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Potent Mutagens Have Positive and Negative Effects on Viral
Fitness of Reovirus *in vitro*

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In

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Potent Mutagens Have Positive and Negative Effects on Viral Fitness of Reovirus *in vitro*

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Due to the inherently error-prone nature of RNA replication, mutations to genomes of RNA viruses occur frequently and accumulate. We hypothesized that RNA versions of nucleoside analogues that increase mutation rates in DNA could cause increases in the mutation rate of a model RNA virus, Reovirus, and decrease the fitness of the virus *in vitro* due to the accumulation of deleterious mutations. After conducting multiple passages of Reovirus in mouse L929 cells in the presence of these potential RNA mutagens in four separate trials, there were not only virus samples with the expected decreased infectivity, but surprisingly, samples with marked increases in infectivity. The life histories of these Reoviruses under mutagenesis may clarify likely consequences of mutagenic antiviral therapy.

Introduction

Reovirus is a non-enveloped, icosahedral (T=13), double stranded RNA (dsRNA) virus that is known to cause gastrointestinal and respiratory infections in young children (Kapikian et al., 1976). It is a generally mild virus in the Western Hemisphere making it an ideal virus to work with. Reovirus is a model dsRNA virus that can be used to study Eigen's theory of error catastrophe, the process of introducing sufficient mutations into a viral genome to render it incapable of carrying out productive infection (Anderson, et al., 2004). This theory led to the

concept of lethal mutagenesis as a way to cure viral infections (Bull, et al., 2007). Lethal mutagenesis involves pushing a within-host population of viruses to extinction by overwhelming it with an elevated mutation rate (Bull, et al., 2007). Increasing the mutation rate of these viruses could exceed the error threshold for viability of a viral population within a host (Anderson et al., 2004). However, error catastrophe can delay or even prevent extinction by shifting the population to genotypes that are robust to mutation (Bull, et al., 2007). Therefore it is possible that some of the introduced mutations could be neutral or even beneficial for the virion.

Studies have shown that select chemical mutagens can be used to artificially increase error rates in a variety of RNA viruses, including vesicular stomatitis virus (VSV), human immunodeficiency virus type 1 (HIV-1), poliovirus type 1, foot-and-mouth disease virus, lymphocytic choriomeningitis virus, Hantaan virus, and hepatitis C virus (Bull, et al., 2007). Since Reovirus is also a RNA virus with a RNA polymerase that lacks a proofreading domain, any mutations introduced into the genome would accumulate and be passed on to subsequent virion progeny. Additionally, linear increases in mutation rate result in exponential reduction in viral fecundity and even slight increases in mutagenesis may be sufficient to drive a viral population into extinction (Graci and Cameron, 2008). Since few broad spectrum antiviral drugs exist today, error catastrophe may represent a possible pathway to broad spectrum antiviral drug development.

In order to test whether we could induce error catastrophe in Reovirus, we utilized RNA analogs of known DNA mutagens namely, 2-Aminopurine, 5-Bromouridine, and 8-Oxoguanosine (Fig. 1A-C). Each mutagen has a different nitrogenous base with which it can bind. 2-Aminopurine most frequently bonds to thymine, but it can also be incorporated opposite cytosine (Sowers, et al., 1986); 5-Bromouridine can bond with guanosine or adenine (Bertoluzza,

et al., 1997), and 8-Oxoguanosine can base pair with cytosine or adenine (Briebe, et al., 2004). These RNA analogs can be incorporated into the viral genome every time there is a replication event.

We began with mutagenic 2-Aminopurine and 5-Bromouridine ribonucleoside media as well as a combination media (2-Aminopurine and 5-Bromouridine). After multiple passages, we substituted the more potent 8-Oxoguanosine for the relatively weak 2-Aminopurine. Ribavirin was also included as a positive control (Fig. 1D). Ribavirin shows antiviral activity against a variety of RNA viruses and is used in conjunction with interferon to treat hepatitis C infections (Crotty, et al., 2000). Ribavirin incorporation induces mutations in viral genomes during multiple rounds of RNA replication in the cell, resulting in a substantial increase in the production of defective viral genomes (Crotty, et al., 2000). Additionally, ribavirin is particularly effective as it competitively inhibits correct nucleotide incorporation with an inhibition constant value in the range of 400 μ M (Crotty, et al., 2000). As a pseudo-base, ribavirin can bind to uracil or cytosine and incorporation opposite uracil is isoteric to that opposite cytosine (Crotty, et al., 2000). Ribavirin is a powerful antiviral and has even been shown to reduce production of infectious virus from poliovirus infected cells to as low as 0.08% (Crotty, et al., 2000). Thus, ribavirin was an ideal positive control for our experiment.

