Targeting the pro-inflammatory factor CCL2 (MCP-1) with Bindarit for influenza A (H7N9) treatment

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Influenza A viruses are important human and animal pathogens. Seasonal influenza viruses cause infections every year, and occasionally zoonotic viruses emerge to cause pandemics with significantly higher morbidity and mortality rates. Three cases of laboratory confirmed human infection with avian influenza A (H7N9) virus were reported in 2013, and there have been several cases reported across South East Asia, and recently in North America. Most patients experience severe respiratory illness, with mortality rates approaching 40%. No vaccine is currently available and the use of antivirals is complicated due to the emergence of drug resistant strains. Thus, there is a need to identify new drugs for therapeutic intervention and disease control. In humans, following H7N9 infection, there is excessive expression of pro-inflammatory factors CCL2, IL-6, IL-8, IFNα, interferon-γ, IP-10, MIG and macrophage inflammatory protein-1β, which has been shown to contribute to fatal disease outcomes in mouse models of infection. In the current study, the potent inhibitor of CCL2 synthesis, Bindarit, was examined as a countermeasure for H7N9-induced inflammation in a mouse model. Bindarit treatment of mice did not have any substantial therapeutic efficacy in H7N9 infection. Consequently, the results suggest that Bindarit may be ill-advised in the treatment of influenza H7N9 infection.

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Plasma levels of IL-6 and IL-8 are sharply upregulated, whereas IL-10, macrophage inflammatory protein-1β, and interferon-γ are increased to intermediate levels.4 In contrast, IL-1β, tumor necrosis factor α (TNFα), and MIP-1α are found only at minimal concentrations. Whether the elevated cytokine and chemokine levels cause or contribute to the severity of H7N9 disease has yet to be determined.4 However, the upregulation of pro-inflammatory cytokines and chemokines, such as the monocyte chemoattractant protein-1 (MCP-1/CCL2), IL-6, IL-8, IFN-α, IP-10, MIG and macrophage inflammatory protein-1β was described in H7N9-infected patients with lung injury and severe pneumonia.2 The cytokine levels in C57BL/6 and BALB/c mice infected with H7N9 (A/Anhui/A/2013 strain) were compared. C57BL/6 mice exhibited more severe lung injury, slower recovery from lung damage, less effective viral clearance, higher levels of CCL2, IL-6 and IL1β, and lower levels of TNFα and interferon-γ than BALB/c mice. These data suggest that TNFα and IFNγ may help to suppress viral gene expression and increase viral clearance, while CCL2 and IL-6 may contribute to lung injury during H7N9 disease.17

The focus of this study was to assess the effects of drug inhibition of the pro-inflammatory factor CCL2, which may have a pathogenic role during H7N9 disease. Bindarit represents a novel class of inhibitor that reduces CCL2 synthesis.18 Bindarit has been successfully used to alleviate virus-induced inflammation in several animal models of disease. For example, Bindarit was efficacious in mouse models of Chikungunya and Ross River virus infections, where it was shown to ameliorate infections and disease.19,20 Furthermore, Bindarit was able to reduce arthritic inflammation without showing any detrimental effect on virus clearance in these animal models of alphavirus infection.21 Therefore, the activity of Bindarit activity was determined to reduce pulmonary and serum CCL2 levels in a mouse model of H7N9 disease. Bindarit selectively inhibits CCL2. CCL2 is a critical mediator of neuroinflammation in myriad disease states, including multiple sclerosis,22 human immunodeficiency virus (HIV)-1-induced encephalitis,23 Guillain-Barré syndrome,24 Alzheimer’s disease,25 ischemia,26 neurotrauma,27 epilepsy,28 neurogenic hypertension29 and alcoholism.30 Bindarit has also been studied for therapeutic intervention for these diseases. The clinical tolerability data for Bindarit in different CCL2-dependent illnesses demonstrated Bindarit safety up to a maximum dose of 2400 mg per day for as long as 6 months, and suggests the potential of Bindarit to be beneficial for a range of diseases.31

This study aimed to explore the role of CCL2 in H7N9 disease and the potential of Bindarit to act as a countermeasure against H7N9-induced pathology in a mouse model. Intriguingly, the survival rate of Bindarit-treated mice was comparable to that of non-treated mice, while weight loss, cellular infiltration and viral titers were considerably increased with Bindarit treatment. Thus, the use of Bindarit as a therapeutic to treat H7N9 disease seems ill-advised.

