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4 *In vitro* anti-influenza A activity of interferon (IFN)- λ 1 combined
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8 with IFN- β or oseltamivir carboxylate
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36 *Keywords:* Influenza A virus; IFN- β ; IFN- λ 1; Oseltamivir carboxylate; Combination therapy
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Abstract

Influenza viruses, which can cross species barriers and adapt to new hosts, pose a constant potential threat to human health. The influenza pandemic of 2009 highlighted the rapidity with which an influenza virus can spread worldwide. Currently available antivirals have a number of limitations against influenza, and novel antiviral strategies, including novel drugs and drug combinations, are urgently needed. Here, we evaluated the *in vitro* effects of interferon (IFN)- β , IFN- λ 1, oseltamivir carboxylate (a neuraminidase (NA) inhibitor), and combinations of these agents against two seasonal (*i.e.*, H1N1 and H3N2) influenza A viruses. We observed that A/California/04/09 (H1N1) and A/Panama/2007/99 (H3N2) isolates were similarly sensitive to the antiviral activity of IFN- β and oseltamivir carboxylate in A549 and Calu-3 cells. In contrast, IFN- λ 1 exhibited substantially lower protective potential against the H1N1 strain (64-1030 fold $\downarrow$, $P < 0.05$), and was ineffective against H3N2 virus in both cell lines. Three dimensional analysis of drug-drug interactions revealed that IFN- λ 1 interacted with IFN- β and oseltamivir carboxylate in an additive or synergistic manner, respectively, to inhibit influenza A virus replication in human airway epithelial cells. Overall, the present study demonstrated that anti-influenza agents with different mechanisms of action (*e.g.*, a NA inhibitor combined with IFN- λ 1) exerted a significantly greater ($P < 0.05$) synergistic effect compared to co-treatment with drugs that target the same signaling pathway (*i.e.*, IFN- β plus IFN- λ 1) *in vitro*. Our findings provide support for the combined use of interferon plus oseltamivir as a potential means for treating influenza infections.

1. Introduction

Influenza virus is one of the most common human respiratory viral infections, with epidemics and pandemics of varying morbidity and mortality occurring each year. About 10-20% of people in the United States experience influenza infection every year, and more than 200,000 individuals per year are hospitalized for complications related to influenza (Nicholson et al., 2003; Wright et al., 2006). As the primary viral cause of pneumonia, influenza virus is one of the ten leading causes of death in the United States (~20,000 per year). Furthermore, occasional interspecies transmission of avian and swine influenza viruses of different subtypes (H1N1, H5N1, H7N7, and recently H7N9) occur periodically and can cause high mortality rates in humans (WHO, 2014; Wright et al., 2006).

Although vaccination remains the best defense against seasonal and pandemic influenza, antiviral drugs provide a critical second line of defense against influenza infection in those instances where a vaccine is not available to generate an immediate immune response. However, our armamentarium of antiviral drugs is currently limited, with only two specific classes of antiviral drugs available to manage influenza: inhibitors of the M2 protein (*e.g.*, amantadine and rimantadine) (Hay et al., 1985; Hayden, 1996; Pinto et al., 1992) and neuraminidase (NA) inhibitors (*e.g.*, oseltamivir, zanamivir, and peramivir) (Lee and Yen, 2012; Monto, 2003, Varghese et al., 1992; von Itzstein et al., 1993). These two drug classes differ not only in their mechanisms of action, but also in their pharmacokinetics, tolerance profiles, and resistance patterns (De Clercq, 2006). The emergence of antiviral drug resistance is a major obstacle to the clinical use of the anti-influenza agents. Almost 100% of the currently circulating H1N1 strains to have emerged from the 2009 pandemic are resistant to adamantane derivatives (Deyde et al., 2007; Mossad, 2009), as are the majority of H3N2 viruses (Bright et al., 2005; Hata et al., 2007)

1 and the most highly pathogenic H5N1 avian viruses (Hurt et al., 2007; Govorkova et al., 2013).
2 Additionally, the unexpected dominance (~98%) of oseltamivir-resistant H1N1 strains between
3 2007 and 2009 demonstrated that NA inhibitor resistance can emerge rapidly and spread
4 worldwide (Hurt et al., 2009; Lackenby et al., 2008). Clearly, the increased antiviral resistance
5 among circulating influenza A viruses adds to the urgent need to develop novel antiviral
6 strategies.

7 New drug development is a long and challenging process, and the prudent combination of
8 established antivirals that target distinct mechanisms of viral replication at the same time is an
9 attractive strategy. Combination therapy offers several advantages over monotherapy, including
10 lower rates of resistance, greater potency and cost-effectiveness, lower total drug dosages, and
11 decreased risk of respiratory complications (Hayden, 1996; Govorkova and Webster, 2010). A
12 number of *in vitro* and *in vivo* studies have verified that the combined use of judiciously chosen
13 antiviral drugs can significantly enhance anti-influenza activity (Govorkova and Webster, 2010
14 [for a review up to 2010]; Nguyen et al., 2012; Smee et al., 2013; Tarbet et al., 2012).