The selected mutagens were present in the media during the entirety of viral infection and replication so as to encourage maximum incorporation. We hypothesized that infectivity would substantially decrease over the course of multiple passages due to the accumulation of deleterious mutations. These experiments aim to evaluate the efficacy of these mutagens as potential effectors of error catastrophe in vitro and as an antiviral strategy.

Materials and Methods

Mutagens and Cells. Murine L929 fibroblast cells were purchased from ATCC and the mutagens were purchased from Berry & Associates, Inc. Ribavirin was purchased from Sigma-Aldrich (St. Louis, Missouri). Cells were maintained in a T75 flask and were split every 3-4 days with trypsin. Mutagens were diluted to a concentration of 100 μM and aliquots were stored in the freezer.

Infection. Growth curves in the presence of various concentrations of candidate mutagens determined that 100 μM of mutagen was the highest concentration that could be used without hindering cell growth (L. Slipher, unpublished observations). 200,000 mouse L929 cells (ATCC) were grown and then incubated with 2mL of media (containing either 100 μM mutagen or unaltered complete DMEM media) in a six well plate for 1 hour then infected with 10 μL of 10^{-2} stock T3 Abney (ATCC, Manassas, VA) or T1 Lang Reovirus (Gift from Dr. Terry Dermody, Vanderbilt, TN).

Harvesting and Reinfection. Media (mutagenic or unaltered) was added two days after infection and the cells were harvested and lysed via freeze thawing on the fourth day. Subsequently 10 μL of the lysate medium was used to infect the next round of cells thereby carrying the accumulated mutations from generation to generation. It should be noted that the T3 strain has a tendency to lose infectivity after ~ 10 passages whereas T1 Lang does not (T. Dermody, unpublished observations).

Endpoint dilution assay. 96 well plates containing 10,000 L929 cells per well were seeded with 100 μL viral lysate ($10^{-4} \rightarrow 10^{-9}$) and after four days, presence of infectious virus was assessed by

appearance of cytotoxic effect. If any evidence of cytotoxic effect was detected, that well was labeled infected.

Antibody Staining of Reovirus Infected Cells. Cells were infected with 4 μ L of 10⁻⁶ Reovirus and allowed to incubate for 2-3 days. The media was then removed and the cells were washed with PBS and fixed with methanol in the freezer for 10 minutes. After removing the methanol, the cells were washed with PBS 1% BSA and the primary antibody (rabbit anti-reovirus) in PBS 1% BSA (1:500) was applied and incubated at 37°C for 2 hours. This solution was then removed, the cells were washed again with PBS 1% BSA, and then the secondary antibody (anti-rabbit-goat IgG) in PBS 1% BSA (1:1000) was applied and allowed to incubate for 1 hour at 37°C. The solution was removed, the cells were washed with PBS 1% BSA and DAB (Diamino benzadine) staining was then applied as directed by manufacturer's instructions (Abcam, Inc.). After 15 minutes the DAB staining was removed and PBS 1% BSA was added to the wells. Infection events were then counted under the microscope. Due to unwanted residual staining with DAB, fluorescent antibody staining was also performed. This required a similar procedure to the DAB staining up until the addition of the secondary antibody (1:1000 AlexaFluor 488 fluorescent anti-rabbit-goat), provided by Dr. Omar Quintero. The secondary antibody solution was applied to the wells and allowed to incubate for 1 hour at 37°C wrapped in aluminum foil (to protect the fluorescent antibody). This solution was then removed and PBS 1% BSA was added to the wells. Infection events were then counted under an inverted fluorescent microscope.

