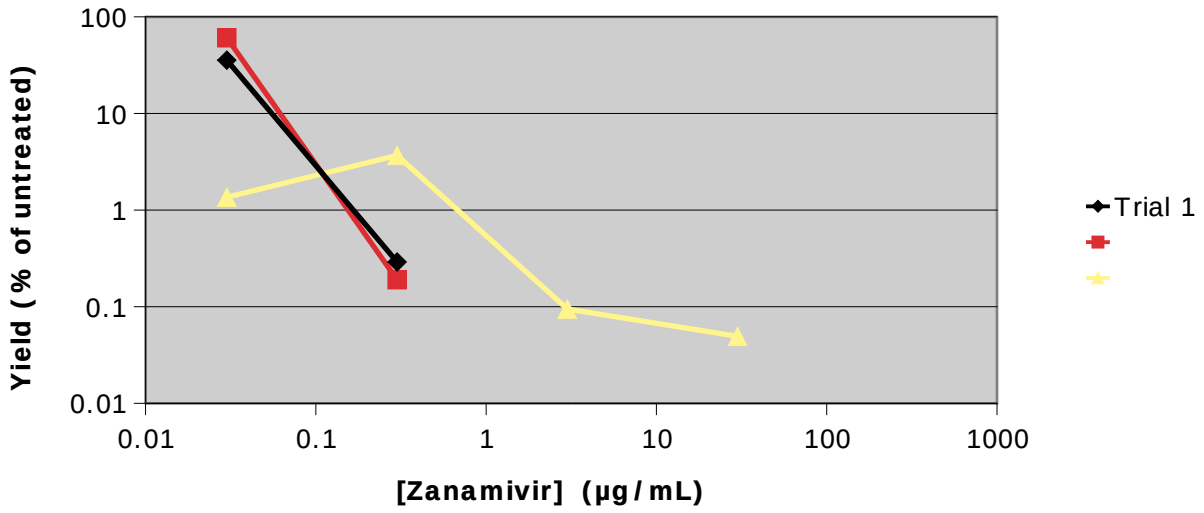


Figure 5: Yield of Influenza A/PR/8/34(H1N1) when in presence of various concentrations of Zanamivir and grown in MDCK cells after 72 hours at 35°C.

