RESEARCH ARTICLE

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Preliminary investigation on the combined effect of S-adenosyl-L-methionine (SAM) and oseltamivir on experimental influenza A virus infection in mice

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ABSTRACT
Influenza is one of the most contagious viral diseases, caused by influenza virus and it affects thousands of people every year. The infection causes changes in the intracellular redox balance, increased production of reactive oxygen species, development of antioxidant deficiency and conditions of oxidative stress. Decreased level of gluthatione during flu is responsible for the severe pathology and complications. The purpose of our studies was to follow the effect of the combination S-adenosyl-L-methionine (SAM) as a precursor of glutathione and the specific neuraminidase inhibitor oseltamivir in influenza infected mice. SAM was given as a single daily dose of 50, 100 and 150 mg/kg, starting from 5 days before infection until day 4th after viral inoculation. Oseltamivir was given in a daily dose of 2.5 mg/kg in two intakes for 5 days, starting from 4th hour before infection. End-point evaluation was 14 day survival rate, average survival time, index of protection, and virus titer in lungs.
The results showed that application of SAM alone did not indicate significant antiviral protection. In mice supplemented with oseltamivir only survival rate was 70%, but combination of oseltamivir and SAM in lower doses led to rising of 90% protection. The present findings suggest that combined therapy of SAM as a precursor of glutathione and the specific inhibitor of influenza virus replication oseltamivir could be effective on modulation of host defence mechanism(s) in low therapeutic doses.

Key words: influenza virus A (H3N2), S-adenosyl-methionine, glutathione, oseltamivir

Introduction

The influenza virus is one of the most infectious human agents, and influenza epidemics appear every year worldwide (Han&Meydani, 2000). The development of the pathogenicity and virulence of the influenza virus is determined by several interacting factors, pooled in two ways: (i) Host factors, as availability of enzymes in host cells which are essential for viral entry and replication; the state of immunocompetence of the individual host; the ability of the immune system to control the viral replication effectively without causing serious collateral damage for the host by its inflammatory response; (ii) Viral factors: restriction of cytopathogenic effects to allow an appropriate balance between viral replication and control by the host; modulation of the immune response to attenuate effective host defence mechanisms (Behrens & Stoll, 2006). As a result of the infection, local lung damages were observed due to viral replication in the cylindrical ciliary epithelium of bronchi and bronchioles, which leads to progressive inflammation of the alveolar cells, bronchopneumonia (viral or combined viral-bacterial), massive bronchitis (including bronchiolitis), and is the major causes of lethal exit (Taubenberger&Morens, 2008). In accordance of recommendations of World Health Organization, antiviral drugs for influenza are two types, based on their modes of action: (i) the neuraminidase inhibitors oseltamivir, zanamivir, peramivir, and related compounds, and (ii) the M2 protein blockers rimantadine-HCl and amantadine-HCl. Although both types of agents have proved their antiviral effectivity, the rate of drug resistance is constantly increasing, especially for M2 blockers (WHO, 2014).

In order to include the main processes involved in influenza pathogenesis, this work was aimed to observe the combined effect of oseltamivir and S-adenosyl-L-methionine against influenza A/Aichi/2/68 (H3N2) virus infection (10xMLD50) in mice.

Materials and Methods

Compounds

Oseltamivir phosphate (the ethyl ester prodrug of oseltamivir) was purchased from Hoffmann-La Roche

(Switzerland). The compound was diluted *ex tempore* in phosphate-buffered saline (PBS) for *in vivo* experiments.

S-adenosyl-methionine (SAM), Sigma Aldrich, was dissolved in sunflower oil for *in vivo* testing.

Virus

Influenza virus A/Aichi/2/68 (H3N2) was obtained from the D. I. Ivanovsky Institute of Virology, Moscow (Russia), adapted to mice, and then propagated in 10-day-old chicken embryos through serial intraallantoic passages.

Animals and treatment

White male mice of the ICR line with body weight 14–16 g were placed in specially designed, well-ventilated acrylic cage containers, with free access to water and food, and maintained in the Animal House facility of the Stephan Angeloff Institute of Microbiology, BAS. During a 3-day acclimation period (prior to experimental onset), they were observed for signs of diseases and/or physical abnormalities. Animal husbandry and experiments were conducted in accordance with the guidelines of Bulgaria's Directorate of Health Prevention and Humane Behaviour toward Animals.

For the purpose of the experiment they were anaesthetized with ether and infected intranasally with 10×LD50 influenza virus strain A/Aichi/2/68 (H3N2). The experimental groups were designed as shown in Table 1.

Mice were observed daily for 14 days for survival after infection. Mean survival time, weight of the groups, index of protection and coefficient of protection were calculated.

