Postinfection A77-1726 Treatment Improves Cardiopulmonary Function in H1N1 Influenza-Infected Mice

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Acute respiratory disease is associated with significant morbidity and mortality in influenza. Because antiviral drugs are only effective early in infection, new agents are needed to treat nonvaccinated patients presenting with late-stage disease, particularly those who develop acute respiratory distress syndrome. We found previously that the *de novo* pyrimidine synthesis inhibitor A77-1726 reversed the influenza-induced impairment of alveolar fluid clearance. Patients with acute respiratory distress syndrome and intact alveolar fluid clearance demonstrate lower mortality than those with compromised fluid clearance. We therefore investigated the effects of treatment with nebulized A77-1726 (67.5 mg/kg) on indices of cardiopulmonary function relevant to the diagnosis of acute respiratory distress syndrome. BALB/cAnNCr mice (8–12 wk old) were inoculated intranasally with 10,000 plaque-forming units/mouse influenza A/WSN/33 (H1N1). Pulse oximetry was performed daily. Alveolar fluid clearance, lung water, and lung mechanics were measured at 2 and 6 days after inoculation in live, ventilated mice by BSA instillation, magnetic resonance imaging, and forced-oscillation techniques, respectively. A77-1726 treatment at 1 day after inoculation delayed mortality. Treatment on Days 1 or 5 reduced viral replication on Day 6, and improved alveolar fluid clearance, peripheral oxygenation, and cardiac function. Nebulized A77-1726 also reversed influenza-induced increases in lung water content and volume, improved pulmonary mechanics, reduced bronchoalveolar lavage fluid ATP and neutrophil content, and increased IL-6 concentrations. The ability of A77-1726 to improve cardiopulmonary function in influenza-infected mice and to reduce the severity of ongoing acute respiratory distress syndrome late in infection suggests that pyrimidine synthesis inhibitors are promising therapeutic candidates for the management of severe influenza.

Keywords: alveolar fluid clearance; pulmonary edema; antiviral agents; lung function; ARDS

Influenza A viruses cause a highly contagious acute respiratory illness in humans (1). Currently, seasonal influenza accounts for 200,000 hospitalizations and more than 36,000 excess deaths per year in the United States alone, and is the eighth leading cause of annual mortality in this country (2). Moreover, the recent

CLINICAL RELEVANCE

Acute respiratory disease is associated with significant morbidity and mortality in influenza. However, a “therapeutic gap” is evident in influenza, with no virus-specific therapies currently available to prevent, retard, or manage the progression of severe disease to acute respiratory distress syndrome (ARDS) in those nonvaccinated subjects who present later in infection, when antiviral agents are no longer effective. We show that the aerosol treatment of influenza-infected mice with the *de novo* pyrimidine synthesis inhibitor A77 delays mortality and significantly improves a variety of indices of cardiopulmonary function pertinent to diagnoses of ARDS in human subjects. Nucleotide synthesis inhibitors such as A77 are therefore promising candidates to fill the “therapeutic gap” in influenza.

pandemic H1N1 “swine influenza” strain (A/California/09) was estimated to have infected around 61 million people in the United States alone between April 2009 and April 2010, resulting in approximately 275,000 hospitalizations and 12,500 excess deaths (3).

At present, annual vaccination is the mainstay of influenza prophylaxis (1). However, rapid influenza virus mutation necessitates annual vaccine reformulation, and current limitations in the speed of vaccine production can prove problematic when novel influenza strains arise (4). Moreover, attitudes toward vaccination in the general population are by no means uniformly positive (5), and vaccination rates in the United States are consequently inadequate to control influenza transmission fully, particularly in high-risk populations (6). Furthermore, around 85% of developing countries have been estimated to have no access to vaccines during influenza pandemics (7).

Given the current limitations of influenza vaccination, a substantial percentage of the population is highly likely to become infected and require treatment during the next pandemic. Neuraminidase inhibitors such as oseltamivir are highly effective at reducing viral replication, disease severity, and person-to-person transmission in influenza. However, the development of viral mutants that are resistant to oseltamivir is increasingly common (8). Moreover, these antiviral agents are not always widely available, can be costly, and may be of only limited use, even in the early stages of infection (9, 10). Many patients present later in disease, and those with severe influenza may go on to develop acute respiratory distress syndrome (ARDS), which is associated with poor prognoses (11). After ARDS has developed, nonspecific (and often costly) inpatient supportive care is the only available treatment option (12, 13). Hence, a “therapeutic gap” in influenza is evident, with no virus-specific therapies available to prevent, retard, or manage the progression of severe disease to ARDS in those nonvaccinated subjects who present later in infection, when antiviral agents are no longer effective. This

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gap may be of particular significance during pandemics, in which large numbers of patients could develop disease severe enough to require hospitalization and care in already overburdened intensive care units (ICUs) (12, 14).