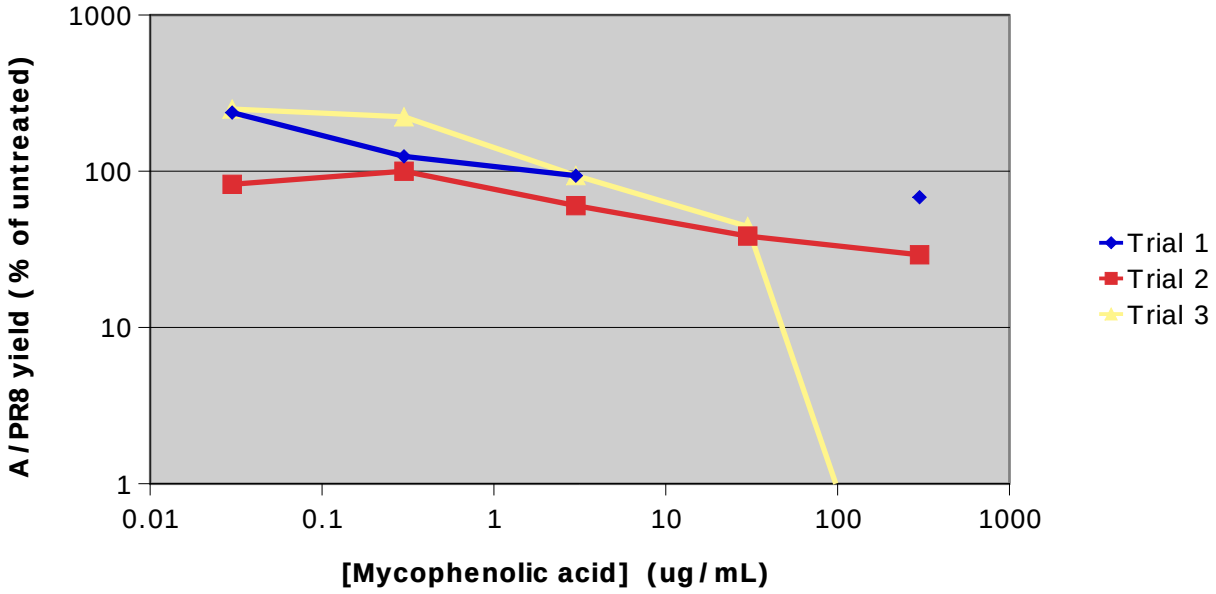
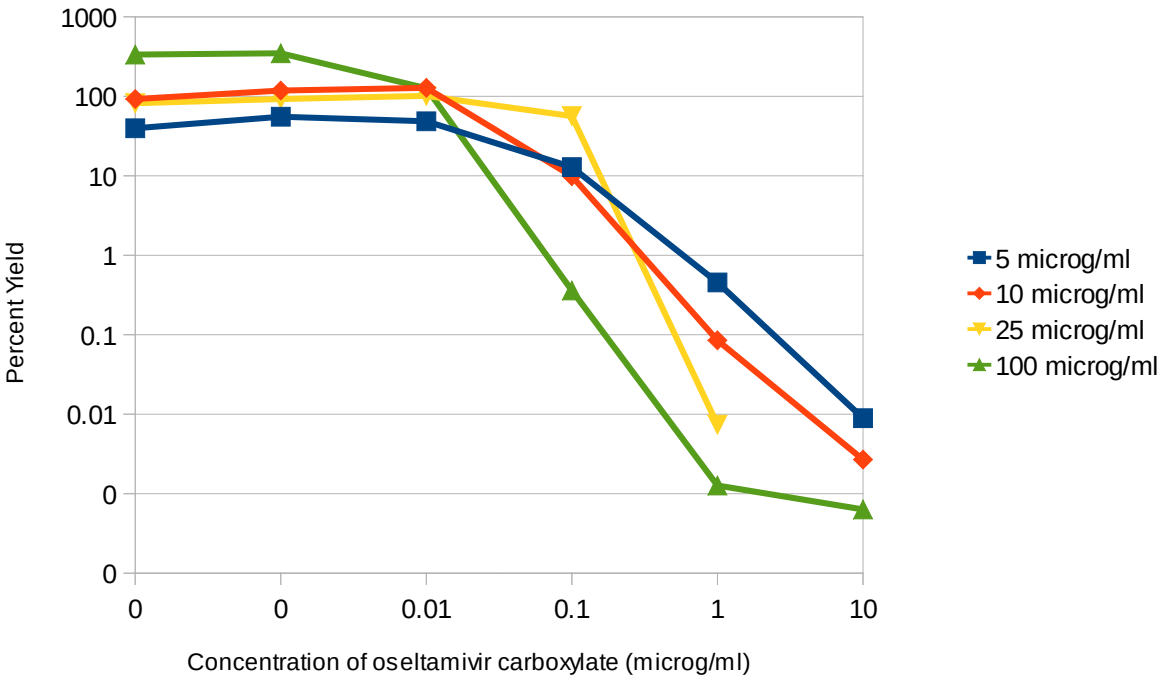
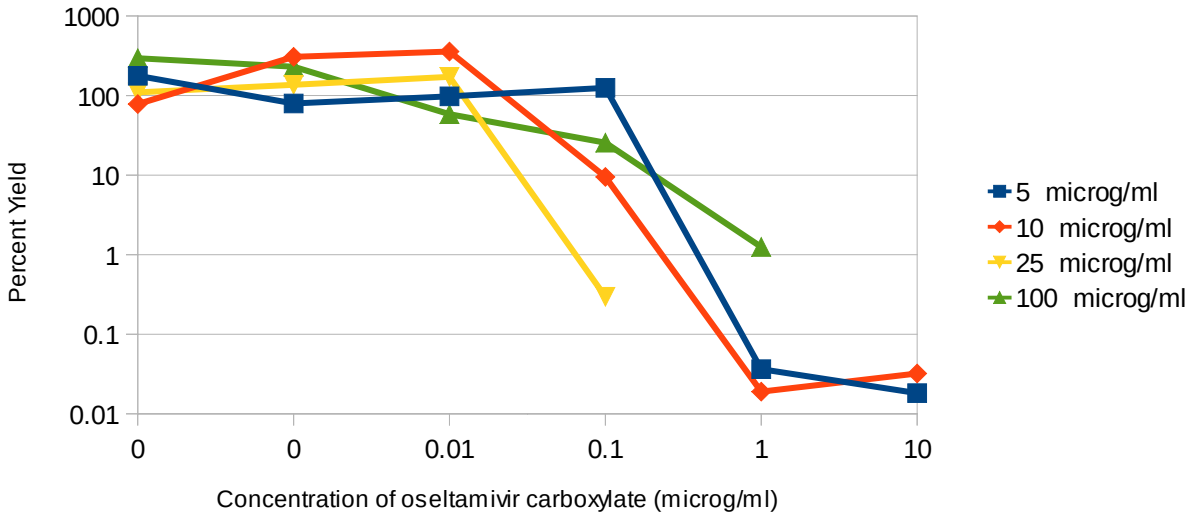


