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Neuraminidase inhibitors reduce nitric oxide production in influenza virus-infected and gamma interferon-activated RAW 264.7 macrophages

Tomas Kačergius[1], Arvydas Ambrozaitis[1], Yuping Deng[2], Stefan Gravenstein[2]

[1]Department of Infectious Diseases, Dermatovenerology and Microbiology, Faculty of Medicine, Vilnius University, LT-08117 Vilnius, Lithuania

[2]Glennan Center for Geriatrics and Gerontology, Department of Internal Medicine, Eastern Virginia Medical School, Norfolk, VA 23507, USA

Correspondence: Tomas Kačergius, e-mail: tomas.kacergius@mf.vu.lt

Abstract:
The influenza virus (influenza) infection causes an intense infiltration of pulmonary tissues by macrophages, which abundantly generate a free radical, nitric oxide (NO) resulting in lung damage. Neuraminidase inhibitors (NIs) restrict influenza virus replication but whether they can suppress NO production within macrophages is unknown. RAW 264.7 macrophages were exposed to interferon-gamma (IFN-γ), live influenza (A/PR/8/34) or a combination of both and were treated with NIs (oseltamivir or zanamivir). Results revealed that the drugs reduced a synergy between influenza and IFN-γ in NO synthesis within the cells at all of the used concentrations (0.01, 0.1, 1 μg/ml). In contrast to zanamivir, this effect occurred in a concentration-dependent manner with oseltamivir treatment. On the other hand, all concentrations of zanamivir significantly suppressed NO production in comparison to that upon the combined exposure only ($p < 0.05$). Both compounds also considerably decreased NO generation in the IFN-γ-stimulated macrophages, and zanamivir in the influenza-infected cells as well. However, neither of the drugs inhibited iNOS mRNA expression in the cells containing these stimulants. Additionally, the data indicate that a prodrug oseltamivir can be activated *in vitro* within the macrophage cultures. These findings are important for designing treatment approaches to limit pulmonary inflammation during influenza infection.

Key words:
influenza virus, interferon-gamma, macrophages, nitric oxide, neuraminidase inhibitors, oseltamivir, zanamivir

Abbreviations: bp – base pair, CPE – cytopathic effect, DMEM – Dulbecco's modified Eagle medium, iNOS – inducible nitric oxide synthase, NI – neuraminidase inhibitor, NO – nitric oxide, NO_2^- – nitrite, OD – optical density, rIFN-γ – recombinant interferon-gamma, RT-PCR – reverse transcriptase-polymerase chain reaction, SEM – standard error of the mean, $TCID_{50}$ – 50% tissue culture infectious dose

Introduction

Influenza remains a serious respiratory infection causing high rates of morbidity and mortality. The vast majority of deaths during the outbreaks of influenza occur because of the resultant pneumonia [17]. In the past decade, several studies in mouse models demonstrated that overproduction of free radicals, including nitric oxide (NO), contributes to the development of pneumonia during influenza virus infection [1, 2]. Pulmonary inflammation is a characteristic feature of the immune response to primary influenza virus infection, and also promotes the production of proinflammatory cytokines and recruitment of macrophages into the lungs. Cytokines, like tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) stimulate the production of inducible nitric oxide synthase (iNOS) leading to NO syn-

thesis in high quantities predominantly within macrophages [5]. Since mouse macrophage influenza virus infection and IFN-γ synergistically increase NO production *via* iNOS induction *in vitro* [4], influenza virus could trigger an excess generation of NO in macrophages *in vivo*, causing pulmonary damage.