We previously found that mice infected with a lethal dose of H1N1 influenza A/WSN/33 exhibited nucleotide-mediated bronchoalveolar epithelial dysfunction (impaired alveolar fluid clearance; AFC) (15). Effective AFC is crucial for efficient gas exchange in the lung (16), and patients with ARDS with intact AFC demonstrate lower morbidity and mortality than those with compromised AFC (17). We therefore hypothesized that, by reducing intrapulmonary nucleotide concentrations, early or late postinfection treatment with the *de novo* pyrimidine synthesis inhibitor A77-1726 (A77) may improve cardiopulmonary function, delay the progression to ARDS, and reduce the severity of ARDS, thereby helping to fill the “therapeutic gap” in influenza.

MATERIALS AND METHODS

Mouse Inoculation

BALB/cAnNCr mice (8–12 wk old) were inoculated intranasally with 10,000 plaque-forming units of H1N1 influenza A/WSN/33 in 50 μ l PBS with 0.1% BSA under light isoflurane anesthesia. Mice were individually marked and weighed daily. Data for each experimental group were derived from a minimum of two independent infections. All procedures were approved by the Institutional Animal Care and Use Committee of Ohio State University.

Administration of Nebulized A77

A77 (EMD Biosciences, La Jolla, CA) was reconstituted in DMSO, and aliquots of stock solution were frozen. Conscious, influenza-infected mice were restrained (SoftRestraints; SciReq, Montreal, PQ, Canada) and then exposed to 67.5 mg/kg nebulized A77 (from the stock solution diluted 1:1,000 in sterile normal saline), using a computer-controlled nose-only aerosol system (inExpose; SciReq). The full A77 dose was delivered to each mouse over a 5-minute period, admixed to room air at a bias flow rate of 2 L/minute.

Magnetic Resonance Imaging of the Lung

Isoflurane-anesthetized mice were imaged using a 9.4-T BioSpec high-field small-animal magnetic resonance imaging (MRI) system (Bruker BioSpin Corp., Karlsruhe, Germany). Respiratory and cardiac-gated axial T1-weighted and T2-weighted images were acquired over the entire lung. The lung region of interest in T1-weighted images was manually outlined by a blinded observer (K.A.P.). Lung volumes were calculated by summing outlined regions of interest from each contiguous slice. Fluid volumes were measured by setting a global threshold in T2-weighted images and summing the segmented voxels within the lung region of interest. For image display purposes (but not data analysis) in Figure 4, T1-weighted and T2-weighted images were overlaid and identified by false color, with image contrast adjusted to improve visualization.

Other Methods

Preparations of viral inocula and measurements of AFC rates, viral titers, cardiopulmonary function, lung water content, lung mechanics, and bronchoalveolar lavage fluid (BALF) cells and mediators were performed as previously described (15). Additional details of all methods are provided in the online supplement.

Statistical Analyses

Descriptive statistics were calculated using InStat version 3.05 (GraphPad, San Diego, CA). Weight-loss curves were compared according to a log-rank (Mantel-Cox) test, using Prism software (GraphPad). $P < 0.05$ was considered statistically significant. All data are presented as means $\pm$ SEM.

RESULTS

A77 Ameliorates the Nucleotide-Mediated Inhibition of AFC in Influenza-Infected Mice

We found previously that influenza A/WSN/33 induced AFC impairment at 2 to 6 days postinoculation (DPI) and the development of lethal primary influenza pneumonia at 6 to 8 DPI (15). In initial proof-of-concept studies, we therefore determined the effects of early (1 DPI) or late (5 DPI) exposure to a single dose of nebulized A77 on AFC at 2 and 6 DPI. AFC rates in untreated, influenza-infected mice were comparable to those in our earlier studies (Figure 1A). Treatment with vehicle (sterile saline + 0.01% DMSO) at 1 DPI exerted no effect on AFC at 2 DPI (not shown). A77 nebulization at 1 DPI almost completely restored normal AFC at 2 but not 6 DPI, whereas A77 treatment at 5 DPI significantly improved AFC at 6 DPI.

AFC inhibition in mice infected with replication-competent influenza virus results from increased BALF nucleotide concentrations (15). In agreement with these findings, the BALF ATP content significantly increased at 2 and 6 DPI (Figure 1B). These effects were reversed by A77 treatment at 1 or 5 DPI.

The addition of the epithelial Na⁺ channel (ENaC) inhibitor amiloride (1.5 mM) to the AFC instillate reversed the increase in AFC observed in A77-treated mice at 2 DPI (Figure 1C). This indicates that A77 increases ENaC-mediated AFC in influenza-infected mice.

