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Antiviral Activity of AV-001 against Influenza and Three Common Cold Viruses

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Abstract

AV-001 is a dietary supplement; it is used as a throat and nose spray composed of natural ingredients, including *Sambucus nigra* (European elder) and eucalyptus with 1, 8-cineole. The aim of this study was to evaluate the antiviral activity of AV-001 against the pathogenic viruses human influenza A viruses H1N1 and H7N9, respiratory syncytial virus, rhinovirus, human coronavirus, and adenovirus. Antiviral activity was evaluated by reduction of the viral-induced cytopathic effect; AV-001 was assessed against a challenge virus in suspension, inoculated onto host cells, and assayed for infectious viral load.AV-001 inactivated human influenza A viruses H1N1 and H7N9, respiratory syncytial virus, rhinovirus, and human coronavirus, by ^{3}7.17 Log_{10}, ^{3}6.42 Log_{10}, ^{3}4.72 Log_{10}, ^{3}4.35 Log_{10} and ^{3}3.92 Log_{10}, respectively. Thus, AV-001 exhibited strong and broad-spectrum antiviral activities and may represent an effective treatment or preventive agent for respiratory viral infection by inactivating the viruses upon their entry into the body.

Keywords: Antiviral activity, H1N1, H7N9, human coronavirus, natural, respiratory rhinovirus, syncytial viru

1. Introduction

Seasonal flu is caused by influenza viruses, which infect the respiratory tract. It is estimated that on average, approximately 5% to 20% of residents in the United States (US) get the seasonal flu, and more than 2,00,000 people are hospitalized for flu-related complications each year [1]. The overall national economic burden of influenza-attributable illness for adults, age 18 years and above is $83.3 billion; direct medical costs for influenza in adults totaled $8.7 billion including $4.5 billion for adult hospitalizations resulting from influenza-attributable illness [2]. With development of resistance to current anti-viral medications [3], as well as pandemics including recent H7N9 [4] and Middle Eastern respiratory virus corona virus (MERS-CoV) [5, 6], there is a need for new safe and effective anti-viral treatments.

In the US, the common cold leads to 75 to100 million physician visits annually at a conservative cost estimate of $7.7 billion per year [6].[6]An estimated 22 to 189 million school days are missed annually due to a cold. As a result, parents missed 126 million workdays to stay home to care for their children. When added to the 150 million workdays missed by employees suffering from a cold, the total economic impact of cold-related work loss exceeds $20 billion per year.

The most common cold viruses include rhinoviruses - causing 10% to 40% of colds; coronaviruses - causing 20% of colds; and Respiratory Syncytial Virus (RSV) - responsible for 10% of colds. Treatments and preventions range from prescription medications to over-the-counter products to disinfectants to face masks to simple hand washing. Americans spend $2.9 billion on over-the-counter drugs and another $400 million on prescription medicines for symptomatic relief. More than one-third of people who saw a doctor received an antibiotic prescription, which has implications for antibiotic resistance. The Center for Disease Control (CDC)

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recommends a yearly flu vaccine as the first and most important step in protecting against flu viruses. However, vaccines have several weakness: 1) production is slow - experts have shown that it may take approximately 3 to 6 months for an effective vaccine to be produced, and an influenza pandemic could be devastating during the early incubation period; 2) Vaccine Effectiveness (VE) is difficult to predict: in January 2013, CDC published interim estimates of the 2012-2013 flu vaccine's effectiveness at preventing medical visits due to laboratory-confirmed flu, and reported an overall VE of 62% [7]; 3) influenza viruses mutate quickly; and there are many different flu viruses, but a flu vaccine only protects against the 3 viruses that a committee suggests will be most common; 4) vaccination against influenza has recently been met with low compliance rates in industrialized countries [8], even among health-care workers [9]; 5) concerns about safety, the possibility of side effects, and the vaccine development process are the primary reasons cited for not receiving the vaccine.