15 Type I interferons (*e.g.*, IFN- α/β) and the more recently discovered type III interferons
16 (IFN- $\lambda 1$, - $\lambda 2$, - $\lambda 3$) are key mediators of the innate immune response against influenza virus
17 infection (Durbin et al., 2000; Hsu et al., 2012; Sheppard et al., 2003; Kotenko et al., 2003).
18 Specifically, influenza A viral components are recognized by *Toll*-like receptors such as TLR3
19 and/or TLR7, and within the cytoplasm by RIG-I and melanoma differentiation associated
20 factor-5 (Crotta et al., 2013; Hsu et al., 2012; Osterlund et al., 2012). Recognition of viral
21 components through these mechanisms up-regulates the expression of type I and type III IFNs,
22 which induce hundreds of IFN-stimulated genes (ISGs), culminating in viral restriction and

activation of the adaptive immune response (Durbin et al., 2000; Kotenko et al., 2003; Durbin et al., 2013).

Several previous studies showed that IFNs exert antiviral activity against influenza virus infection. Osterlund *et al.* demonstrated that both seasonal and 2009 pandemic H1N1 influenza A viruses are sensitive to the antiviral actions of type I (IFN- α/β) and type III (IFN- $\lambda 1$ and - $\lambda 3$) IFNs in human monocyte-derived dendritic cells and macrophages (Osterlund et al., 2010). Conversely, in the human epithelial A549 cell line, 2009 pandemic H1N1 virus was less sensitive than the human A/Puerto Rico/8/34 (H1N1) influenza strain to several IFN- α subtypes (Scagnolari et al., 2011). Pretreatment with IFN- α/β significantly suppressed the replication of H1N1 virus (Beilharz et al., 2007) and most importantly of highly pathogenic H5N1 avian influenza viruses both *in vitro* and *in vivo* (Szretter et al., 2009). In a guinea pig model, human recombinant IFN- α , administered intranasally, significantly reduced lung and nasal wash titers of a reconstructed 1918 pandemic H1N1 virus as well as a contemporary H5N1 strain (van Hoeven et al., 2009). Additionally, type III IFN- λ s were found to exert variable degrees of antiviral activity *in vivo* against influenza A and B viruses (Mordstein et al., 2008, 2010), especially in mouse lungs (Sommereyns et al., 2008).

The anti-influenza protective potential of type III IFNs in models that resemble human airway epithelium, the primary cellular target of influenza virus infection, has not been thoroughly assessed. In the present study, we compared the anti-influenza A activities of IFN- $\lambda 1$ (a type III IFN), IFN- β (a type I IFN), and the NA inhibitor, oseltamivir carboxylate, in two distinct human airway epithelial cell lines (*i.e.*, A549 and Calu-3 cells). We further tested whether IFN- $\lambda 1$ functions additively, synergistically, or antagonistically with these compounds against influenza A infection *in vitro*.

2. Materials and methods

2.1. Compounds

Recombinant IFN- β and IFN- λ 1 were obtained from R&D Systems (Minneapolis, MN). The NA inhibitor, oseltamivir carboxylate (the active metabolite of oseltamivir [3R,4R,5S]-4-acetamido-5-amino-3-[1-ethyl propoxy]-1-cyclohexane-1-carboxylic acid), was kindly provided by Dr. Matthew J. Memoli at the National Institute of Allergy and Infectious Diseases, Bethesda, MD.

2.2. Viruses and cells

Human influenza A/California/04/09 (H1N1) and A/Panama/2007/99 (H3N2) viruses, which represent the antigenically dominant strains that circulated in humans during 2009–2014 and 1999, respectively, were kindly provided by Dr. Robert G. Webster at St. Jude Children's Research Hospital, Memphis, TN. Stock viruses were prepared by one passage in the allantoic cavities of 10-day-old embryonated chicken eggs for 48 h at 37°C, and aliquots were stored at –70°C until used. All experimental work was performed in a biosafety level 2 laboratory approved for use with these strains by the U.S. Department of Agriculture and the U.S. Centers for Disease Control and Prevention.

Madin-Darby canine kidney (MDCK), human alveolar basal epithelial (A549) cells and cells derived from human bronchial submucosal glands (Calu-3) were obtained from the American Type Culture Collection (Manassas, VA) and were maintained as described previously (Hsu et al., 2012; Ilyushina et al., 2008).