Figure 6: Yield of Influenza A/PR/8/34(H1N1) when in the presence of various concentrations of Mycophenolic acid and grown in MDCK cells after 72 hours at 35°C.

MPA does appear to inhibit the virus by at least ten fold in one trial. However, the standard deviation of the trials is so high that it cannot be said that they are significantly different from one another. Higher concentrations of MPA may also need to be tested.


1000
100
10
1
0.1
0.01
0
0
0
0
0.01
0.1
1
10
Percent Yield
Concentration of oseltamivir carboxylate (microg/ml)
5 microg/ml
10 microg/ml
25 microg/ml
100 microg/ml


1000
100
10
1
0.1
0.01
Percent Yield
0
0
0.01
0.1
1
10
Concentration of oseltamivir carboxylate (microg/ml)
5 microg/ml
10 microg/ml
25 microg/ml
100 microg/ml

IC90	8.3E-1	2.8E-1	4.8E-1	6.9E-3
NY55 IC50	2.9E-1	2.9E-3	7.5E-2	8.1E-2
IC90	5.4	1.8E-2	8.0E-2	2.6E-1

WST-1 Viability Assay

The purpose of performing the WST-1 assay is to determine cellular toxicity of the anti-viral drugs. If an anti-viral shows an ability to reduce viral titre but also has a high toxicity the low titre may be explained by cell death. The WST-1 assay was also to be used to calculate "selectivity indices" (dose that causes 50% cytopathic effect divided by dose that attenuates virus replication 50%; selectivity indices are used as a measure of selective toxicity; the higher the index, the safer the drug). Unfortunately, the assay failed and with no time to repeat it, no results were obtained. However, when used as an agent for immunosupppresion, MMF is given in doses much higher than tested here so we may speculate that the cell toxicity is low.

Discussion

Preliminary results from assays such as those described above generally provide very "clear-cut" responses. If an anti-viral agent has any activity, it usually reduces progeny production by more than 10-fold. This was expected and confirmed in the screening trials for the agents currently used against influenza. It should be noted that there was large variability in the results for the trials. For example, the calculated IC50 for oseltamivir had a range of over 100 fold. This made it very hard to determine if in fact MPA was making a difference in the combination trials.

We expect that combining two different anti-virals that affect different portions of the replication cycle will have an additive or synergistic effect on virus attenuation. However, the results of the combination trials show little potential of MPA to enhance the ability of oseltamivir. To be noted, only one trial of each concentration and virus strain was completed successfully. More replicate trials are needed before any definitive conclusions can be made.

As a drug that has already been used in other areas, MPA also provides the luxury of testing higher doses, as those tested in lab are well below that used therapeutically. If higher doses result in improved viral inhibition and high selectivity indices, MPA would be worthwhile studying further.

Future work

Due to the time constraints not all aims of the project were completed. For a more well-rounded experiment there are a number of tasks left to be completed. These include replicating the WST-1 assays and trouble-shooting why plates did not show usable results, repeating the experiment with different influenza subtypes (such as two seasonal H1N1 viruses, A/Brisbane and A/New Caledonia and the contemporary pandemic swine-origin H1N1, SOIV), repeating the experiment in different cell lines (HEK293 human embryonic kidney and fibroblastic HeLa

cells), measuring the progeny viral protein by ELISA and the viral RNA by quantitative ("real-time") PCR. In addition, only one combination of anti-virals was tested in this project. It would be worthwhile to try other combinations with MPA to help round out its ability. For example, it would be interesting to see the combination of ribavarin and MPA, as both are IMPDH inhibitors. If MPA derived its anti-viral activity from its ability to inhibit IMPDH then we should expect an additive response. However, if a synergistic response was seen we may conclude that it works through some other mechanism.

Conclusion

This study concludes that MPA does have anti-viral activity against at least one strain of influenza A virus. However, it appears not to potentiate the activity of the neuraminidase inhibitor, oseltamivir. More work is needed to make any firm conclusions.

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