The cystic fibrosis transmembrane conductance regulator (CFTR) Cl[−] channel inhibitor CF_{inh}-172 (100 μ M) reduces AFC in uninfected mice, but improves AFC in untreated animals at 2 DPI by blocking increased Cl[−] secretion (15). As in uninfected mice, CF_{inh}-172 reduced AFC in A77-treated animals at 2 DPI (Figure 1C). Under Cl[−]-free conditions, which maximize the electrochemical gradient for epithelial Cl[−] secretion, A77 treatment restored normal AFC at 2 DPI (Figure 1D), indicating that A77 restores normal CFTR function in influenza-infected mice.

Because of the significant effects of early and late postinfection A77 therapy on AFC, we investigated its impact on other aspects of murine lung function, and in particular those relevant to the diagnosis and assessment of ARDS in human subjects.

A77 Prolongs Survival, despite Increasing Postinfection Weight Loss and Only Limited Effects on Influenza Replication

Treatment with nebulized A77 at 1 DPI increased median time to death from 6 to 7 days, although it did not prevent mortality (Figure 2A). Despite this protective effect, A77 treatment at 1 DPI increased the rate of weight loss in infected mice (Figure 2B). At 6 DPI, body weight was 3% lower in A77-treated animals, although this difference may not be of major clinical importance. Weight loss at 6 DPI was not significantly altered by treatment with nebulized A77 at 5 DPI.

Lung homogenate influenza titers did not differ between untreated and A77-treated mice at 2 DPI (Figure 2C), but were reduced at 6 DPI in mice treated with A77 at 1 or 5 DPI. However, this effect was modest (< 0.5 log).

A77 Improves Cardiopulmonary Function

Carotid arterial O₂ saturation (SaO₂) declined very significantly by 6 DPI (Figure 3A), without a compensatory increase in respiratory rate (Figure 3B). Heart rate (Figure 3C) and cardiac contractility (pulse distension; Figure 3D) declined progressively over the course of infection. Similar myocardial dysfunction was reported previously in patients hospitalized for both seasonal and “swine flu” influenza (18, 19).

A77 at 1 DPI (A77-Tx Day 1) on Day 2 AFC in Cl^- -free (Na^+ gluconate) BSA ($n = 7$ per group). Dotted lines indicate mean values for each parameter in uninfected mice. * $P < 0.05$ and *** $P < 0.0005$, versus uninfected mice. † $P < 0.1$, † $P < 0.05$, and # $P < 0.0005$, versus infected, untreated mice (UNTx) at the same postinfection time point.

Treatment with nebulized A77 (67.5 mg/kg) at 1 DPI moderately improved SaO_2 and significantly increased heart rate and cardiac contractility at 2 DPI. A77 treatment at 1 or 5 DPI significantly improved SaO_2 , heart rate, and pulse distension at 6 DPI.

A77 Ameliorates ARDS-Like Bilateral Airspace Disease

In human patients, the presence of bilateral infiltrates on chest X-ray is considered an essential criterion for a diagnosis of ARDS (20, 21). However, neither radiographic nor computed tomographic imaging provides sufficient resolution and contrast for the assessment of pulmonary edema in mice (22). We therefore used a modified MRI method for the accurate and unambiguous quantification of lung volume and water content throughout both lungs during the course of infection (23). Because MRI imaging was performed under moderate isoflurane anesthesia, animals could recover, allowing us to follow the progression of edema formation and changes in its spatial distribution during the course of infection on an individual animal basis. As expected, a water signal (from T2-weighted scans) was virtually absent in uninfected mice (mean signal $< 0.1\%$ of total lung volume, as determined from T1-weighted scans of thoracic structures). Influenza infection resulted in significant pulmonary edema at 2 to 6 DPI (Figures 4A and 4C). At 2 DPI, heterogeneous foci of fluid-filled tissue were present in both lungs in a perihilar distribution (not shown). By 6 DPI, these regions had coalesced and expanded to the point where large sections of the lung and, in some mice, entire lung lobes became completely fluid-filled (Figure 4C). Comparable heterogeneously distributed areas of collapsed and overdistended lung tissue have been reported in computed tomographic scans of lungs in patients with ARDS (24). In contrast, after A77 treatment at 1 DPI, T2-weighted (water) signals were almost completely absent from the lungs at both 2 and 6 DPI (Figures 4A and 4D).

Influenza induced a significant increase in total lung volume at 6 DPI (Figure 4B), most likely as a result of increased alveolar recruitment (25). However, the mean volume of aerated

lungs did not significantly increase (from $520 \pm 14 \text{ mm}^3$ in uninfected mice to $577 \pm 30 \text{ mm}^3$ at 6 DPI). Lung volumes at 2 and 6 DPI were normal in mice treated with A77 at 1 DPI (Figure 4B).

A77 Attenuates the Development of Pulmonary Edema

Influenza infection resulted in a significant increase in lung wet/dry weight at 2 and 6 DPI (Figure 5A), which correlated very significantly ($P < 0.005$) with the percentage of fluid-filled lung volume measured by MRI. A77 treatment at 1 but not 5 DPI blocked the influenza-induced increase in lung water content.