2.3. Infectivity of influenza A viruses

The infectivity of A/California/04/09 (H1N1) and A/Panama/2007/99 (H3N2) viruses in MDCK cells was determined by plaque assay and expressed as log₁₀ plaque-forming units (PFU)

per milliliter (Hayden et al., 1980). Briefly, confluent MDCK cells were incubated at 37°C for 1 h with 10-fold serial dilutions of virus. The cells were then washed and overlaid with minimal essential medium (MEM) containing 0.3% bovine serum albumin, 0.9% Bacto agar, and 1 µg/ml l-[tosylamido-2-phenyl]ethylchloromethylketone-treated trypsin. After 3 days of incubation at 37°C, the cells were stained with 0.1% crystal violet in 10% formaldehyde solution, and the PFU per milliliter were determined.

2.4. In vitro susceptibility assay

The antiviral activities of IFN-β, IFN-λ1 and oseltamivir carboxylate, alone and in combination, were determined by reduction of the cell-associated virus yield using an enzyme-linked immunosorbent assay (ELISA). Confluent monolayers of A549 or Calu-3 cells in 96-well plates were pretreated with antiviral agents at a range of concentrations (IFN-β and/or IFN-λ1, 0.00001 to 1000 ng/ml for 24 h; oseltamivir carboxylate 0.00004 to 9700 ng/ml for 1h). After pretreatment, cells were overlaid with 2× drug(s)-containing medium (50 µl/well), infected with influenza virus at a multiplicity of infection (MOI) of 0.1 PFU/cell, and incubated for 24 h at 37°C (or 48 h for the experiments with oseltamivir carboxylate used alone or in combination). Virus replication was determined by measuring viral nucleoprotein on the surface of infected cells. The percent inhibition of virus replication was calculated from the absorbance values determined at 490 nm on a microplate reader (Bio-Rad Laboratories, Hercules, CA) after correction for the absorbance values of uninfected cultures. The absorbance values for the control wells (without drugs) were considered to indicate 0% inhibition of virus replication. At least three or four independent experiments were performed to determine the 50% effective concentrations (EC₅₀) values as the concentration of single compound required to reduce the cell-associated virus yield by 50% relative to that of untreated wells.

2.5. Extracellular virus yield reduction assay

The extracellular virus yield reduction assay was performed as described previously in 24-well plates containing confluent A549 or Calu-3 cells (Govorkova et al., 2004). The concentrations of IFN- β and IFN- $\lambda 1$ tested ranged from 0.001 to 100 ng/ml. The concentrations of oseltamivir carboxylate ranged from 0.004 to 9700 ng/ml. IFNs or oseltamivir carboxylate (alone or in combination) were added to the 24-well plates for 24 h or 1 h, respectively. After pretreatment, the cells were overlaid with 2 $\times$ drug(s)-containing medium (100 μ l/well), infected with influenza virus at a MOI of 0.1 PFU/cell, and incubated for 24 h at 37°C (or 48 h for the experiments with oseltamivir carboxylate used alone or in combination). Virus yields were determined by measuring the 50% tissue culture infectious dose (TCID₅₀) values of culture supernatants 72 h after inoculation of MDCK cells. The drug concentration that caused a 50% decrease in the TCID₅₀ titer in comparison to control wells without drug was defined as EC₅₀. The results of two independent experiments were averaged.

2.6. Cytotoxicity

The effect of the compounds on the growth of uninfected A549 and Calu-3 cells in 96-well plates was determined by using CellTiter Aqueous One solution (Promega, Madison, WI). Briefly, a confluent monolayer of cells was overlaid with MEM containing IFN- β , IFN- $\lambda 1$ and oseltamivir carboxylate, individually or in combination, at each concentration tested in the study. After 48 h of incubation at 37°C, the plates were incubated with CellTiter Aqueous One solution for 2 h at 37°C. The absorbance of drug-exposed and untreated control cell samples was measured according to the manufacturer's protocol. Experiments were performed in triplicate under each condition.

2.7. Quantitative reverse transcription-PCR (qRT-PCR) of IFN-stimulated genes (ISGs)

Quantification of changes in gene expression was carried out by qRT-PCR analyses of individual ISGs (*i.e.*, *MX1*, *OAS1*, *IRF7*, *IFIT1*, and *IFIT3*). Total cellular RNA was isolated using RNeasy Minikits (Qiagen, Germantown, MD), treated with DNase, and 1 µg of the purified RNA was reverse-transcribed to cDNA with Quantiscript reverse transcriptase (Qiagen). The cDNA was mixed with RT² SYBR[®] green qPCR Mastermix (Qiagen) and qPCR was performed using the ViiA[™] 7 system (Applied Biosystems, Carlsbad, CA). Changes in gene expression levels were analyzed using ViiA[™] 7 software v.1.2.2 (Applied Biosystems), and the results were expressed as the mean-fold increase relative to the untreated control gene expression levels after normalization to the housekeeping gene, *GAPDH*. Graphing and statistical analysis of qPCR results were performed using Prism 5.0 (GraphPad Software, San Diego, CA). Values represent the mean ± S.D. of triplicate determinations.