Indirect ELISA. 96 well microtiter plates were seeded with 50 μ L of various concentrations of experimental and purified Reovirus (10⁻² \rightarrow 10⁻⁸). The plate was then covered in adhesive plastic wrap and incubated overnight at 4°C. The solution was removed and the wells were washed with

200 μ L of PBS. The solution was removed and replaced with 200 μ L blocking buffer (PBS 1% BSA), the plate was then rewrapped and incubated overnight at 4°C. The solution was removed and the plate was washed twice with PBS. 100 μ L of primary antibody (1:500 rabbit-anti-reovirus) was added and the plate was rewrapped and incubated for 2 hours at room temperature. The solution was then removed and washed twice with PBS. 100 μ L of secondary antibody (1:1000 rabbit-anti-goat IgG) was then added, the plate rewrapped and incubated for 1 hour at room temperature. The solution was removed and the plate was washed twice with PBS. 100 μ L of the TMB substrate was then added (turned wells blue) as per the manufacturer's instructions (Abcam, Inc.) and allowed to incubate for one minute at room temperature. Following the incubation, 100 μ L of 0.12M HCl was added (turned wells yellow) and the plate was read at an optical density of 450nm.

Results

To assess the effect of the candidate mutagens, 2-Aminopurine, 5-Bromouridine, and 8-Oxoguanosine, we grew Reovirus in murine L929 cells in their presence through multiple passages and then determined the infectivity of each virus sample. Our preliminary experiments with T3 Abney Reovirus and 2-Aminopurine and 5-Bromouridine showed that all conditions initially exhibited a marked decrease in infectivity compared to the untreated virus samples (Fig. 2 and 3). The magnitude of the decrease in infectivity was smaller for 2-Aminopurine than 5-Bromouridine. The initial decrease after two passages was substantial (-10^{-2} for 2-Aminopurine and -10^{-3} for 5-Bromouridine). However, this initial decrease was followed by a marked gain after passage 3 (Fig. 3). The magnitude of the increase or rebound in infectivity resulted in the

same threshold of infectivity with both 2-Aminopurine and 5-Bromouridine reaching a maximum TCID₅₀ value of 10⁹/0.1mL. The time it took to reach a TCID₅₀ of 10⁹/0.1mL differed for each mutagen with 2-Aminopurine rebounding to the maximal level by passage 3 and 5-Bromouridine at passage 5.

In order to better understand this phenomenon, we performed the same experiment, but replaced T3 Abney with the more stable strain of Reovirus, T1 Lang as T1 is hypothesized to have a lower endogenous mutation rate than T3 (T. Dermody, unpublished observations). We also replaced 2-Aminopurine with the stronger mutagen 8-Oxoguanosine. Since 8-Oxoguanosine has been shown to be a strong DNA mutagen, we hypothesized it would have a similar effect on RNA (Briebe, et al., 2004). These experiments yielded similar results where all conditions, each mutagen and the combination treatment, exhibited a decline in infectivity until passage 3 where they all regained infectivity and sometimes surpassed the wildtype (Fig. 4). Again, the magnitude in both decrease and subsequent recovery was not the same for each mutagen. 8-Oxoguanosine exhibited a decrease in infectivity before 5-Bromouridine (passage 2 vs. passage 3 respectively). Additionally, 8-Oxoguanosine had a final TCID₅₀ of 10⁹/0.1mL at passage 5 whereas 5-Bromouridine had a final TCID₅₀ value of 10⁸/0.1mL. While the primary T1 experiment had an unusual beginning, (the wildtype i.e. no nuc, and all conditions gained infectivity before losing and then regaining infectivity whereas in all other experiments all conditions lost infectivity in the first and second passages) the trend of a decrease in infectivity followed by a marked gain in infectivity could still be observed.

We then decided to test the two Reovirus strains in tandem and added ribavirin as a positive control. We observed the same trend in both viruses, but we did find that ribavirin showed complete inhibition of infection with T3 and near complete inhibition in T1 (Table 1 and

Fig. 5). In the T1 experiment, Ribavirin was able to prevent infection in all viral dilutions except 50% of the wells in the highest viral concentration (10^{-4}). In order to better assess the effect of this phenomenon we performed an indirect ELISA in between harvesting and infection events in order to control for the concentration of virus between passages. Antibody staining was substituted for end point dilution assays in order to more accurately quantify the infectivity of the progeny virions. End point dilution assays can only determine if a well is infected or not, whereas antibody staining allows the experimenter to count individual infection events, which gives a more quantitative assessment of viral infectivity across multiple dilutions. While end point dilution assays may reveal that all of the wells in 10^{-4} and 10^{-6} are infected, antibody staining would help discern the level of infectivity between the conditions. For example, there may be 200 infection events in 10^{-4} and only 75 in 10^{-6} revealing a difference in infectivity, but an end point dilution assay would only be able to mark both conditions as infected. Unfortunately, due to unwanted residual staining, these experiments could not be properly assessed.