Improved protection against influenza and other viruses may be obtained with a product that is: 1) wide spectrum (inactivates viruses regardless of the type); 2) specifically targets the locations where viruses infect and spread (nose and throat); 3) easily deployable (obtained 'over the counter' and used at home daily); 4) proven safe (contains ingredients that have a long history of safe use across worldwide populations). Many natural products have been shown to have antiviral activity

AV-001 is a direct-contact throat and nose spray for daily use in the year-round protection against all major classes of the influenza virus as well as additional viruses. AV-001 is a combination of natural ingredients, including *Sambucus nigra*, the main active ingredient. This spray is designed to directly inhibit infection by blocking viruses at its points of entry: the nose and throat. AV-001works via mechanisms that are distinct from the current standard of care for anti-virals, and is intended to provide a rapid onset of action, while the long history of safety of the ingredients allows an expanded treatment window.

In this study, the virucidal effectiveness of AV-001 was measured by assaying the potential to inactivate virus in suspension. AV-001 was evaluated against several pathogenic viruses, including human influenza A viruses (H1N1 and H7N9), rhinovirus type 14, adenovirus type 2, RSV, and human coronavirus (229E strain) in suspension following the standards in the ASTM International E1052-11 method, "Standard Test Method to Assess the Activity of Microbicides against Viruses in Suspension."

2. Materials and Methods

2.1 Composition of AV-001

AV-001 is a dietary supplement that contains a mixture of standardized herbal extracts, including purified extract from the berries of *Sambucus nigra* (European elder) and eucalyptus with 1, 8-cineole.Other ingredients include methyl salicylate, thymol, and L-Menthol.

2.2 Cells and Virus Strains

MCR-5 cells (ATCC CCL-171), A549 cells (ATCC CRL-185), MOCK cells (ATCC CCL-34), and HeLa cells (Diagnostic Hybrids CCL-2), were cultured in growth medium consisting of Eagle's Minimal Essential Medium (E-MEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% sodium bicarbonate, and 1% HEPES, at 37°C in a 5% CO2 incubator. H1-HeLa cells (ATCC CRL-1958) was cultured in RPMI-1640 medium supplemented with 10% FBS, 1% sodium bicarbonate, and 1% HEPES at 37°C in a 5% CO2 incubator.

Human coronavirus, Strain 229E (ATCC VR-740), Adenovirus Type 2 (ATCC VR-846), Respiratory Syncytial Virus, (ATCC VR-26), and Rhinovirus Type 14 (ATCC VR-284) were purchased from ATCC. Human Influenza A Virus (H1N1, AlCalifornia/04/09) was purchased from Charles River Laboratories. Virus stocks of the influenza A virus (H1N1) were propagated and quantified in MDCK cells, adenovirus in A549 cells, RSV in HeLa cells, coronavirus in MRC-5 cells, and rhinovirus in H1-HeLa cells. All virus stocks were stored at −70°C until used.

2.3 Virucidal Activity

AV-001 was evaluated against a challenge virus in suspension. For each run, a 2.7 ml aliquot of AV-001 was spiked with 0.3 ml of the virus suspension and mixed thoroughly. At the completion of each contact time, an aliquot of the reaction mixture was pulled and immediately mixed with an equal volume of neutralizer,

and further quenched by dilution with medium. The quenched sample was serially diluted with medium in tenfold increments and inoculated onto host cells to assay for infectious virus. The inoculated host system was incubated and read for quantity of infectious virus.

The residual infectious virus in the test and controls was detected by viral-induced effect (CPE). Selected dilutions of the neutralized inoculum/test article mixture were added to cultured cell monolayers at a minimum of 4 wells per dilution per sample. Inoculated plates were incubated at 36 ± 2ºC in 5 ± 1% CO_2 for 4-6 days for influenza A viruses (H1N1 and H7N9), 14–18 days for RSV, 5–7 days for coronavirus, 6–9 days for rhinovirus, and 11–14 days for adenovirus, and then examined for presence of infectious virus by CPE. The titer of the virus (log_{10} $TCID_{50}$/ml) was calculated using the Spearman-Karber formula [10, 11] or Poisson distribution when no virus was detected [12].