2.8. Statistical analysis and synergy determinations

Susceptibility of influenza A viruses to IFN-β, IFN-λ1, and oseltamivir carboxylate in A549 and Calu-3 cells was compared by an unpaired two-tailed *t*-test. The levels of ISGs expression were compared by analysis of variance (ANOVA). A probability value of 0.05 was prospectively chosen to indicate that the findings of these analyses were not the result of chance alone.

The combination data from the *in vitro* studies were analyzed with MacSynergy[™] II software provided by Dr. Mark N. Prichard (Prichard and Shipman Jr., 1990; Prichard et al., 1992). Theoretical additive interactions were calculated from dose-response curves for each drug used individually. This calculated additive surface was then subtracted from the experimentally determined dose-response surface to give regions of non-additive interactions. The confidence

intervals around the experimental dose-response surface were used to evaluate the data statistically, and the volume of peaks was calculated and used to quantify the volume of synergy (or antagonism) produced. The guidelines for volumes of synergy determinations expressed as $\text{unit}^2 \%$ ($\text{unit} \times \text{unit} \times \%$) at a 95% confidence level were as follows: 0–25, insignificant; 25–50, minor but significant; 50–100, moderate; >100, strong synergy or antagonism. Synergy plots were generated at the 95% confidence limit.

3. Results

3.1. Susceptibility of influenza A viruses to IFN- β , IFN- $\lambda 1$, and oseltamivir carboxylate

We tested the sensitivity of influenza A/California/04/09 (H1N1) and A/Panama/2007/99 (H3N2) viruses to treatment with IFN- β , IFN- $\lambda 1$, or oseltamivir carboxylate as single agents. We first evaluated the antiviral activity of these agents by cell ELISA, which determines the level of drug inhibition of cell-associated virus in A549 and Calu-3 cells (Table 1 and Supplementary Figure 1). The mean EC_{50} values for both isolates ranged from 0.2 to 2.9 ng/ml for IFN- β in both cell lines. Relative to IFN- β , IFN- $\lambda 1$ was ~64 to 1667-fold less effective ($P < 0.05$) against H1N1 and H3N2 viruses, with 50% inhibition of H3N2 virus yield being reached at the highest concentrations tested (0.5–1 $\mu\text{g/ml}$). The sensitivity of the two viruses to oseltamivir carboxylate did not differ significantly in each cell line ($\text{EC}_{50} \approx 0.1$ –4.3 ng/ml, Table 1 and Supplementary Figure 1). We also assayed the antiviral activity of IFN- β , IFN- $\lambda 1$, and oseltamivir carboxylate by virus yield reduction assay, which allows detection of the extracellular virus yield in cell culture supernatants. We observed that the EC_{50} values determined by virus reduction assay (data not shown) and cell ELISA were comparable for all compounds tested.

3.2. Antiviral activity of IFN- β and IFN- λ 1 combination against influenza A viruses

We next assessed the antiviral efficacy of combined treatment with IFN- β plus IFN- λ 1 over a range of concentrations of each compound against H1N1 and H3N2 isolates *in vitro*. Data on the inhibition of influenza H1N1 and H3N2 cell-associated virus yield were plotted in three dimensions to form a response surface (Fig. 1), and the mode of IFNs interaction was characterized by the regression model (Prichard and Shipman Jr., 1990; Prichard et al., 1992). This model provides a quantitative measure of the total synergy (or antagonism) of a drug combination in terms of synergy (or antagonism) volumes, which represent the cumulative synergy and/or antagonism across all concentrations for all the compounds in combination (Prichard and Shipman Jr., 1990; Prichard et al., 1992).

The overall interaction between IFN- β and IFN- λ 1 in inhibiting cell-associated yield of H1N1 (Figs. 1A and 1C) and H3N2 (Figs. 1B and 1D) viruses was additive. However, some pockets of minor to moderate synergy or antagonism were observed for certain combinations of the two IFNs (Fig. 1 and Table 2). The combined action of the two IFNs reached maximum synergy against the H1N1 variant with an IFN- β concentration of 0.00001 ng/ml and an IFN- λ 1 concentration of 0.1 (A549 cells) or 0.0001 ng/ml (Calu-3 cells) (Figs. 1A and 1C). Against the H3N2 isolate, maximal IFN- β –IFN- λ 1 combined inhibition was observed with IFN- β at 0.00001 ng/ml and IFN- λ 1 at 10 ng/ml (A549) or 0.1 ng/ml (Calu-3) (Figs. 1B and 1D). Although the modes of action of the IFN- β –IFN- λ 1 combination did not differ from each other against both influenza viruses in both cell lines studied (Fig. 1), synergy volumes for both isolates were higher in A549 than in Calu-3 cells (*i.e.*, moderate vs. minor, Table 2). This could be explained by the fact that possible regions of synergy ranged over a broader range of IFN- β

concentrations against both the A/California/04/09 (H1N1) and A/Panama/2007/99 (H3N2) strains in A549 cells compared to the Calu-3 cell line (Table 2).