We attempted to sequence the genomes of both less infectious (pre passage 3) and more infectious (post passage 3) harvested virus samples from each treatment condition. We converted the RNA genome to DNA and performed PCR to amplify the genomes. When the samples were placed in the nanodrop they revealed a medium to high concentration of DNA. However, when the samples were run on a 1% agarose gel at 120V for 45 minutes, no bands were visible. Unfortunately, we were not able to obtain viable DNA and therefore were unable to sequence the genome and locate where the mutations occurred and what type of mutations were present.

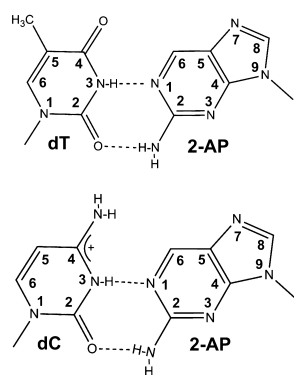


Fig. 1A. 2-Aminopurine (Reha-Krantz, et. al, 2011).

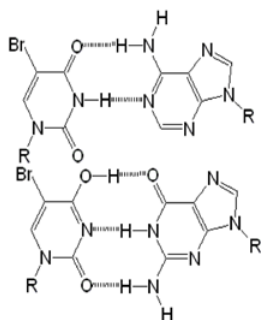


Fig. 1B. 5-Bromouridine (Bertoluzza, et al., 1997).

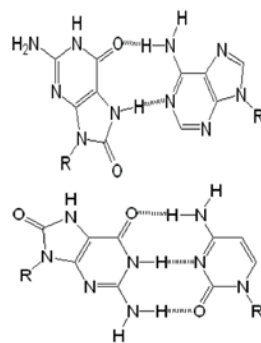


Fig. 1C. 8-Oxoguanosine (Briebe, et al., 2004).

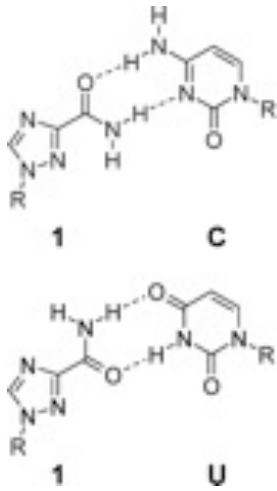


Fig. 1D. Ribavirin (Moriyama, et. al, 2008).

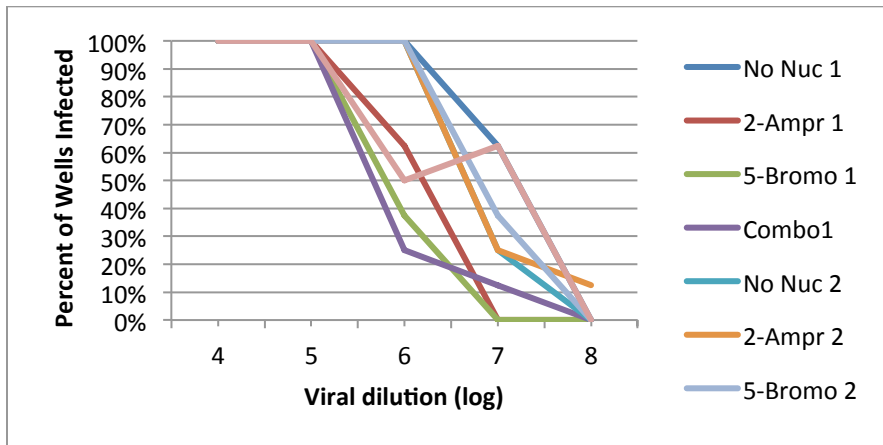


Fig. 2. Viral Dilution vs. Percent of Wells infected. Endpoint dilution assay Passage 1 T3-Abney. All conditions exhibit a relative decrease as the virus gets more dilute with almost all conditions (except 2-Aminopurine 2 at 12.5%) reaching 0% infected wells by 10^{-8} .