2.4 Controls

Controls included an input viral load control, neutralizer effectiveness/viral interference control, a cytotoxicity control, a media negative control, and a virus stock titer control. The neutralizer effectiveness/viral interference control was performed in order to determine if residual active ingredients were present after neutralization and if it interfered with virus infectivity. A 2.7 ml aliquot of each test article was mixed thoroughly with 0.3 ml of medium (in lieu of the challenge virus), held for contact time, and then neutralized. The neutralized sample was further quenched by dilution with medium. The sample was then serially diluted in tenfold increments using dilution medium. Each dilution was divided into 2 portions, one for neutralizer effectiveness/viral interference control, and the other for cytotoxicity control. For the neutralizer effectiveness/viral interference control, 0.1 ml of a low tittered virus was added to 4.5 ml of each dilution of the solution, held for a period equivalent or greater than the contact time, then used to inoculate host cells. For the cytotoxicity control, the sample obtained from the neutralizer effectiveness/viral interference control run were inoculated onto host cells and incubated. The condition of the host cells was recorded at the end of the incubation period. For the input load control, a 2.7 ml aliquot of medium (in lieu of AV-001) was mixed thoroughly with 0.3 ml of the challenge virus, held for contact time, and then neutralized. The neutralized sample was further quenched by dilution with medium. The quenched sample was serially diluted with dilution medium in tenfold increments and selected dilutions were inoculated onto host cells to assay for infectious virus. The virus control results were used as the input viral load and compared with AV-001 treatment results to evaluate viral reduction by AV-001. For the media control, at least 4 wells were inoculated with media in each assay to demonstrate that cells remained viable and media was sterile throughout the assay. For the virus stock titer control, an aliquot of the virus was serially diluted and inoculated directly onto host cells to confirm the appropriate titer.

3. Results

In order to determine the virucidal effectiveness of AV-001, each virus was incubated with AV-001, as described above. Samples were titrated by 50% Tissue Culture Infectious Dose ($TCID_{50}$) endpoint assay using the appropriate host cell system for each virus. The viral load (log_{10} $TCID_{50}$) was calculated by adding the viral titer (log_{10} $TCID_{50}$/ml) to the log_{10} (the concentration of the volume in ml times the volume correction). The volume correction accounted for the neutralization of the sample post contact time. The log_{10} reduction factor was then calculated by subtracting the output viral load (log_{10}) from the input viral load (log_{10}). AV-001 inactivated all viruses tested, with the exception of adenovirus type 2, when exposed for 5 minutes at 20ºC (Table 1).

Table 1: Virucidal Effectiveness of AV-001

	Log_{10} *$TCID_{50}$ Initial Viral Load	Log_{10} *$TCID_{50}$ Output Viral Load	Log_{10} Reduction Factor	Percent Inactivation
Influenza A H1N1 Charles River A/ California/04/09	8.78	£ 1.61	³ 7.17	³ 99.99%
Influenza A H7N9 Anhui A/ Anhui/1/2013 H7N9	8.03	£ 1.61	³ 6.42	³ 99.99%
Rhinovirus	6.96	£ 2.61	³ 4.35	³ 99.99%
Respiratory Syncytial Virus	7.33	£ 2.61	³ 4.72	³ 99.99%
Coronavirus	5.53	£ 1.61	³ 3.92	³ 99.99%
Adenovirus	5.83	5.83	0	0

*TCID50 – 50% Tissue Culture Infective Dose.

The "Initial Viral Load" represents the virus units (Log_{10} $TCID_{50}$) recovered after mixing and holding the virus in medium; the "Final Viral Load" represents the virus units (Log_{10} $TCID_{50}$) recovered after mixing and holding the virus in AV-001.

The viral stock titer control for each assay confirmed the appropriate titer used in the experiment, and sufficient amount of virus was recovered for the virus recovery control (data not shown). In the cell viability control wells, no virus was detected, the cells remained viable, and the media were sterile. In all neutralizer effectiveness/viral interference control wells tested, virus was detected. Viral-induced CPE was distinguishable from test article-induced toxicity in all cases. Thus, all of the controls met the criteria for a valid test.

