Nanoviricides As Anti-Influenza Agents

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ABSTRACT

Nanoviricides® anti-influenza agents are designed to mimic a natural host cell receptor to which all influenza A viruses bind and infect host cells. A nanoviricide is composed of a flexible polymer backbone with virus-specific ligands attached. The attachment of multiple ligands to a single polymer chain coupled with the fact that multiple polymer chains make up a single micelle leads to a very high avidity for the nanomicelle binding to the virus. A nanoviricide micelle may bind to a virus particle because of the specific interactions between the nanoviricide ligands and the glycoproteins on the virus surface. Binding of the influenza virus to the nanomicelle is believed to neutralize and engulf the virus, rendering it incapable of infection.

Anti-influenza nanoviricides® were evaluated for efficacy and potency *in vivo* using a highly lethal model, allowing the survival lifetimes to be directly used for rank ordering of efficacy. One million virus particles of Influenza A Strain A/WS/33 (H1N1) were aspirated directly into the lungs of mice. A repeat "booster" infection was performed at 22 hrs. This influenza model was designed to be uniformly fatal in 100% of the infected, untreated animals within 5 days after infection. Treatment with both the anti-influenza nanoviricides and Tamiflu® (Roche) commenced 24 hours after the first viral infection. Tamiflu was administered orally twice daily at 20mg/kg (i.e. 40mg/kg/day) while the anti-influenza nanoviricides were intravenous injections at 100mg/kg every 48 hrs. The animals treated with the best of the optimized, anti-influenza nanoviricides survived greater than twice as long (18.1 days) as opposed to the animals treated with Tamiflu (7.8 days) The increased survival was associated with a reduction in lung viral load measured at 4 days after virus infection. The most effective anti-influenza agent tested demonstrated a greater than 15-fold greater viral load reduction as compared to Tamiflu. Thus, nanoviricides represent potential anti-influenza therapeutic agents with a novel mechanism of action; further optimization is in progress.

Keywords: Anti-influenza therapeutic agents, nanomicelles, virus neutralization

1 INTRODUCTION

Current antiviral therapies suffer from limited efficacy, incomplete coverage due to genetic heterogeneity of the virus, rapid emergence of virulent, readily transmissible, drug resistant mutants, and/or side effects. There are a number of stages in the viral life cycle that represent potential targets for the development of antiviral therapies: 1) viral attachment and entry into the cell; 2) uncoating of the virus; 3) transcription of viral mRNA; 4) translation of viral mRNA; 5) replication of viral DNA or RNA; 6) maturation of viral proteins; 7) assembly of viral particles; 8) budding or release of mature virus. The current approved antiviral drugs exploit many of these targets as their mechanisms of action. Most of the current anti-viral strategies are based on inhibition or modulation of the intracellular biochemical pathways, mediated by both viral and host cell enzymes for the production of new virus particles[1-6].

Unlike the current antiviral strategies, NanoViricides nanotechnology possesses potent antiviral efficacy by targeting the mechanisms of cell attachment or cell binding of viruses. A nanoviricide is composed of a flexible polymer backbone with virus-specific ligands attached. The attachment of multiple ligands to a single polymer chain coupled with the fact that multiple polymer chains make up a single micelle leads to a very high avidity for the nanomicelle binding to the virus. A nanoviricide micelle may bind to a virus particle because of the specific interactions between the nanoviricide ligands and the glycoproteins on the virus surface. Binding of the virus to the nanomicelle is believed to neutralize and engulf the virus, rendering it incapable of infection[7]. The results reported herein show the anti-viral efficacy of nanoviricides in a murine model of influenza virus infection in which the virus infection is uniformly lethal.

2 MODEL

Male Balb/C mice, 6-7 weeks old and weighing between 18 to 22 gms, were infected with Influenza A virus strain A/WS/33/ (H1N1); 10,000 viral particles were infused into the animal as a nasal spray at time 0 and at 23 hrs after the initial viral infection. Mice were sorted into ten groups of twenty two. Group A mice were infected but untreated control animals. Group B received saline, 100 µl i.v., as a Negative Treatment Control and Group C, received Oseltamivir p.o. as a Positive Treatment Control. Groups D,E,F,G,H,I,& J received the proprietary Nanoviricides, NV5-34-63-1, NV5-34-63-2, NV5-34-63-3, NV5-34-63-4, NV5-34-63-5, NV5-34-63-6, NV5-34-63-7, respectively. The nanoviricides were given i.v. in 100 µl of a 20 mg/ml solution per animal (~50 mg/kg) on days 1,3,5,7,9,10,12,15 and 18 after the first virus inoculation by the nasal infection). Group B mice were treated on the same days as

the Nanoviricides-treated mice. The Group C, Oseltamivir-treated mice were administered Oseltamivir, 20 mg/kg p.o. 2x daily for the duration of the study, beginning 24 hrs after the first virus inoculation.