3.3. Effect of optimal IFN- β –IFN- λ 1 combination on expression of ISGs

Our dose-response curves revealed that IFN- β and IFN- λ 1 reached maximum synergy at 0.00001 ng/ml for IFN- β combined with different concentrations of IFN- λ 1 (0.0001–10 ng/ml) against influenza A viruses (Fig. 1). Therefore, we next examined the ability of one of the optimal IFN- β –IFN- λ 1 combinations to induce expression of several ISGs, including *MX1*, *OAS1*, *IRF7*, and *IFIT1/3*, in A549 and Calu-3 cells at 24 h post-treatment (Fig. 2). IFN- β at 0.00001 ng/ml failed to significantly increase the expression levels of all ISGs examined compared to untreated control cells, except for *MX1* in Calu-3 ($P < 0.05$). In contrast, treatment with IFN- λ 1 at 10 ng/ml significantly up-regulated expression of all of the ISGs in A549 cells, and significantly increased the expression of the *IRF7* and *IFIT1* genes in Calu-3 cell line ($P < 0.05$, Fig. 2). Importantly, we observed that after combined treatment with IFN- β plus IFN- λ 1 at 10 ng/ml, the levels of induction of all ISGs tested were significantly higher compared to those in untreated cells ($P < 0.05$), except *IFIT3* in Calu-3. In addition, IFN- β combined with IFN- λ 1 did not decrease the ISG expression levels, and significantly amplified expression of *IFIT1*, *IFIT3* and *OAS1* and *IFIT1* in A549 and Calu-3, respectively, compared with that in the cells treated with either IFN alone ($P < 0.05$, Fig. 2). Taken together, our results indicated that optimal IFN- β –IFN- λ 1 combination was more efficient at amplifying ISG expression than single IFN treatment *in vitro*.

3.4. Antiviral activity of oseltamivir carboxylate plus IFN- λ 1 combination against influenza A viruses

Since the IFN- β –IFN- λ 1 interaction was additive with a few pockets of minor/moderate synergistic activity against H1N1 and H3N2 influenza viruses (Table 2), we next evaluated the interaction between NA inhibitor, oseltamivir carboxylate, and IFN- λ 1 against both isolates in A549 and Calu-3 cells. As shown in Fig. 3, the mode of interaction between oseltamivir carboxylate and IFN- λ 1 differed from that of IFN- β combined with IFN- λ 1 (Fig. 1). Moreover, our results showed that the patterns of drug interactions appeared to be different against the two isolates in A549 cells (Figs. 3A and 3B) and also between the two cell lines against A/Panama/2007/99 (H3N2) virus (Figs. 3B and 3D).

Within the range of concentrations tested, maximum synergy against the H1N1 strain occurred at less than 0.04 ng/ml oseltamivir carboxylate combined with an IFN- λ 1 concentration of 1 ng/ml in A549 cells (Fig. 3A). In contrast, the strongest synergistic effect against the H3N2 isolate occurred at a relatively high oseltamivir carboxylate concentration (970 ng/ml), regardless of IFN- λ 1 concentration, and at the highest IFN- λ 1 concentration (100 ng/ml) in combination with oseltamivir carboxylate concentrations < 9700 ng/ml (Fig. 3B). Furthermore, in Calu-3 cells, synergistic activity was observed across a wide range of concentrations of both compounds against A/California/04/09 (H1N1) and A/Panama/2007/99 (H3N2) viruses, namely, at concentrations $\square$ 0.00004 or $\square$ 0.00097 ng/ml for oseltamivir carboxylate and $\square$ 0.01 or $\square$ 0.1 for IFN- λ 1, respectively (Figs. 3C, 3D, and Table 2). The interaction of oseltamivir carboxylate with IFN- λ 1 reached maximum strong synergy ($\approx$ 70.2 unit² %) against H1N1 and H3N2 viruses when oseltamivir carboxylate at 0.004 or 0.0097 ng/ml was combined with the highest dose of IFN- λ 1 (100 ng/ml), respectively. However, regions of weak to strong antagonistic interaction,

with volumes of antagonism ranging from -0.6 to -55.7 ng/ml² %, were also observed (Figs. 3C, 3D, and Table 2). In addition, total volumes of synergy and antagonism for both isolates tested differed significantly in A549 cells ($P < 0.05$), but were comparable in the Calu-3 cell line (Table 2).