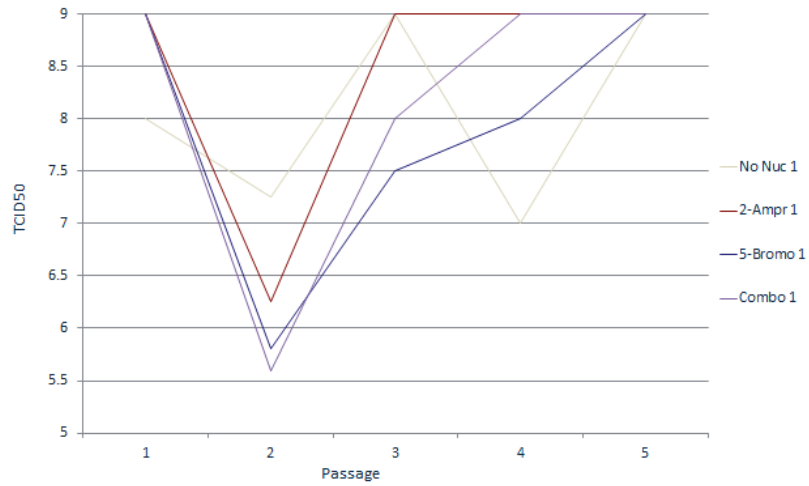


Fig. 3. Passage vs. TCID50 Type 3 Abney Reovirus (T3). A marked gain in infectivity occurs at passage 3 for all conditions including the wildtype which then decreases in infectivity while the experimental conditions show a complete recovery and reach their initial level of infectivity.

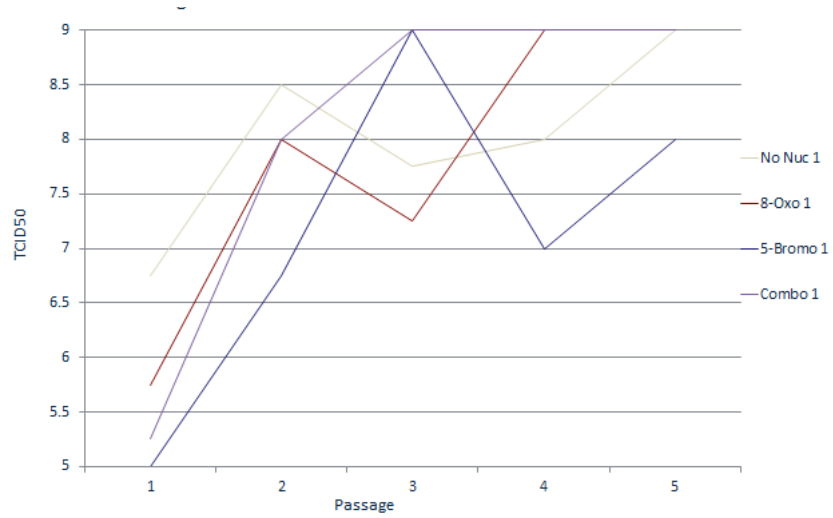


Fig. 4. Passage vs. TCID50 Type 1 Lang Reovirus (T1). A marked gain in infectivity occurs after passage 3 for all experiment conditions. 8-Oxoguanosine was substituted for 2-Aminopurine in this and subsequent experiments.