Table 1. *Experimental groups and drug supplementation.*

Groups	Treatment
I.	Healthy, non-infected animals (n=15);
II.	IVI (n=15);
III.	IVI plus oseltamivir 2.5 mg/kg daily in two intakes, *per os* (n=15);
IV.	IVI plus SAM 50 mg/kg, *i.p.* (n=15);
V.	IVI plus SAM 100 mg/kg, *i.p.* (n=15);
VI.	IVI plus SAM 150 mg/kg, *i.p.* (n=15);
VII.	IVI plus oseltamivir 2.5 mg/kg and SAM 50 mg/kg, *i.p.* (n=15);
VIII.	IVI plus oseltamivir 2.5 mg/kg and SAM 100 mg/kg, *i.p.* (n=15);
IX.	IVI plus oseltamivir 2.5 mg/kg and SAM 150 mg/kg, *i.p.* (n=15);

IVI – influenza virus infection;
Oseltamivir was given in a dose of 2.5 mg/kg daily in two intakes, per os, for 5 days, starting 4 h before infection and for the subsequent 4 days after infection;
SAM was supplemented intraperitoneally, once a day starting 5 days before infection and for the subsequent 4 days after infection.

Determination of body weight, mean survival time (MST), protection coefficient (PC), and protection index (PI)

Body weight was measured on the days 1th, 4th, 7th, and 12th.

Mean survival time was calculated according to the formula:

$MST = \sum[f(d - 1)]/n$

where *f* is number of mice recorded dead on day *d* (the survivors on day 14 were included in the calculation) and N is number of mice in a group (Grunert et al., 1965).

Protection coefficient (PC) was calculated as percentage mortality in placebo group divided by percentage mortality in the drug-treated group.

PC (%) = % mortality in placebo group/ % mortality in the drug-treated group.

Protection index (PI) was evaluated by the equation:

PI (%) = (PC - 1/PC)*100 (Galabov et al., 2006).

Results

The effect of SAM and oseltamivir alone and in combinations on the cumulative mortality rate is shown in Figure 1. SAM only, administrated in different doses could not affect significantly this parameter. Oseltamivir in a dose of 2.5 mg/kg showed 30% mortality rate. Supplementation of infected mice with SAM only in doses of 50, 100, and 150 mg/kg could not protect them. Combination therapy of oseltamivir with SAM 150 mg/kg showed higher mortality

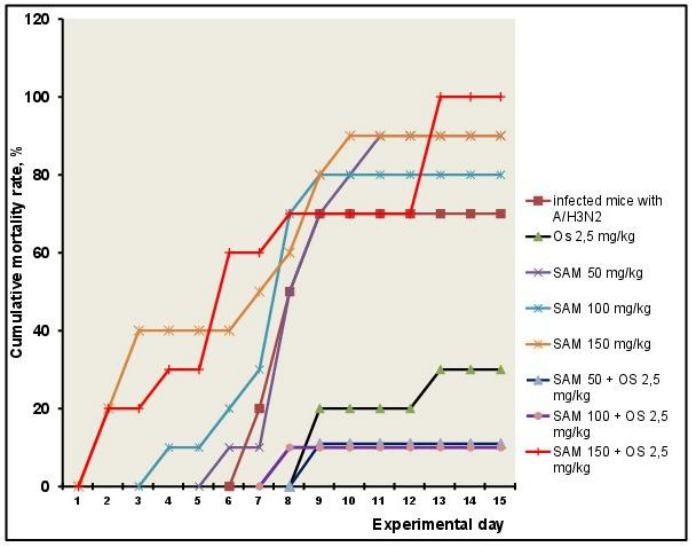

Figure 1. *Effect of SAM, oseltamivir, and their combination on cumulative mortality rate of influenza virus infected mice, monitored until 14th day after inoculation.*

rate than the infected but not treated animals. As seen on the Figure 1, combining oseltamivir with SAM in doses of 50 and 100 mg/kg showed the lowest mortality rate of 10%.

The changes in the body weight of the mice is shown in Fiugure 2. Infected and non-treated mice lost about 20% of their weight. Mice that were supplemented with SAM 50 mg/kg showed similar result. In all other groups there were no significant changes in their body weight.

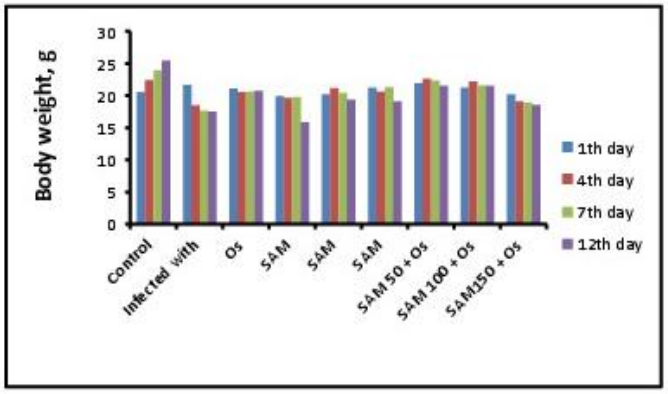

Figure 2. *Changes in the body weight of influenza virus infected mice, monitored until 14th day after inoculation.*

The effect of SAM and oseltamivir alone and in combinations on MST, PC and PI are shown in Table 2.