The development of pulmonary edema in untreated mice was accompanied by a significant increase in BALF protein and lactate dehydrogenase at 6 DPI (Figures 5B and 5C). Treatment with nebulized A77 at 1 or 5 DPI exerted no effect on these markers of bronchoalveolar epithelial integrity.

A77 Improves Lung Mechanics

Total lung resistance increased significantly at 2 and 6 DPI (Figure 6A). In addition, airway hyperresponsiveness to the bronchoconstrictor methacholine was present at 2 DPI (Figure 6B). Both dynamic and static lung compliance declined significantly at 2 and 6 DPI (Figures 6C and 6D, respectively). These alterations in the mechanical properties of small airways and lung parenchyma in influenza-infected mice are qualitatively and quantitatively comparable to those observed in human ARDS (21).

Treatment with A77 at 1 DPI normalized total lung resistance and reversed airway hyperresponsiveness at 2 DPI. A77 administration at either time point also reduced airway resistance at 6 DPI. A77 treatment at 1 DPI improved dynamic compliance at 2 but not 6 DPI, and mice treated with A77 at 5 DPI demonstrated normal dynamic compliance at 6 DPI. However, A77 treatment exerted no effect on static lung compliance. These findings suggest that A77 primarily improves lung compliance by reducing resistance to airflow rather than parenchymal stiffness.

Figure 1. A77 ameliorates nucleotide-mediated inhibition of alveolar fluid clearance. (A) Effect of treatment (Tx) with nebulized A77 (67.5 mg/kg) at 1 day post-inoculation (DPI) (A77-Tx Day 1) on baseline alveolar fluid clearance rate (AFC) at 2 DPI ($n = 7$) and 6 DPI ($n = 15$), or A77 treatment at 5 DPI (A77-Tx Day 5) on AFC rate at 6 DPI ($n = 6$), compared with untreated animals ($n = 12$ per time point). (B) Effect of treatment with nebulized A77 (67.5 mg/kg) at 1 DPI (A77-Tx Day 1) on bronchoalveolar lavage fluid (BALF) ATP content at 2 DPI ($n = 5$) and 6 DPI ($n = 9$), or A77 treatment at 5 DPI (A77-Tx Day 5) on AFC at 6 DPI ($n = 10$), compared with untreated animals ($n = 5$ per time point). (C) Effect of treatment with nebulized A77 at 1 DPI (A77-Tx Day 1) on Day 2 AFC in the presence of 1.5 mM amiloride (AMIL) or 100 μM cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor-172 (CF-inh), compared with untreated animals ($n = 7$ –9 per group). (D) Effect of treatment with nebulized

Figure 2. A77 prolongs survival, despite increasing postinfection weight loss and only limited antiviral effects. Effect of treatment of influenza-infected mice with nebulized A77 (67.5 mg/kg) at 1 DPI (A77-Tx DAY 1) or 5 DPI (A77-Tx DAY 5) on (A) mortality ($n \geq 10$ per group), (B) body weight (BWT; percent change from baseline; $n > 15$ per group, including data from mice that died before 6 DPI), and (C) influenza virus replication (log titer in whole-lung homogenates; $n \geq 5$ per group). *** $P < 0.0005$, versus untreated mice (UNTx). $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.005$, versus untreated mice at the same postinfection time point. FFU, focus-forming units.

A77 Decreases Pulmonary Histopathology

Influenza A virus infection resulted in mild bronchiolitis at 2 DPI, which was not significantly impacted by A77 treatment at 1 DPI (Figures E1A and E1B in the online supplement). Marked necrosuppurative bronchopneumonia, alveolar histiocytosis, and perivascular lymphocyte cuffing were evident by 6 DPI in untreated mice (Figure E1C). The severity of histopathologic lesions at this time point was significantly ameliorated by A77 treatment, particularly when administered at 1 DPI (Figures E1D and E1E).

A77 Reduces BALF Neutrophil Concentrations

Tissue pathology in untreated mice was accompanied by a significant increase in BALF total cell counts at 2 and 6 DPI (Figure 7A). By 6 DPI, neutrophils accounted for approximately 70% of all cells in the BALF (Figure 7B). BALF alveolar macrophage and lymphocyte counts also increased significantly by 6 DPI (Figures 7C and 7D, respectively). Treatment

with nebulized A77 at 1 DPI reduced total cell counts at 6 DPI by attenuating the neutrophilic response. Neutrophils constituted only 36% of total BALF cells in this group. A77 administration at 5 DPI exerted a significantly greater effect on BALF neutrophil counts at 6 DPI than did treatment at 1 DPI. In mice treated with A77 at 5 DPI, neutrophils constituted only 12% of total BALF cells at 6 DPI. In contrast, A77 exerted no effects on the magnitude or kinetics of mononuclear leukocyte responses.