The study was based on evaluating a limited number of samples across a number of different viruses for which AV-001 could be considered a treatment. While not specifically designed for hypothesis testing, the results are discussed from a statistical perspective. First and foremost, such studies are used to show that an effect can be demonstrated and this study provided strong evidence of effect in all of the samples except for the adenovirus. Count data are frequently evaluated using the Poisson distribution and if one assumes the baseline is representative of the true distributional parameter, the follow-up viral load reductions (>98.4%) are *statistically* extremely unlikely. Data on each of the six viruses considered were collected in duplicate and averaged together. As screening evaluations for influenza are non-specific, we considered the mean and associated 95% CI for the mean for the Log_{10} viral load reduction for these strains based on the averaged results. Even with a limited sample size the mean was 4.43 and the 95% two-sided CI was (1.80, 7.06). This corresponds to a lower bound on the reduction value that is still greater than 98.4%.

4. Discussion

The goal of this study was to determine the potential antiviral activity of AV-001 against several viral pathogens known to cause infection in humans. AV-001 inactivated all viruses tested, with the exception of adenovirus type 2, when exposed for 5 minutes at 20ºC, indicating that AV-001 can offer effective viral inactivation against a wide spectrum of harmful viruses on a daily basis. AV-001 may also complement the seasonal influenza vaccine by attacking the virus at the point of entry, the nasal–pharyngeal cavity. In addition, AV-001 may be more accessible in the case of pandemic outbreak during the 4–6 month period of time when vaccines must be produced after a pandemic viral strain is identified.

From a clinical perspective, it is well known that it is often difficult to diagnose the exact virus causing upper respiratory and common cold symptoms. The additional virucidal activity of AV-001 against the other viruses tested (rhinovirus, respiratory syncytial virus and human coronavirus) is an added benefit with potential significant clinical implications.

The mechanism of action of AV-001 includes several possibilities based on the ingredients. Elderberry extract, one component of AV-001, is known to contain *Sambucus nigra* Agglutinin (SNA). SNA is a lectin that avidly binds to α2-6 sialic acid receptors, which are the same receptors that viruses, such as influenza, use to gain cellular entry [13]. By blocking these receptors in the nose and throat, cellular entry of the virus is less effective. The function of the influenza virus surface protein hemagglutinin may be affected by AV-001 so that is does not bind as effectively to the α 2-6 sialic acid receptors on the cell surface. The other active viral surface protein, neuraminidase, would be left unaffected and could destroy the cellular sialic acid binding receptors. Eventually, the cellular viral receptors would be either blocked or destroyed while the virus is disabled due to surface protein conformation change to prevent binding to any viable sialic acid receptors. Viral cellular entry would be inhibited giving the immune system valuable time to respond. Eucalyptus is a known remedy for cold and flu, and has also been shown to have an effect on lipid-coated viruses such as Herpes Simplex Virus 1 (HSV-1) [14]. The exact mechanisms and contribution of each component needs rigorous research and further studies.

The test results showed no activity of AV-001 against the adenovirus. There are several possible reasons for this. First, the adenovirus is not a lipid coated virus but rather a heavily fortified icosahedral virus with multiple long spike projections. This icosahedral structure is particularly strong due to not only the 240 hexon capsomers and 12 penton capsomers forming the icosahedron matrix but also minor capsid proteins that stabilize nonequivalent

interactions between hexons allowing the same hexon capsomer to be used in four different chemical environments on the surface of the capsid [15]. The adenovirus does not dissociate in acidic environment of the endosome but rather waits for nuclear entry to dissociate. Only one adenovirus (Ad37 in subgroup D) out of the hundred or so sub-types has a sialic acid component on the fibrous spike [16]. Therefore the sialic acid blocking effect of the test product AV-001 will not be effective on the majority of adenovirus sub-types.

These data suggest both a treatment (therapeutic) and preventative strategy due to the 5-minute efficacy in inactivating several viruses. This work leads the way to further investigations such as the viral load reduction in time, identifying AV-001 concentrations required to elicit an effect, and other evaluations that would inform the use of AV-001 as a potential agent to prevent the spread of a viruses. We suggest that populations begin using AV-001 at the first signs of viral infections in their communities, as well as after first signs of infection. An extensive GLP safety study in rodents at *14 times the normal dosage has been completed with no adverse effects.* Clinical trials are in process to show the most effective timing and duration of treatment, however due to the safe nature of the ingredients, the possibility of adverse effects is minimal.

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