These data point out the relevance and potential benefits of alternative therapeutic strategies acting specifically through the inhibition of iNOS expression. Antiviral agents provide a reasonable starting point for such an approach, i.e. suppression of virus replication and virus spread within the respiratory tract by antivirals could indirectly cause a reduction in iNOS expression and NO synthesis. A new class of anti-influenza drugs, the neuraminidase inhibitors (NIs) – zanamivir (inhaled) and oseltamivir (oral), selectively inhibit viral neuraminidase, and consequently interfere with the release of progeny virions from the infected cells, retarding virus dissemination through the lungs [15]. In the mouse model experiments, the NIs demonstrated a high efficacy in reducing pulmonary virus titers, lung consolidation and mortality scores of the influenza virus-infected mice [9, 13, 14]. However, it is unknown whether NIs are able to inhibit NO generation specifically within macrophages during influenza virus infection. Therefore, in the present study, we investigated the capacity of NIs (oseltamivir and zanamivir) to suppress NO production in influenza virus-infected and IFN-γ-activated RAW 264.7 macrophage cell line *in vitro* by limiting the virus' effect.

Materials and Methods

Cell culture

Murine RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection, Manassas, VA, USA. Media and components (including antibiotics) for cultivation of the cell culture were purchased from Invitrogen Corp., Grand Island, NY, USA. Adherent RAW 264.7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (1.25 μg/ml). The cells were cultured in a humidified incubator at 37°C and 5% CO_2. The number of passages did not exceed twenty. Cell viability was evaluated by trypan blue dye exclusion.

Influenza virus, and determination of the virus infectivity titer

The mouse-adapted influenza A/Puerto Rico/8/34 (A/PR/8/34) (H1N1) virus, a kind gift of Dr. Bradley S. Bender (College of Medicine, University of Florida, Gainesville, FL, USA), was propagated in the allantoic cavities of 10-day-old embryonated chicken eggs as previously described [6]. For the determination of influenza A/PR/8/34 virus infectivity titer, confluent monolayers of Madin-Darby canine kidney (MDCK) cells were inoculated with the virus stock of serial 10-fold dilutions. The 50% tissue culture infectious dose ($TCID_{50}$) was evaluated according to the extent of viral cytopathic effect (CPE) in monolayers after 96 h. The virus infectivity titer was calculated using Kärber method [6], and it was determined as $10^{8.4}$ $TCID_{50}$/ml.

Antiviral drugs

Oseltamivir ((3R,4R,5S)-4-acetylamino-5-amino-3(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid, ethyl ester, phosphate (1:1)) and zanamivir (5-(acetylamino)-4-[(aminoiminomethyl)-amino]-2,6-anhydro-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid) were obtained from Roche Laboratories Inc., Nutley, NJ, USA, and GlaxoSmithKline, Research Triangle Park, NC, USA, respectively. It should be noted that the concentrations of NIs used in the experiments were selected on the basis of 50% inhibitory concentrations for influenza A/PR/8/34 virus replication *in vitro*, which are 0.35 μM or 0.1 μg/ml (1 μM = 0.284 μg/ml) for oseltamivir carboxylate and 0.3 μM or 0.1 μg/ml (1 μM = 0.33 μg/ml) for zanamivir as indicated in the literature [14], and also available online at http://www.rocheusa.com/products/tamiflu/pi.pdf; http://us.gsk.com/products/assets/us_relenza.pdf.

Experimental design

For three independent experiments, RAW 264.7 cells were seeded at 1×10^6 cells/well in 6-well tissue culture plates containing 5 ml of serum-free DMEM/well, and they were incubated for 18 h. Then, the cells were exposed in triplicate fashion to mouse recombinant (r) IFN-γ (1 ng/ml), purchased from Sigma-Aldrich Co., St. Louis, MO, USA, live influenza A/PR/8/34 virus (infectivity titer of 10^6 $TCID_{50}$/ml) or a combination of influenza virus plus rIFN-γ in the

presence of oseltamivir (in the first experiment) and zanamivir (in the second experiment) at the concentrations of 0.01, 0.1 and 1 μg/ml. Unexposed cells and cells treated only with the NIs served as controls. The incubation was continued for an additional 24 h under standard conditions (37°C and 5% CO_2). In the third experiment, differently from the previous experiment, zanamivir was added to RAW 264.7 macrophage cultures after 2 h of their exposure to rIFN-γ, influenza virus or a combination of both, and the total incubation time was 18 h. Afterwards, nitrite levels in the macrophage culture supernatants were measured, and cells were harvested for RNA isolation and iNOS mRNA detection by reverse transcriptase (RT)-PCR.