3.5. Cytotoxicity

In the present study, we observed that neither IFN- β , IFN- λ 1, nor oseltamivir carboxylate, was cytotoxic regardless of cell type. The 50% cytotoxic concentrations (TC₅₀s) as single agents were $\square$ 500 ng/ml and $\square$ 10 μ g/ml for the interferons and oseltamivir carboxylate, respectively. Similarly, no cytotoxicity was observed when these agents were used in combination (data not shown).

4. Discussion

The present study evaluated the effectiveness of IFN- λ 1, alone or in combination with either IFN- β or the NA inhibitor, oseltamivir carboxylate, against H1N1 and H3N2 influenza isolates in two human epithelial cell lines: the human lung adenocarcinoma epithelial cell line, A549, and Calu-3 cells, which are derived from human bronchial submucosal glands. These cell lines functionally recapitulate different parts of human airway epithelium, and thus provide an ideal *in vitro* model for analysis of the antiviral action of type III IFN- λ 1 as well as for the evaluation of the modes of type III IFN–type I IFN and type III IFN–NA inhibitor interactions against influenza infection.

Previous studies demonstrated that although type I and type III IFNs bind to distinct cell membrane receptors, they trigger strikingly similar responses leading to the up-regulation of multiple ISGs. The most important distinction between type I and type III IFNs is the cellular

distribution of their corresponding receptors. Receptors for type I IFNs are expressed on virtually all cell types; whereas, receptors for type III IFNs are expressed primarily on epithelial cells of both the respiratory and gastrointestinal tracts (Donnelly et al., 2011; Mordstein et al., 2008, 2010). Therefore, type III IFN- λ s act in a cell-specific manner by binding to receptors that are pivotal to the activation of innate immunity particular to the respiratory mucosa, the primary entry site for influenza virus.

In the present study, we observed that A/California/04/09 (H1N1) and A/Panama/2007/99 (H3N2) isolates were equally sensitive to the antiviral activity of type I IFN- β in A549 and Calu-3 cells. In contrast, type III IFN- λ 1 exhibited substantially lower protective potential against the H1N1 strain (64-1030 fold $\downarrow$, $P < 0.05$) and was ineffective against the H3N2 virus in both cell lines. This finding indicated that, although IFN- λ 1 may play a role in the antiviral defense of human epithelial cells, IFN- λ 1-mediated anti-influenza activity is strain-specific, and IFN- λ 1 alone may not be adequate to control viral infection in the human respiratory tract. The mechanism by which IFN- β imparts antiviral activity superior to that induced by IFN- λ 1 against influenza A viruses is not clear, but may reflect differences in the relative levels of the corresponding cell surface receptors, the kinetics of ISG expression, and/or the receptor binding affinities. It remains to be determined if the observed differences between H1N1 and H3N2 strains in sensitivity to type I versus type III IFNs extends to other influenza viruses. Additional studies are required to determine if the A/Panama/2007/99 (H3N2) isolate induces a novel mechanism that counteracts the antiviral activity of the host type III IFN- λ 1 response.

In this study, we found that the combination of IFN- β plus IFN- λ 1 or IFN- λ 1 plus an NA inhibitor can mediate a greater or lesser antiviral effect than treatment with these agents as single

1 compounds *in vitro*. Our three-dimensional approach facilitated a complete analysis of all drug
2 concentrations tested, and is one of the most suitable models for the assessment of drug
3 interactions (Prichard and Shipman Jr., 1990; Prichard et al., 1992). Based on the calculated
4 synergistic volumes, our results clearly indicated that anti-influenza agents with different
5 mechanisms of antiviral action (*i.e.*, a NA inhibitor combined with type III IFN- λ 1) exerted
6 substantially higher ($P < 0.05$) synergistic effect compared to co-treatment with drugs that target
7 similar cell signaling pathways (*i.e.*, type I IFN combined with type III IFN). Indeed, we
8 observed that IFN- β and IFN- λ 1 interacted principally in an additive manner, including a few
9 pockets of minor and moderate synergy/antagonism, in inhibiting both cell-associated and
10 extracellular (data not shown) virus yield of influenza A/California/04/09 (H1N1) and
11 A/Panama/2007/99 (H3N2) strains in A549 and Calu-3 cells. This simple additivity suggested
12 that the antiviral mechanisms of type I and type III IFNs were independent of each other in the
13 cell culture systems tested. However, we did observe a few instances where minor synergism
14 was likely attributable to more potent amplification of ISG expression by the IFN- β –IFN- λ 1
15 combinations compared to single IFNs.