A77 Alters BALF Cytokine and Chemokine Responses

In untreated mice, influenza induced profound increases in BALF IFN- α , IL-6, keratinocyte cytokine (KC), monocyte chemotactic protein-1 (MCP-1/CCL2), regulated upon activation, normal T cell expressed and secreted (RANTES/CCL5), and TNF- α concentrations at 2 DPI (Table 1). Moderate increases in other cytokines (IL-1 β , IFN- γ , IL-10, and IL-12) were also detected. A77 treatment resulted in an approximately 40% increase in mean IFN- α , KC, and TNF- α concentrations at 2 DPI,

Figure 3. A77 improves cardiopulmonary function in conscious mice. Effects of treatment of influenza-infected mice with nebulized A77 (67.5 mg/kg) at 1 DPI (A77-Tx DAY 1) or 5 DPI (A77-Tx DAY 5) on (A) carotid arterial O₂ saturation (S_O2; percentage), (B) respiratory rate (RR; breaths/minute), (C) heart rate (HR; beats/minute), and (D) pulse distension (PD; μ m) ($n > 10$ per group). Dotted lines indicate mean values for each parameter in uninfected mice. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$, versus uninfected mice. $^{\dagger}P < 0.05$ and $^{\#}P < 0.0005$, versus untreated mice (UNTx) at the same postinfection time point.

Figure 4. A77 ameliorates bilateral air-space disease. Effects of treatment of influenza-infected mice with nebulized A77 (67.5 mg/kg) at 1 DPI (A77-Tx DAY 1) on (A) lung water content (percentage of total lung volume) at 2 DPI ($n = 7$) and 6 DPI ($n = 4$), compared with untreated animals ($n = 6$ per time point); $< 0.1\%$ T2-weighted (edema) signal was detected in uninfected mice ($n = 8$), and (B) total lung volume (cm³) in the same experimental groups. Dotted line indicates mean lung volume in uninfected mice. (C) Representative Day 6 midthoracic magnetic resonance image from an untreated, influenza-infected mouse. T1-weighted images (body tissues) are pseudocolored red. T2-weighted images (edema) are pseudocolored green. LV, left ventricular lumen. (D) Representative Day 6 midthoracic magnetic resonance image from an influenza-infected mouse treated with nebulized A77 at 1 DPI. All mice were anesthetized with isoflurane

and imaged using an 11.7T BioSpec high-field small-animal magnetic resonance imaging system with respiratory and cardiac gating. Images were adjusted in brightness, contrast, and color balance for a more uniform appearance. These adjustments do not obscure, eliminate, or misrepresent any information presented in the original scans. *** $P < 0.0005$, compared with uninfected mice. † $P < 0.05$, versus untreated mice (UNTx) at the same postinfection time point.

together with a 3-fold rise in IL-6 concentrations. These changes were accompanied by statistically significant increases in BALF IL-1 β , IL-10, and IL-12 concentrations, and a reduction in IFN- γ . However, the concentrations of these latter cytokines remained low.

At 6 DPI, BALF IFN- α , KC, RANTES, and TNF- α declined significantly relative to 2 DPI in untreated mice, whereas IFN- γ and IL-10 content markedly increased. A77 treatment at either 1 or 5 DPI increased mean BALF IL-6 and IL-10 concentrations at 6 DPI by more than 2- and 3-fold, respectively. Both treatments also increased MCP-1, RANTES, and TNF- α concentrations, although TNF- α concentrations were very low at

this time point. Finally, A77 treatment at 5 DPI reduced IFN- γ content at 6 DPI, whereas A77 treatment at 1 or 5 DPI did not alter BALF concentrations of other cytokines at 6 DPI.

DISCUSSION

Annual vaccination and antiviral drugs are the mainstays of influenza prophylaxis and therapy (1). However, antiviral drugs are only effective early in infection. Patients who present with severe influenza later in infection often progress to ARDS, which is associated with poor prognoses (11). A significant fraction of patients hospitalized for severe primary influenza pneumonia

Figure 5. A77 attenuates the development of pulmonary edema. Effect of treatment of influenza-infected mice with nebulized A77 (67.5 mg/kg) at 1 DPI (A77-Tx DAY 1) or 5 DPI (A77-Tx DAY 5) on (A) lung water content (wet/dry weight ratio; $n = 6$ –9 per group), (B) BALF protein content (μ g/ml; $n = 6$ –10 per group), and (C) BALF lactate dehydrogenase (LDH) content (mU/ml; $n = 5$ –7 per group). Dotted lines indicate mean values for each parameter in uninfected mice. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$, versus uninfected mice. † $P < 0.05$ and # $P < 0.0005$, versus untreated mice (UNTx) at the same post-infection time point.