Nitrite determination

Nitrite (NO_2^-) level, an index of cellular NO production, was determined in macrophage culture supernatants using a modified Griess reagent (Sigma-Aldrich Co., St. Louis, MO, USA) as recommended by the manufacturer. The optical density (OD) at 540 nm was measured using a microplate reader-spectrophotometer ($PowerWave_X$; Bio-Tek Instruments Inc., Winooski, VT, USA). Nitrite concentrations were calculated using the KC4 statistical program, version 2.5 (Bio-Tek Instruments Inc., Winooski, VT, USA), by comparing OD of nitrite in samples with respective OD of standard solutions of sodium nitrite prepared in DMEM. The nitrite detection limit was 0.24 μmol/ml.

RNA isolation and iNOS mRNA detection by semiquantitative RT-PCR analysis

The total RNA was isolated using Trizol reagent according to the instructions provided by Invitrogen Corp., Grand Island, NY, USA. iNOS mRNA was detected using one-step reaction with the Access RT-PCR System kit (Promega Corp., Madison, WI, USA). The murine primers for iNOS and β-actin mRNAs detection were selected on the basis of the published nucleotide sequences: 5'-GTCAACTGCAAGAGAACGGAGAAC-3' (iNOS forward primer); 5'-GAGCTCCTCCAGAGGGTAGG-3' (iNOS backward primer); 5'-TGGAATCCTGTGGCATCCATGAAAC-3' (β-actin forward primer); 5'-TAAAACGCAGCTCAGTAACAGTCCG-3' (β-actin backward primer) [3, 18]. Murine β-actin mRNA expression was used as an internal control. RT-PCR was performed with 1 μg of total RNA from each sample in 25 μl reaction volumes according to the manufacturer's instructions. RT-PCR was carried out in a thermal cycler (GeneAmp PCR System 9600; Perkin-Elmer Applied Biosystems Corp., Norwalk, CT, USA) under the following conditions: first strand cDNA was synthesized at 48°C for 45 min (1 cycle) and then denatured at 94°C for 2 min (1 cycle); followed by 35 cycles at 94°C for 30 s (denaturation), 55°C for 1 min (annealing), and 68°C for 45 s (extension); final extension was performed at 68°C for 7 min (1 cycle). 30% of the iNOS (because to better detection of the band resulting from the influenza virus exposure) and 10% of the β-actin total reaction products were subjected to electrophoresis. The amplified PCR products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide, and then gels were photographed under UV transillumination. 100 bp DNA ladder (Invitrogen Corp., Grand Island, NY, USA) was used as a marker for identification of sizes of iNOS and β-actin amplified products. Bands corresponding to iNOS and β-actin products were quantified by densitometry using Scion Image software, version Beta 3b (available at http://www.scioncorp.com), and values (relative OD units) were expressed as the iNOS/β-actin ratio.

Statistical analysis

Data are expressed as the means ± standard error of the mean (SEM). Statistical evaluation was performed with SPSS program, version 10.0, using *t*-test for independent samples (two-tailed) for comparison of means. A p value less than 0.05 was considered statistically significant.

Results

With exposure to influenza virus only, the measured nitrite levels in RAW 264.7 macrophage cultures were slightly above the nitrite detection limit during the first experiment (Fig. 1). These levels did not exceed significantly the nitrite levels in unexposed cell cultures ($p > 0.05$). Consequently, treatment of the influenza virus-infected macrophages with oseltamivir did not clearly affect nitrite formation (Fig. 1). In the second experiment, nitrite generation was under detection limits within unexposed cell cultures but the virus caused a significant elevation of nitrite levels versus the nitrite detection limit ($p < 0.05$) (Fig. 1).