Each animal was sacrificed when the animal reached the predetermined endpoint. The mice were monitored until death on 21 days (considered to be full recovery) for the following parameters: Survival (4hr intervals when there is marked body weight change, body weight measured daily until marked change occurs, when it will be every 4 hrs.), and clinical signs taken every two hours. As and when animals became moribund they were euthanized by carbon dioxide inhalation. The lung tissues were collected, weighed, and divided in two halves, one preserved in formalin and from the other half used to prepare a tissue homogenate for lung viral load determination. All surviving animals were euthanized on day 21. At 84, 96,108 hrs four animals per group per time point were sacrificed and the lungs were removed, weighed and half of the lung tissue for histology, while the remaining lung tissue was frozen at -80°C for viral load determination.

3 RESULTS

The survival results are shown in Figure 1. The Group A infected, untreated control mice and Group B saline-treated mice all died with a mean survival of 120 hrs. Similarly, the Group J, NV-5-34-63-7 treated NNVC mice, the vehicle control group, all died with a mean survival of approximately 120 hrs. The Oseltamivir-treated mice exhibited a prolonged survival, 187 hrs, while mice treated with NV-5-34-63-1 to NV-5-34-63-6 showed substantially improved survival as compared to the positive control Oseltamivir, ranging from 300 hrs to 434 hrs. The mice treated with NV-5-34-63-4 displayed the best survival.

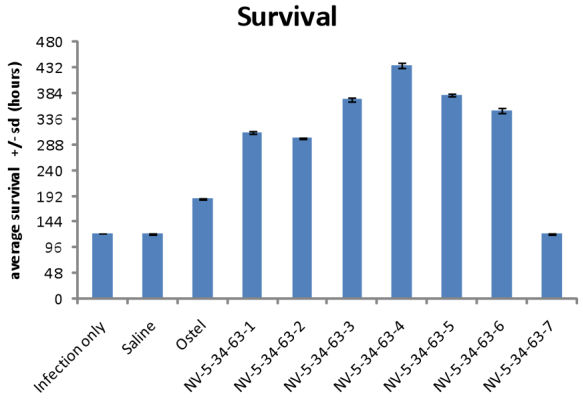

Figure 1. Average survival in hours of treated or untreated infected mice.

The p values from Student's t test of survival of each treatment group vs. the infected, untreated control group showed that the oseltamivir and NV-5-34-63-1 to NV-5-34-63-6 groups all were statistically significant, $p < 0.001$, whereas the saline and vehicle control, NV-5-34-63-7, were not statistically different than the infected, untreated group.

As shown in Figure 2, body weight began to decline in the infected, untreated controls (Group A), the saline-treated controls (Group B) and in Group J NV-5-34-63-7 by 72 hrs and continued to decline until death. The Oseltamivir-treated mice maintained body weight through 72 hrs and declined from 96 hrs onward until death. Similar to the survival results, the mice treated with NV-5-34-63-1 through NV-5-34-63-6 (Groups D-I) maintained their body weight substantially longer, from 216 hrs (day 9) for NV-5-34-63-1 to 360 hrs (day 15) for NV-5-34-63-4 and declined thereafter until death. For all groups, the onset of reduced water and food consumption generally coincided with the onset of body weight loss.

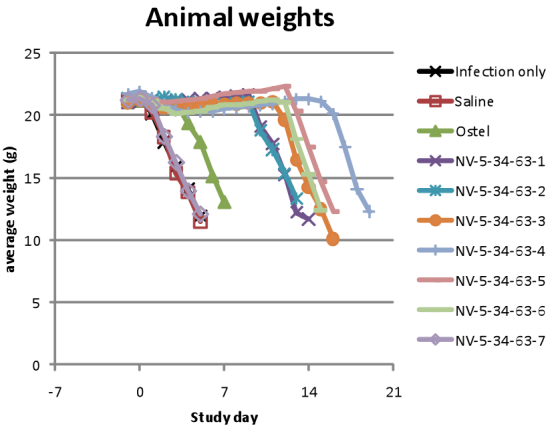

Figure 2. Weights of animal of treated or untreated infected mice.

Influenza virus infection in these animals produced characteristic influenza lung lesions composed of inflammatory cells, edema, hemolysis, and necrosis of lung tissue. Analysis of formalin-fixed, H&E stained sections of these lungs using a dissecting microscope reveals that these lesions appear as plaques. In the infected, untreated group as well as the saline-treated and vehicle-treated, NV-5-34-63-7 groups the lung lesions were severe at an early stage, 84 hrs and progressed. As shown in Figure 3, both plaque number and area were increased. In the oseltamivir group, the plaque number was low at 84 hrs and increased at 108 hrs. In the nanoviricide-treated groups, Groups NV-5-34-63-1 to NV-5-34-63-6 plaque number and plaque area was low at 84 hrs and was not increased at 108 hrs.