Figure 6. A77 improves lung mechanics. Effects of treatment of influenza-infected mice with nebulized A77 (67.5 mg/kg) at 1 DPI (A77-Tx DAY 1) or 5 DPI (A77-Tx DAY 5) on (A) baseline total lung resistance (R_{BASAL} ; cm H₂O · second/ml), (B) maximal airway resistance after the nebulization of 50 mg/ml methacholine (R_{MAX} ; cm H₂O · second / ml), (C) dynamic lung compliance (C_{DYN} ; ml/cm H₂O × 10), and (D) static lung compliance (C_{ST} ; ml/cm H₂O × 10). All mice were anesthetized with valium/ketamine and mechanically ventilated on a FlexiVent computer-controlled piston ventilator (Scireq Respiratory Equipment, Montreal, PQ, Canada), with 8 ml/kg tidal volume, at a frequency of 150 breaths/minute, against 3 cm H₂O positive end-expiratory pressure ($n = 5-7$ per group). Dotted lines indicate mean values for each parameter in uninfected mice. ** $P < 0.005$ and *** $P < 0.0005$, compared with uninfected mice. † $P < 0.005$ and # $P < 0.0005$, versus untreated mice (UNTx) at the same postinfection time point.

during the “swine flu” pandemic developed ARDS (26), and histopathologic lesions consistent with this syndrome have been described retrospectively in patients infected with highly pathogenic H5N1 (“bird flu”) and 1918 “Spanish flu” influenza A strains (27, 28). The only treatment option available for ARDS consists of nonspecific, supportive ICU care (12, 13). An important “therapeutic gap” therefore exists, and novel drugs are needed to either delay the onset of ARDS or reduce its severity in this cohort of influenza-infected patients. Such drugs could reduce the potential for ICU overload in pandemic situations (29, 30), maintaining the ability of the healthcare system to provide adequate care to all who require it (12, 14).

Previously, we demonstrated that influenza induces rapid, nucleotide-mediated AFC impairment (15). As proof-of-concept experiments for the present study, we confirmed this effect, and

demonstrated that early or late postinfection treatment with the *de novo* pyrimidine synthesis inhibitor A77 could improve AFC and reduce BALF nucleotide concentrations at both 2 and 6 DPI. Because patients with ARDS with intact AFC demonstrate lower morbidity and mortality than those with compromised AFC (17), we investigated whether A77 may exert additional beneficial effects in influenza-infected mice. We found that, despite only a limited antiviral effect, A77 exposure at 1 DPI delayed mortality and resulted in prolonged improvements in SpO_2 , cardiac function, pulmonary edema, lung mechanics, and histopathology, thereby retarding the progression to ARDS. Moreover, these beneficial effects of A77 treatment persisted for up to 5 days, possibly because this drug can bind with high affinity to proteins in the alveolar lumen or within respiratory cells (<http://products.sanofi.us/arava/arava.html>). Most importantly, however, A77 treatment

Figure 7. A77 reduces BALF neutrophil concentrations in influenza-infected mice. Effects of treatment of influenza-infected mice with nebulized A77 (67.5 mg/kg) at 1 DPI (A77-Tx DAY 1) or 5 DPI (A77-Tx DAY 5) on BALF numbers of (A) total cells, (B) neutrophils (PMNs), (C) alveolar macrophages (AMs), and (D) lymphocytes (Lymphs) ($n \geq 10$ per group). All cell counts are expressed as $\times 10^6$ /ml. Dotted lines indicate mean values for total cells and alveolar macrophage counts in uninfected mice (not shown for neutrophils and lymphocytes because counts for both cell types were less than 10^4 /ml in uninfected mice). * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$, versus uninfected mice. † $P < 0.005$ and # $P < 0.0005$, versus untreated mice (UNTx) at the same postinfection time point.

TABLE 1. NEBULIZED A77-1726 MODULATES BRONCHOALVEOLAR LAVAGE FLUID CYTOKINE AND CHEMOKINE RESPONSES IN INFLUENZA-INFECTED MICE