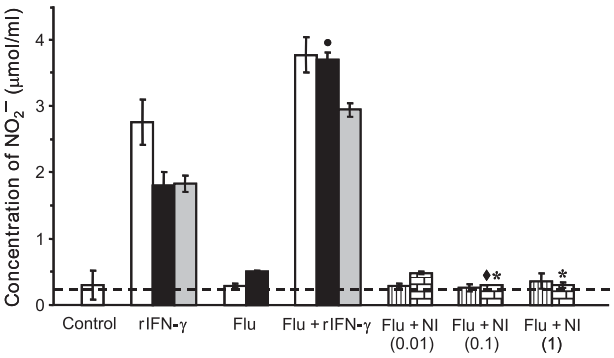

Fig. 1. Nitrite levels in RAW 264.7 macrophage cultures after stimulations with rIFN-γ, influenza virus (Flu) or their combination, and the effect of neuraminidase inhibitors (NIs) in the influenza virus (Flu)-exposed cells. □ – control data from the first experiment (24 h); ■ – control data from the second experiment (24 h); ▩ – control data from the third experiment (18 h); ▥ – effect of oseltamivir in the first experiment; ▦ – effect of zanamivir in the second experiment. Control represents unexposed cells. The dashed line crosses Y axis at the point of 0.24 μmol/ml indicating the NO_2^- detection limit. Concentrations of the NIs are indicated in μg/ml. Values are the means ± SEM of triplicates in a single experiment. ◆ SEM is not presented because of the equal values in three measurements from three separate wells. • $p < 0.05$ compared to rIFN- and influenza virus (Flu); * $p < 0.05$ compared to influenza virus (Flu)

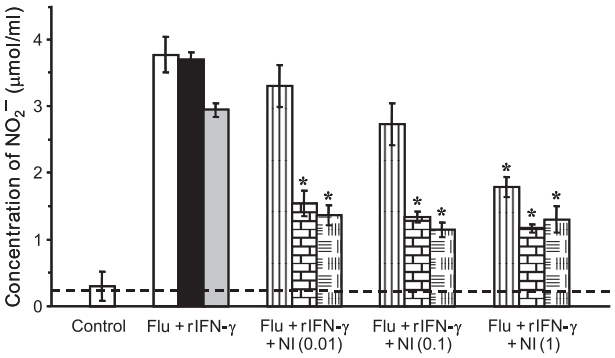

Fig. 3. The effect of neuraminidase inhibitors (NIs) on nitrite formation in RAW 264.7 macrophage cultures exposed to the combination of influenza virus (Flu) plus rIFN-γ. □ – control data from the first experiment (24 h); ■ – control data from the second experiment (24 h); ▩ – control data from the third experiment (18 h); ▥ – effect of oseltamivir in the first experiment; ▦ – effect of zanamivir in the second experiment; ▤ – effect of zanamivir in the third experiment. Control represents unexposed cells. The dashed line crosses Y axis at the point of 0.24 μmol/ml indicating the NO_2^- detection limit. Concentrations of the NIs are indicated in μg/ml. Values are the means ± SEM of triplicates in a single experiment. * $p < 0.05$ compared to the combination of influenza virus (Flu) plus rIFN-

The presence of zanamivir in the influenza virus-infected macrophage cultures reduced formation of nitrite by 40% at the concentrations of 0.1 and 1 μg/ml as compared to nitrite concentrations in the cell cultures exposed only to influenza virus (Fig 1). In contrast, during the third experiment neither unexposed nor influenza virus-infected macrophages generated a detectable amount of nitrite, and the addition of zanamivir did not affect that as well. Noteworthy, treatment of the unexposed macrophages with oseltamivir or zanamivir by itself did not significantly stimulate production of nitrite versus the nitrite detection limit and unexposed cells in all experiments ($p > 0.05$) (data not shown).