16 In contrast, strong synergistic interactions between oseltamivir carboxylate and IFN- λ 1
17 that target distinct viral and host pathways, respectively, were observed by cell ELISA and by
18 virus yield reduction assay (data not shown). One possible mechanism for the strong synergy
19 observed between these antiviral agents may involve a decrease in the virus replication rate
20 following exposure to the NA inhibitor. This provides additional time for greater induction of
21 ISG expression by IFN- λ 1, leading to an enhanced antiviral effect against influenza virus
22 infection. Of interest are the observed antagonistic effects of certain combinations of high
23 concentrations of oseltamivir carboxylate with low doses of IFN- λ 1 against both H1N1 and

1 H3N2 influenza isolates in Calu-3 cells. The reason for this unexpected antagonism can be
2 explained by analysis of the raw data, which showed that high doses of the NA inhibitor reduced
3 the virus yield of both influenza viruses to the maximum extent, making any additional antiviral
4 effect of IFN- λ 1 undetectable by cell ELISA. Because it is as important to identify combinations
5 that result in antagonistic interactions as it is to identify those that result in synergistic drug
6 interactions, evaluation of the NA inhibitor plus IFN- λ 1 combination against a number of virus
7 subtypes and strains is desirable by different assays *in vitro*.

8 Because *in vitro* antiviral assays in cell culture are not completely reliable predictors of
9 drug interactions and dose requirements *in vivo*, preclinical studies in appropriate animal models
10 are required to test the potential anti-influenza efficacy of the drug combinations that we
11 evaluated in this study. A clinical trial (#NCT01146535) to assess the efficacy and safety of IFN-
12 α in combination with oseltamivir has been initiated, and should provide important data on the
13 use of this combination therapy against seasonal influenza. In addition, recombinant human IFN-
14 λ 1 has been evaluated clinically as a potential treatment for chronic hepatitis C (Muir et al.,
15 2010; Ramos, 2010). The initial phase-1 results demonstrated that IFN- λ 1 is well tolerated, and
16 can induce antiviral activity comparable to that induced by IFN- α with fewer adverse effects
17 (Muir et al., 2010). A recent study by Friborg and colleagues showed that the combination of
18 IFN- λ 1 plus one or more direct-acting antiviral drugs was much more effective at inducing
19 inhibition of hepatitis C replication *in vitro* than treatment with IFN- λ 1 alone (Friborg et al.,
20 2013). Our findings indicate that IFN- λ 1 combined with oseltamivir carboxylate might hold
21 promise as a novel therapeutic strategy against influenza infection. This unique combination of a
22 biologic antiviral agent (IFN) plus a viral inhibitory drug (oseltamivir) may provide a more

1 effective means to treat influenza-infected patients, and possibly decrease the chance of
2 development of viral resistance to the NA inhibitor.

3

Acknowledgments

We are grateful to Mark N. Prichard for providing the MacSynergy II software program.

We thank Dr. Robert G. Webster for providing influenza A/California/04/09 (H1N1) and A/Panama/2007/99 (H3N2) viruses and monoclonal anti-NP antibodies. We also thank Dr. Matthew J. Memoli (NIAID) for providing oseltamivir carboxylate, Alexander I. Ilyushin for computer support, and Dr. Harry Smith (FDA) for editorial assistance.

This study was supported in part by a Senior Postgraduate Research Fellowship Award to N.A.I. from the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration.

This study was also supported by a grant from the FDA Medical Countermeasures initiative program to R.P.D.

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Figure legends

Figure 1. Drug-drug interactions defined by three-dimensional response plots for IFN- β combined with IFN- $\lambda 1$. A549 or Calu-3 cells were infected with influenza A/California/04/09 (H1N1) virus (A and C) or with A/Panama/2007/99 (H3N2) virus (B and D), respectively, and treated with combinations of both agents (IFN- β and IFN- $\lambda 1$). Reduction of the cell-associated virus yield was measured by cell ELISA, and data from three independent experiments were used for the analysis. The X and Y axes show the concentrations (in ng/ml) of IFN- β and IFN- $\lambda 1$, respectively. The Z axis shows the calculated drug-drug interactions (*i.e.*, synergy ($Z \geq 0$), antagonism ($Z < 0$), or additivity ($Z = 0$)) at the 95% confidence level).

Figure 2. Effect of treatment with IFN- β (0.00001 ng/ml), IFN- $\lambda 1$ (10 ng/ml) or their combinations on induction of individual ISGs (*i.e.*, *MX1*, *OAS1*, *IRF7*, *IFIT1*, and *IFIT3*) in A549 (A) and Calu-3 (B) cells. *, $P < 0.05$ compared with the results for the mock-treated cells, one-way ANOVA; °, $P < 0.05$ compared with the results for the cells treated with IFN- β , one-way ANOVA; •, $P < 0.05$ compared with the results for the cells treated with IFN- $\lambda 1$, one-way ANOVA.