	Day 0	Day 2		Day 6		
	UNT _x	UNT _x	A77-Tx Day 1	UNT _x	A77-Tx Day 1	A77-Tx Day 5
IFN- α	17 $\pm$ 1	449 $\pm$ 92	603 $\pm$ 20*	24 $\pm$ 3	34 $\pm$ 6	23 $\pm$ 2
IFN- γ	<1	125 $\pm$ 23	25 $\pm$ 4*	6,073 $\pm$ 518	6,322 $\pm$ 372	2,086 $\pm$ 502*
IL-1 β	3 $\pm$ 1	49 $\pm$ 8	78 $\pm$ 4*	19 $\pm$ 3	30 $\pm$ 5	20 $\pm$ 2
IL-6	7 $\pm$ 1	472 $\pm$ 58	1,220 $\pm$ 67 [†]	566 $\pm$ 76	1,372 $\pm$ 126 [†]	1,303 $\pm$ 179 [†]
IL-10	5 $\pm$ 1	43 $\pm$ 5	109 $\pm$ 9 [‡]	614 $\pm$ 109	2,030 $\pm$ 204*	744 $\pm$ 90
IL-12	9 $\pm$ 2	39 $\pm$ 5	85 $\pm$ 4*	37 $\pm$ 5	52 $\pm$ 4	31 $\pm$ 3
KC	36 $\pm$ 5	2,260 $\pm$ 198	3,089 $\pm$ 118*	333 $\pm$ 94	555 $\pm$ 67	370 $\pm$ 34
MCP-1 (CCL2)	<1	2,560 $\pm$ 170	2,187 $\pm$ 261	2,026 $\pm$ 196	2,845 $\pm$ 95 [†]	2,608 $\pm$ 107 [‡]
RANTES (CCL5)	<1	424 $\pm$ 60	571 $\pm$ 100	159 $\pm$ 11	310 $\pm$ 44 [‡]	232 $\pm$ 24
TNF- α	2 $\pm$ 1	478 $\pm$ 79	689 $\pm$ 25*	29 $\pm$ 8	77 $\pm$ 18 [‡]	41 $\pm$ 5

Definition of abbreviations: CCL2, chemokine (C-C motif) ligand 2; CCL5, chemokine (C-C motif) ligand 5; KC, keratinocyte cytokine; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated on activation, normal T cell expressed and secreted; Tx, treated; UNT_x, untreated mice.

All concentrations are expressed as pg/ml ($n \geq 9$ per group).

* $P < 0.05$, versus untreated mice at the same time point.

[†] $P < 0.005$, versus untreated mice at the same time point.

[‡] $P < 0.0005$, versus untreated mice at the same time point.

late in infection (at 5 DPI) also resulted in significant improvements in SaO₂, pulmonary edema, airway mechanics, and neutrophil infiltration at 6 DPI, by which time severe lung injury and ARDS are already present. We know of no available drugs with similar efficacy. ICU mainstays such as corticosteroids and β -adrenergic agonists generally exert very limited beneficial effects on lung function after the onset of severe viral acute lung injury (12). A77 is therefore a promising candidate agent to bridge the current “therapeutic gap” in influenza, and may be of particular value in the management of late-stage disease.

Treatment with A77 at 1 DPI significantly delayed mortality, although it did not prevent the ultimate demise of influenza-infected mice and actually increased the rate of body weight loss. Based on these criteria, which are commonly used as outcome measures in murine influenza studies, our findings may be interpreted to indicate limited A77 efficacy. However, the infection of human subjects does not induce the extremely rapid, severe, and probably intrinsically lethal weight loss observed in influenza-infected mice ($> 30\%$ in 6 days). Moreover, preadmission changes in body weight are not considered to be of clinical significance in the management of human patients with ARDS (12). In addition, weight loss and mortality in mice have been shown to be poorly correlated with human ARDS outcomes, and are often not of predictive value in determining the clinical efficacy of novel therapeutic agents (31). The rapid onset of severe anorexia and cachexia after influenza infection by no means negates the utility of the mouse as a model for most aspects of influenza pathogenesis. However, this rapid onset may constitute an intrinsic limitation of this species as a model for predicting the effects of novel therapeutic agents on ARDS severity and survival in human patients with severe disease. We therefore focused our studies on the effects of A77 treatment on indices of murine cardiopulmonary function that reflect those used to diagnose and determine the severity of ARDS in patients.

For a diagnosis of ARDS to be proposed in the ICU, a patient must exhibit a specific set of clinical signs that are indicative of severe lung dysfunction and injury: the acute onset of significant hypoxemia (decreased PaO₂ or SaO₂, which are strongly correlated in humans) (32) in the presence of bilateral chest X-ray infiltrates, and the absence of left atrial hypertension, together with either predisposing lung disease or decreased lung compliance (20, 21). Experimentally, additional predictors of poor ARDS outcomes in patients have been identified, including impaired AFC (17). Readouts of lung function analogous to those

used for diagnoses of ARDS in humans are now considered to be highly relevant and appropriate indices of acute lung injury in murine models, primarily because of their translational relevance (31). Using these readouts, we found that cardiopulmonary dysfunction and the severity of lung injury in influenza-infected mice were ameliorated by both early and late postinoculation single-dose A77 treatment. Because both caloric and fluid intake can easily be controlled in an ICU, the improvements in cardiopulmonary function imparted by A77 treatment may be sufficient to improve survival rates significantly in patients with severe influenza, irrespective of any effects of A77 on the rate of weight loss in influenza-infected mice.

The development of severe disease in mice infected with highly pathogenic influenza strains has been associated with increased viral replication rates (33), the induction of a “cytokine storm” (34–36), and excessive neutrophil infiltration (37–40). A77 improved cardiopulmonary function without significant antiviral effects, which implies that the severity of lung dysfunction in influenza may be independent of viral replication. Nevertheless, early postinfection treatment with a combination of oseltamivir and A77, or the use of a pyrimidine synthesis inhibitor with intrinsic antiviral activity such as A3 (41), may prove to exert enhanced beneficial effects in our model.