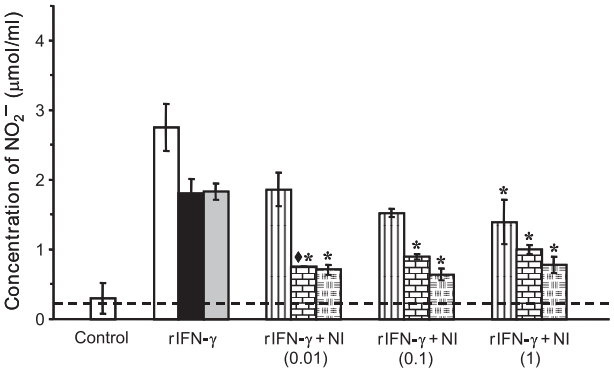

Fig. 2. The effect of neuraminidase inhibitors (NIs) on nitrite formation in the rIFN-γ-stimulated RAW 264.7 macrophage cultures. □ – control data from the first experiment (24 h); ■ – control data from the second experiment (24 h); ▩ – control data from the third experiment (18 h); ▥ – effect of oseltamivir in the first experiment; ▦ – effect of zanamivir in the second experiment; ▤ – effect of zanamivir in the third experiment. Control represents unexposed cells. The dashed line crosses Y axis at the point of 0.24 μmol/ml indicating the NO_2^- detection limit. Concentrations of the NIs are indicated in μg/ml. Values are the means ± SEM of triplicates in a single experiment. ♦ SEM is not presented because of the equal values in three measurements from three separate wells. * $p < 0.05$ compared to rIFN-γ

Incubation of RAW 264.7 macrophages with rIFN-γ significantly increased nitrite formation compared to the nitrite detection limit and unexposed cells ($p < 0.05$) (Fig. 2). The presence of NIs in rIFN-γ-activated macrophage cultures markedly reduced nitrite production compared to the cells stimulated with rIFN-γ alone (Fig. 2). However, this effect of NIs did not occur in a concentration-dependent manner.

The combined influenza virus plus rIFN-γ stimulation of RAW 264.7 macrophages increased nitrite production well over that from exposure to either component alone (Fig. 1). Incubation of the cells containing this combination with NIs led to a substantial reduction in nitrite amounts (Fig. 3). Oseltamivir suppressed nitrite formation in a concentration-dependent fashion, whereas zanamivir did not have such effect. The highest concentration of oseltamivir (1 μg/ml) reduced nitrite levels by approximately 53% in com-

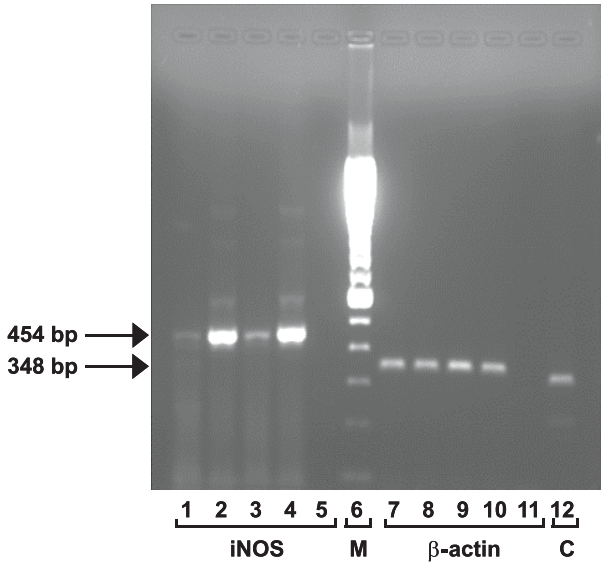

Fig. 4. Detection of iNOS mRNA expression by RT-PCR analysis in RAW 264.7 macrophages at 24 h after exposure to rIFN-γ, influenza virus or their combination. The numbers represent lanes: 1 and 7 – for unexposed cells; 2 and 8 – for exposure to rIFN-γ; 3 and 9 – for exposure to influenza virus; 4 and 10 – for exposure to the combination of influenza virus plus rIFN-γ; 5 and 11 – negative control for RT-PCR performed with sterile nuclease-free water substituted for the RNA template; 12 (C) – positive control for RT-PCR performed with the RNA template from kit (323 bp); 6 (M) – molecular weight marker (100 bp DNA ladder). Arrows indicate the expected sizes of amplified products (454 bp for iNOS, and 348 bp for β-actin)

parison to the nitrite levels upon stimulation with the combination, and the respective concentration of zanamivir – by 68% (in the second experiment) and 57% (in the third experiment).