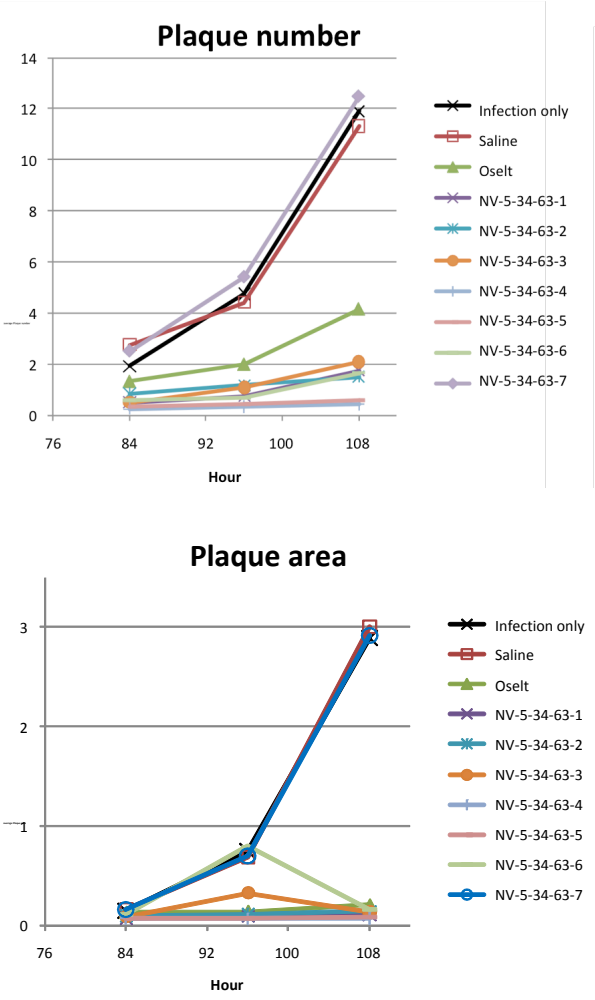

Figure 3. Average number of histologic lung plaques (top) or histologic lung plaque area (bottom) from untreated and treated influenza infected mice.

Pulmonary virus load was measured in lung homogenates at these same 84, 96 and 108 hr time points. As shown in Figure 4, in infected, untreated mice as well as in saline-treated and vehicle-treated, NV-5-34-63-7, mice the viral load was elevated at 84 hrs and continued to increase over the 96 and 108 hr time points. Oseltamivir- and NV-5-34-63-6-treated mice had lower viral load at all time points. At 108 hrs viral load was reduced 2-fold in both groups as compared to the infected, untreated mice. In contrast, mice treated with NV-5-34-63-1 to NV-5-34-63-5 displayed markedly reduced lung viral loads at 84 hrs with little increase at 96 and 108 hrs. For the best compounds, NV-5-34-63-4 and NV-5-34-63-5, the viral load was roughly 15-fold lower than that for the infected untreated mice. Similar results were obtained at dilutions of 1:2000, 1:5000 and 1:10,000.

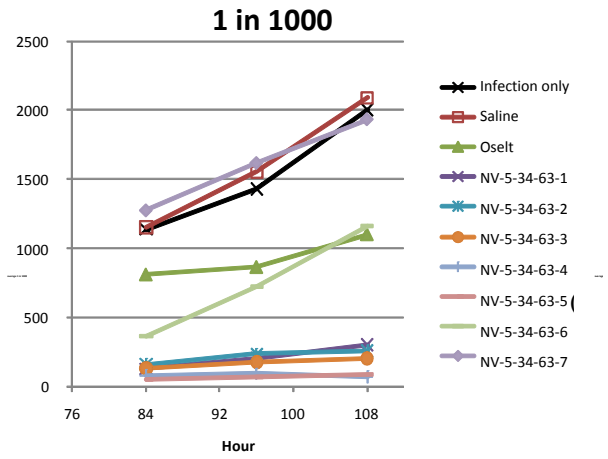

Figure 6. Results of *in vitro* viral plaque assay of lung homogenates. Average number of plaques in homogenates diluted 1:1000.

4 DISCUSSION

The results of the present study show that treatment with nanoviricide anti-viral agents in a lethal influenza mouse model provided significant protection. Control infected mice all died within 5 days; oseltamivir-treated (tamiflu) mice survived a mean of 7.8 days. In contrast the best nanoviricide-treated mice survived 18.1 days. This increased survival of nanoviricide-treated mice was accompanied by protection of body weight loss. More importantly, nanoviricide treatment provided marked protection against the development of virus-induced lung lesions. These characteristic influenza lung lesions, composed of inflammatory cells, edema, hemolysis, and necrosis of lung tissue, were significantly reduced in both number and size. Lung damage is a very important part of the pathology of influenza virus infection. In humans uncontrolled lung damage can lead to pneumonia, and in severe cases of influenza potentially death,. Consistent with the protection against lung damage, lung viral load was markedly reduced in the nanoviricide-treated mice. Thus, the best nanoviricide anti-influenza agents markedly increased survival in this lethal infection model and this survival increase was accompanied by protection against virus-induced lung damage and by a marked reduction in viral load. We believe nanoviricides represent potential anti-influenza therapeutic agents with a novel mechanism of action; further optimization is in progress.

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