The contribution of a “cytokine storm” to the pathogenesis of ARDS associated with severe influenza remains unclear. Hypercytokinemia has been associated with fatal outcomes in human H5N1 influenza (42), yet TNF- α or IL-6 knockout mice are not protected against H5N1 disease (35, 43). We found little to no quantitative or temporal correlation between most BALF cytokines and chemokines and improved cardiopulmonary function in A77-treated mice. For example, A77 administration at 1 or 5 DPI resulted in comparable improvements in cardiopulmonary function at 6 DPI, despite the opposing effects of these treatments on BALF IFN- γ and IL-10. Relative to untreated control mice, A77 nebulization at 1 DPI increased IL-10 but not IFN- γ concentrations at 6 DPI. In contrast, exposure to A77 at 5 DPI increased IFN- γ but not IL-10 concentrations on Day 6. However, BALF IL-6 concentrations increased at both 2 and 6 DPI after A77 treatment at 1 or 5 DPI. Hence, A77-induced improvements in cardiopulmonary function were consistently associated with elevated IL-6 concentrations; this was not the case for any other cytokine analyzed. Taken together, our data indicate that cardiopulmonary dysfunction is not the result of a generalized “cytokine

storm,” although it may be associated with changes in the relative levels or kinetics of individual cytokine responses to influenza infection.

The functional consequences of altered cytokine profiles in A77-treated mice remain unclear. IL-6 is predominantly proinflammatory, and is often correlated with poor ARDS outcomes in humans (44). However, its effects are pleiotropic, and IL-6 can even be lung-protective in some lung injury models (45–47). Therefore, without further research, we cannot determine whether the relationship between elevated IL-6 concentrations and improved cardiopulmonary function in A77-treated mice is correlative or causative. Likewise, the approximately 40% increase in mean BALF TNF- α concentrations at 2 DPI in A77-treated mice may be of concern, particularly given the potential of this cytokine to exacerbate epithelial injury and capillary leakage (48). However, in our previous studies, we did not find any increase in BALF protein concentrations or alveolar barrier permeability to instilled FITC-albumin tracers at this time point (15).

Improved cardiopulmonary function in A77-treated mice was associated with reduced BALF neutrophil counts at both 2 and 6 DPI. Although these findings are only correlative, they suggest that the beneficial effects of A77 on lung function may in part result from an attenuation of the neutrophil response. Previous studies showed that neutrophils and neutrophil-derived reactive oxygen species are involved in the pathogenesis of influenza-induced lung injury, although it remains unclear whether they are detrimental or protective in influenza (37, 49, 50). Importantly, reactive species can alter both endothelial permeability and respiratory epithelial cell ion transport. The abrogation of the neutrophil response may therefore contribute to the beneficial effects of A77 on lung function. However, in earlier studies, we did not find evidence of elevated reactive species generation in our model (15).

Nucleotide-mediated AFC inhibition occurred before the onset of severe cardiopulmonary dysfunction, suggesting that AFC inhibition and the consequent pulmonary edema comprise the primary insults induced by influenza. Alveolar edema reduces the available surface area for gas exchange and increases diffusion distance, resulting in peripheral hypoxemia and subsequent cardiac dysfunction. Likewise, edema fluid will dilute surfactant (lowering dynamic compliance) and reduce the effective diameter of small airways (increasing airway resistance). The compression of small airways by adjacent fluid-filled alveoli and interstitial tissues will further increase resistance. The ability of A77 to reverse almost all the detrimental effects of influenza infection supports this hypothesis, because such multiple beneficial effects would be unlikely unless this drug affects a single upstream event in a cascade of cardiopulmonary dysfunction. However, A77 could not prevent bronchoalveolar epithelial damage, suggesting that a loss of epithelial integrity may instead be a consequence of viral or immune-mediated cytopathologic effects.

In conclusion, we have shown that the early or late postinfection aerosol treatment of influenza-infected mice with the *de novo* pyrimidine synthesis inhibitor A77 delays mortality and significantly improves a variety of indices of cardiopulmonary function pertinent to diagnoses of ARDS in human subjects. These include SaO_2 , myocardial contractility, lung water content, and lung mechanics. In addition, the beneficial effects of this drug are associated with reduced neutrophil infiltration and elevated BALF IL-6 concentrations. Our findings suggest that A77 has the potential to retard the progression to ARDS in influenza-infected human patients during a prolonged period (5 days or more). More importantly, A77 or related agents (41) may be of particular value in reducing the severity of lung dysfunction in late-stage influenza, when treatment is currently limited to supportive measures. Hence, nucleotide synthesis inhibitors such as A77 are promising candidates to fill the current “therapeutic gap” in influenza.

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