RT-PCR analysis confirmed that influenza virus, rIFN-γ and their combination stimulated expression of iNOS gene in RAW 264.7 macrophages during the first and second experiments (Fig. 4 represents results of the second experiment). According to densitometry, the combination of influenza virus plus rIFN-γ elevated iNOS mRNA levels more than each component separately (data not shown). Contrarily, in the third experiment, iNOS mRNA was not detected upon exposure to influenza virus, and its expression was at the same level in response to either rIFN-γ or the combination (data not shown). However, the presence of NIs in the cell cultures exposed to influenza virus or rIFN-γ did not reduce iNOS mRNA expression in all experiments (data not shown). The same was observed after stimulation with the combination of influenza virus plus rIFN-γ in the presence of NIs (Fig. 5A, B represents results of the first and third experiments). In addition, neither of the NIs itself induced iNOS gene, as the iNOS mRNA expression was at the similar level as compared with unexposed cells (data not shown).

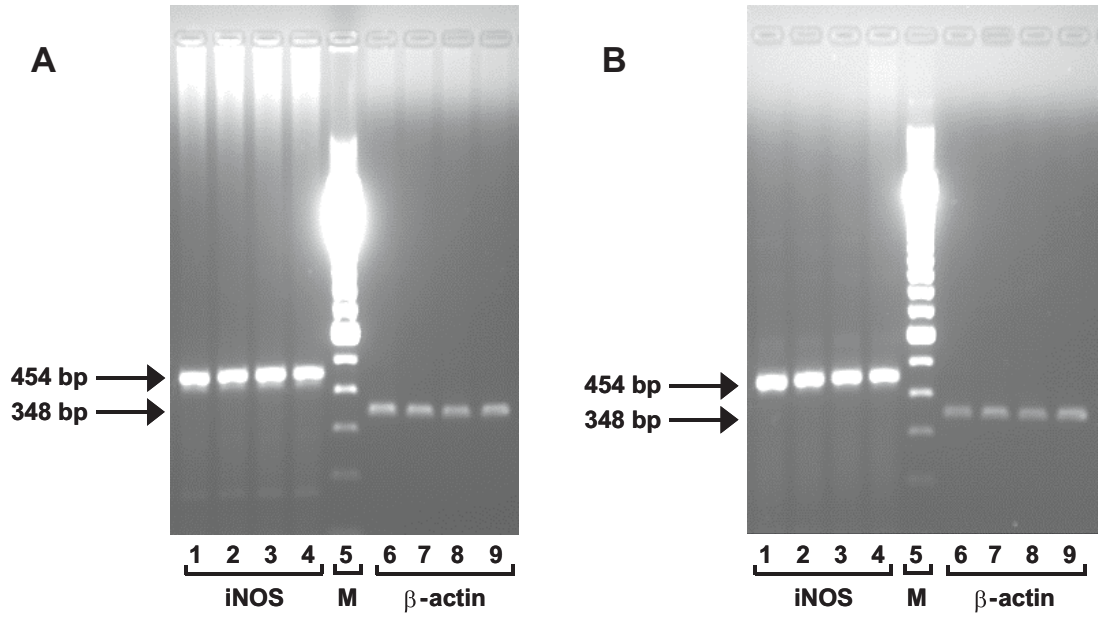

Fig. 5. Detection of iNOS mRNA expression by RT-PCR analysis in RAW 264.7 macrophages under exposure to the combination of influenza virus (Flu) plus rIFN-γ in the presence of neuraminidase inhibitors (NIs). **A** – gel photograph taken at 24 h after exposure to Flu + rIFN-γ in the presence of oseltamivir; **B** – gel photograph taken at 18 h after exposure to Flu + rIFN-γ in the presence of zanamivir. The numbers represent lanes: 1 and 6 – for exposure to Flu + rIFN-γ; 2 and 7 – for exposure to Flu + rIFN-γ + NI (0.01 μg/ml); 3 and 8 – for exposure to Flu + rIFN-γ + NI (0.1 μg/ml); 4 and 9 – for exposure to Flu + rIFN-γ + NI (1 μg/ml); 5 (M) – molecular weight marker (100 bp DNA ladder). Arrows indicate the expected sizes of amplified products (454 bp for iNOS, and 348 bp for β-actin)

It should be highlighted that, during these experiments, the influenza virus caused infection in RAW 264.7 macrophage cultures which decreased density of the cells with occurrence of the viral CPE. Furthermore, the virus in combination with rIFN-γ augmented such processes to a greater extent in the cell cultures. Treatment with the NIs just moderately prevented macrophages from these influenza virus-induced effects.