Figure 3. Drug-drug interactions defined by three-dimensional response surfaces for oseltamivir carboxylate combined with IFN- $\lambda 1$. A549 or Calu-3 cells were infected with influenza A/California/04/09 (H1N1) virus (A and C) or with A/Panama/2007/99 (H3N2) virus (B and D), respectively, and treated with combinations of both compounds. Reduction of the cell-associated virus yield was measured by cell ELISA, and data from three independent experiments were

1 used for the analysis. The X and Y axes show the concentrations (in ng/ml) of oseltamivir
2 carboxylate and IFN- λ 1, respectively. The Z axis shows the calculated drug-drug interactions
3 (*i.e.*, synergy ($Z \geq 0$), antagonism ($Z < 0$), or additivity ($Z = 0$)) at the 95% confidence level.

Table 1. Inhibitory activities of IFN- β , IFN- λ 1 and oseltamivir carboxylate on influenza A viruses in A549 and Calu-3 cells

Viruses	Mean EC ₅₀ $\pm$ S.D. ^a					
	A549			Calu-3		
	IFN- β	IFN- λ 1	Oseltamivir carboxylate	IFN- β	IFN- λ 1	Oseltamivir carboxylate
A/California/04/09 (H1N1)	0.2 $\pm$ 0.1	205.9 $\pm$ 9.2	3.0 $\pm$ 0.3	0.7 $\pm$ 0.1	45.0 $\pm$ 8.4	0.1 $\pm$ 0.1
A/Panama/2007/99 (H3N2)	0.3 $\pm$ 0.1	$\approx$ 500.0	4.3 $\pm$ 0.5	2.9 $\pm$ 0.5	$\approx$ 500.0	0.7 $\pm$ 0.1

^a Sensitivity of influenza viruses to IFN- β , IFN- λ 1, and oseltamivir carboxylate in ELISA. EC₅₀ was determined as the concentration of the compound (ng/ml) necessary to reduce cell-associated virus yield by 50% relative to control infected untreated wells. Values are mean $\pm$ standard deviation (S.D.) from three independent experiments.

Table 2. Effects of IFN- β –IFN- λ 1 and oseltamivir carboxylate–IFN- λ 1 combinations on the yield of cell-associated influenza H1N1 and H3N2 viruses *in vitro*

Virus strain	Drug combination	A549			Calu-3		
		Synergy dose range ^a	MacSynergy volumes ^b		Synergy dose range	MacSynergy volumes	
			Synergy	Antagonism		Synergy	Antagonism
A/California/04/09 (H1N1)	IFN- β + IFN- λ 1	IFN- β (ng/ml): 0.001 – 0.00001, 0.1	85.1	-5.7	IFN- β (ng/ml): 0.00001, 0.0001, 1	29.7	-31.8
		IFN- λ 1 (ng/ml): 0.00001, 0.0001, 0.01, 0.1, 10			IFN- λ 1 (ng/ml): 0.0001, 0.01 – 10		
	Oseltamivir carboxylate + IFN- λ 1	Oseltamivir carboxylate (ng/ml): 0.00004 – 0.04, 4 – 400	181.6	-25.8	Oseltamivir carboxylate (ng/ml): 0.00004 – 400	642.1	-374.1
		IFN- λ 1 (ng/ml): 0.00001, 0.0001, 0.01 – 100			IFN- λ 1 (ng/ml): 0.01 – 100		
A/Panama/2007/99 (H3N2)	IFN- β + IFN- λ 1	IFN- β (ng/ml): 0.0001, 0.00001	60.2	-67.0	IFN- β (ng/ml): 0.00001	27.0	0.0
		IFN- λ 1 (ng/ml): 0.00001, 0.001, 10, 100			IFN- λ 1 (ng/ml): 0.0001, 0.1, 1, 100		
	Oseltamivir carboxylate + IFN- λ 1	Oseltamivir carboxylate (ng/ml): 0.00097 – 970	325.7	-123.1	Oseltamivir carboxylate (ng/ml): 0.00097 – 970	516.1	-323.1
		IFN- λ 1 (ng/ml): 0.00001 – 1, 100			IFN- λ 1 (ng/ml): 0.1 – 100		

^a Possible ranges of synergy as shown on Figures 1 and 2.

^b The data were analyzed by using the MacSynergy II software program, provided by Mark N. Prichard (Prichard and Shipman Jr., 1990; Prichard et al., 1992). Theoretical additive interactions were calculated from the dose-response curves for each drug used individually. The confidence intervals around the experimental dose-response surface were used to evaluate the data statistically, and the volumes of the peaks were calculated and used to quantify the volume of synergy (or antagonism) produced. The guidelines for the volumes of the synergy determinations, expressed as unit² (ng/ml $\times$ ng/ml $\times$ %) at a 95% confidence level, were as follows: 0 to 25, insignificant synergy or antagonism; 25 to 50, minor but significant synergy or antagonism; 50 to 100, moderate synergy or antagonism; >100, strong synergy or antagonism. Synergy plots were made at the 95% confidence limit.

Figure 1A)

B)

C)

D)

Figure 2A)

B)

Figure 3A)

B)

C)

D)