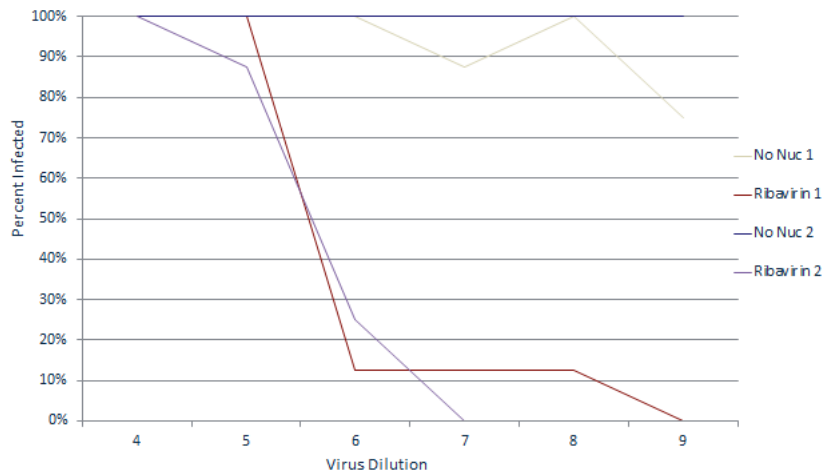


Fig. 5. Infectivity of Ribavirin Treated Reovirus Infected Cells vs. Control. Virus Dilution vs. Percent of Wells Infected T1 Passage 3. Ribavirin completely inhibits infection while the wildtype never has less than 75% of wells infected and does so more rapidly than the control (no nuc) conditions.

Table 1. Tissue culture infectious dose (TCID50) per 100 μ L values. Ribavirin vs. Wildtype. Passage (P) and viral strain (T1 or T3).

P1	T1	T3	P2	T1	T3
No Nuc 1	$\geq 10^9$	$10^{6.5}$	No Nuc 1	$10^{5.75}$	$10^{8.5}$
Ribavirin 1	$10^{5.5}$	$< 10^4$	Ribavirin 1	10^4	$< 10^4$
No Nuc 2	$\geq 10^9$	10^7	No Nuc 2	$10^{5.75}$	$\geq 10^9$
Ribavirin 2	$10^{5.5}$	$< 10^4$	Ribavirin 2	$< 10^4$	$< 10^4$

Discussion

While we cannot say that we in fact induced error catastrophe in Reovirus, the mutagens did cause a substantial decrease in infectivity before passage 3 for both viral strains. However, the most intriguing part of the experiments was the consistent yet unexpected gain in infectivity after passage 3 in both T1 Lang and T3 Abney Reovirus. However this is not entirely astonishing as Bull et al. (2007) documented that error catastrophe can actually shift a viral population to genotypes that are robust to mutation. Sequencing the genomes of less infectious and more infectious samples, as well as comparing them to the wildtype would help elucidate what type of mutations occurred and where they were incorporated into the genome. This would help clarify what parts of the Reovirus genome are important for infectivity and this information could be used to help engineer more effective antiviral strategies.

The mutagens acted as a selection pressure applied to the virus in vitro. Since the mutagens were present at all times of the viral life cycle they were constantly inserting into the genome. Virions that accumulate deleterious mutations die off and do not reproduce. However, virions that accumulate neutral or advantageous mutations not only survive, but reproduce at a high rate due to reduced competition for host cells as a result of a smaller viral population. We are in fact selecting for virions with advantageous or neutral mutations by this process which may explain the gain in infectivity after passage 3. It is possible that by passage 3, all of the viruses that accumulated deleterious died off and the virions with advantageous or neutral mutations are reproducing and drastically increasing their population. These “super” virions propagate more super virions which cause the overall virus infectivity to rise and return to their original level or in some cases, surpass it.

While Ribavirin is known to be effective at combating multiple RNA viruses, its effects on Reovirus have never been assessed. Our experiments demonstrated that Ribavirin has very strong antiviral properties against both T3 and T1 Reovirus and is a potent inhibitor of Reovirus infection. Ribavirin is able to reduce the infectivity of both strains of Reovirus by many orders of magnitude, and can cause a reduction as high as -10^5 (Table 1). Ribavirin was able to completely prevent infection from developing in the T3 experiments and only allowed infection to occur in 50% of the highest viral dilution wells for T1. Thus, we can conclude that Ribavirin, at 100 μ M, can effectively combat both T3 and T1 Reovirus infection in murine L929 cells. This is novel in that no other literature has documented the effect of Ribavirin on Reovirus infection.

Since mutations take time to accumulate it makes sense that this gain was not observed in passage 1 or 2. It is possible that the threshold for deleterious mutations (whether it be a few in particularly important spots or multiple throughout the genome) cannot be reached before passage 3 as not enough reproductive events have occurred. However, we cannot know exactly what transpired in regards to mutations until we sequence the genomes of both less and more infectious virions and compare them to each other and the wildtype sequence.

While the candidate mutagens did decrease Reovirus fitness *in vitro* their effect was only temporary as all treatment conditions except Ribavirin experienced a gain in infectivity and inevitably returned to or surpassed their previous levels of infectivity.

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