Discussion

This study demonstrated a synergistic effect between influenza virus and IFN-γ in NO production within RAW 264.7 macrophages reduced by neuraminidase inhibitors. Unexpectedly, we observed that oseltamivir and zanamivir suppressed NO generation in macrophages stimulated with IFN-γ alone.

The response of RAW 264.7 macrophages to influenza virus and rIFN-γ differed in terms of nitrite formation and iNOS mRNA expression. This different response modulation was related to the number of tissue culture cell passages, explaining the discrepant inductivity of iNOS gene and unequal nitrite levels in response to influenza virus and rIFN-γ.

Both drugs – oseltamivir and zanamivir, have the same mechanism of antiviral action but different chemical structure and metabolism, and both suppressed NO synthesis. Noteworthy, we used oseltamivir, an ethyl ester prodrug, instead of oseltamivir carboxylate in the experiments to find out whether it can be metabolized *in vitro* within macrophage cultures. Zanamivir is in the active form, whereas oseltamivir is converted to the active form – oseltamivir carboxylate by carboxylesterases (predominantly by hepatic as well as blood esterases) under *in vivo* conditions [7, 16]. These broad-spectrum enzymes are also present in human macrophages and, as the non-specific esterases, in mouse macrophages [10, 12]. Therefore, finding of the reduced nitrite levels in the presence of oseltamivir indicates that this drug was transformed into the active form likely by esterases expressed in the macrophages. For the *in vivo* system, it may provide more efficient metabolism of the drug not just in the liver but in other tissues as well (e.g., blood monocytes and tissue macrophages).

Interestingly, the zanamivir experiments show that reduction in NO synthesis within macrophages is independent of the initial stage of influenza virus infection. Zanamivir addition to the cell cultures prior and after 2 h of the combined influenza virus plus rIFN-γ exposure resulted in the inconsiderable differences between nitrite concentrations. Although the total incubation time was controlled, it is likely the time (either 18 or 24 h) did not vary enough to significantly cause the differences among nitrite levels especially considering the concentration-independent effect of the drug. Influenza virus uses other receptors to infect macrophages than to enter epithelial cells [11]. In this respect, the viral neuraminidase and its inhibition with NIs may not play a pivotal role for the early stage of influenza virus infection in macrophages as distinct from the epithelial cells [8]. The reduction of nitrite production within influenza virus-infected and rIFN-γ-activated macrophage cultures by zanamivir determined during the third experiment suggests that this effect occurred at the late stage of infection.

We propose the NIs suppressed nitrite formation in rIFN-γ-stimulated macrophage cultures by either interfering with the attachment of rIFN-γ to its receptors and/or affected certain steps of cellular protein synthesis on ribosomes, including iNOS. Alternatively, the absence of NIs' inhibitory activity on iNOS mRNA expression may relate to insufficient drug concentrations. It would be reasonable to increase the NI concentrations in future experiments. Nevertheless, the data demonstrate that the limitation of both influenza virus and rIFN-γ effects within the combined exposure using NIs led to the decrease in NO production in the macrophages.

Accordingly, we conclude that the NIs (oseltamivir and zanamivir) reduce NO generation in RAW 264.7 macrophages exposed to rIFN-γ alone or to a combination of influenza virus plus rIFN-γ. This would mean that the NIs possess two activities – antiviral (inhibition of the virus infection) and anti-inflammatory (suppression of NO production). However, additional study is needed to understand the mechanism by which the NIs suppress synthesis of NO within macrophages during influenza virus infection.

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