Table 2. *Effect of the combination of oseltamivir and SAM on MST, PC (%) and PI (%) of albino mice infected with influenza virus A/Aichi/2/68 (H3N2) at 10XMLD50.*

Experimental groups	MST	PC	PI
I. Controls	14		
II. Mice infected with A (H3N2)	8.8		
III. Oseltamivir 2.5 mg/kg	11.9	2.3	56.5
IV. SAM 50 mg/kg	8.4	0.77	0
V. SAM 100 mg/kg	7.6	0.875	0
VI. SAM 150 mg/kg	6.9	0.77	0
VII. Oseltamivir 2.5 mg/kg+SAM 50 mg/kg	12.5	7	85.71
VIII. Oseltamivir 2.5 mg/kg+SAM 100 mg/kg	12.5	7	85.71
IX. Oseltamivir 2.5 mg/kg+SAM 150 mg/kg	4.3	0.7	0

In the group of infected with influenza A Aichi (H3N2) but non treated mice the MST was 8.8 days. SAM did not have any protective activity. In all three doses - 50, 100 and 150 mg/kg (respectively groups IV, V and VI) the MST was low and PI was 0. In the highest dose of SAM - 150 mg/kg MST was lowest – 6.9 days. PC in these three groups was almost similar. In a dose of 100 mg/kg MST rose to 7.6, days. In the lowest dose of 50 mg/kg it was 8.4 days.

In the group treated with oseltamivir MST rose to 11.9 days. PC was 2.3 and PI was 56.5. Highest MST, PC and PI were found in the combinations of SAM 50 mg/kg with oseltamivir 2.5 mg/kg (group VIII) and SAM 100 mg/kg and oseltamivir 2.5 mg/kg (group IX). In both groups MST was 12.5 days. PC was 7 and PI- 85.71.

In group X - combination of SAM in a dose of 150 mg/kg and oseltamivir 2.5 mg/kg MST was very low – 4.3 days. PC was 0.7 and PI was 0.

Discussion

In this work, our attention was focused on the combined effect of SAM as a precursor of gluthatione synthesis and oseltamivir as neuraminidase inhibitor in experimental influenza A virus infection in mice. Our experimental results showed that monotherapy with oseltamivir and SAM in different doses do not exhibit enough good protection on examined parameters. Combined effect of oseltamivir and SAM in doses of 50 mg/kg and 100 mg/kg b. w. decreases the MST level about 10 %, PC two times, and PI about 55 %, compared with oseltamivr (Table II). Combination of oseltamivir with higher dose of SAM (150 mg/kg) showed very low protective effect on these parameters. On cumulative mortality rate of influenza virus infected mice best protection showed the comedication of oseltamivir with 50 mg/kg SAM (Figure 1). There are no significant changes in the body weight of influenza virus infected mice, in all experimental groups, monitored until 14th day after inoculation (Figure 2).

A review of the scientific literature shows that the big problem of the application of specific inhibitors, including oseltamivir, leads to high percentage of viral resistance (Galabov et al., 2006). Oseltamivir is a potent and selective inhibitor of the neuraminidase enzyme of the influenza A and B viruses. This enzyme is responsible for cleaving the sialic acid residues on newly formed virions and plays an essential role in the release and spread of progeny virions (Kamps & Hoffman, 2006).

It is an indisputable fact that low levels of glutathione are involved in all disease states, as well in influenza infections. GSH is the most abundant low-molecular-weight thiol, and GSH/glutathione disulfide is the major redox couple in animal cells. The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes - glutamylcysteine synthetase and GSH synthetase. Compelling evidence shows that GSH synthesis is regulated primarily by glutamylcysteine synthetase activity, cysteine availability, and GSH feedback inhibition. (Wu et al., 2003).

The anti-influenza activity of GSH has also been demonstrated in an in vivo experimental model. In particular,

the addition of GSH to the drinking water of influenza infected mice inhibited viral titer in the trachea and lungs (Cai et al., 2003). These effects were the result of GSH-C4's interference with maturation of the viral glycoprotein hemagglutinin (HA), a process that is largely mediated by the redox-sensitive activities of host-cell oxidoreductase-protein disulfide isomerase (Amatore, 2006). Prophylactic agents are administered before virus inoculation to prevent damage.

In antiviral therapy supplementation with drugs which have a function of precursors of important biomolecules, means the ability to protect the vital biomolecules from virus-induced disorders. The modern approach to understanding the mechanism of the antiflu effect of the combination of SAM and oseltamivir should, therefore, be directed towards exploring its possible role in preventing oxidative damage as well as on the promotion of healing process by cell's prevention. The anti-flu agents should be responsible for a significant improvement of the quality of life not only symptomatically but also at a functional level.

The advantages of usage of the combination of specific influenza virus inhibitors and precursors of important biomolecules could be summarized as follows: (i) decreased drug- resistance development; (ii) lower doses of the drugs, which means lower toxicity; (iii) good protection of cell membranes against oxidative damages caused by the influenza virus infection.

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