RESULTS

Bindarit treatment reduces CCL2 gene expression in lung epithelial cells after IAV infection

To evaluate the effectiveness of Bindarit in reducing CCL2 production, lung epithelial cells were infected (MOI = 0.1) with A/California/04/09 (A/CA; H1N1) virus and simultaneously treated with Bindarit. Quantitative reverse transcription PCR (RT-qPCR) was used to investigate the effect of Bindarit on CCL2 gene expression during infection with A/CA (H1N1), a representative, currently circulating IAV subtype, in a human epithelial cell (A549) line. CCL2 was considerably upregulated by 3.21-fold (P < 0.01) in A/CA (H1N1)-infected A549 cells compared to mock-infected controls at 24 h pi (Figure 1). When A549 cells were treated with Bindarit (100 μM), CCL2 gene expression was significantly (P < 0.01) reduced to a level comparable to mock-infected controls.

Bindarit treatment does not protect mice from lethal avian IAV H7N9 infection

The ability of Bindarit to protect mice from lethal H7N9 infection was investigated. Mice were intranasally (i.n.) infected with a lethal dose of H7N9 (10^5 PFU) and treated with Bindarit (70 mg kg^-1) twice daily.
starting at day 1 post-infection (pi). Mice were monitored for weight loss and survival. Bindarit treatment had no detectable impact on weight loss or survival of mice. Mice in both groups lost body weight to a similar extent, reaching a 25% reduction in body weight by day 5 (Figure 2a). Mortality typically occurred between day 5 and 10 pi for untreated mice and between days 7 and 9 for Bindarit-treated mice (Figure 2b). No significant (P < 0.1) difference was noted in the survival rate between the two groups.

Bindarit treatment does not affect lung pathology in mice infected with a lethal dose of avian IAV H7N9

The effect of Bindarit on lung inflammation and pathology was investigated in mice infected with H7N9. Mice were i.n. infected with a lethal dose of H7N9 (10^5 PFU) and orally treated with Bindarit (70 mg kg^-1) twice daily starting at day 1 pi. Mice were killed at day 4 pi and lungs were collected for histopathology. Lungs in both groups, mock- and Bindarit-treated, showed moderate to severe necrotic bronchitis and bronchiolitis. The peribronchiolar and perivascular infiltrations was mild to moderate for both groups and animals showed mild to severe alveolitis (Figures 3c and d). Two mock-treated mice infected with H7N9 developed hemorrhage, a feature that was not observed in Bindarit-treated animals. Taken together, there were no substantial changes in histopathology when mice were orally treated with Bindarit (70 mg kg^-1). All mice showed a lung score of 3 (Table 1).

Bindarit treatment is associated with increased pulmonary cellular infiltration after lethal avian IAV H7N9 infection

To investigate the effects of Bindarit on pulmonary cellular infiltration, flow cytometry was performed using the bronchoalveolar lavage (BAL) fluid from infected mice. A significant (P < 0.001) increase in total number of leukocytes was observed in mice orally treated with Bindarit (70 mg kg^-1) after lethal infection with H7N9 at day 4 pi (Figure 4a). Interestingly, the number of alveolar macrophages was increased after Bindarit treatment (Figure 4b); despite its known ability to reduce production of the monocyte attractant factor MCP-1/CCL2. Furthermore, the influx of eosinophils and CD8+ T-cells was also increased in Bindarit-treated mice (Figures 4c and f).

Bindarit treatment is associated with weight loss after sub-lethal infection with H7N9

No differences in weight loss were observed between mock- and Bindarit-treated mice after i.n. lethal H7N9 infection (10^5 PFU). The lethal challenge may have been overwhelming and therefore the effect of Bindarit insufficient. Therefore, a study using a sub-lethal dose of virus was performed. Mice were infected 10^2.7 PFU with a sub-lethal dose of H7N9 and orally treated with Bindarit (70 mg kg^-1) twice daily starting at day 1 pi. Mice in both groups lost body weight until day 4 pi. However, on days 5, 6 and 8, Bindarit-treated mice showed a considerable increase in weight loss compared to mock-treated control mice (Figure 5a).
Bindarit treatment was associated with an increase in lung viral titers after sub-lethal H7N9 infection

In order to investigate the role of CCL2, the level of lung viral clearance was determined when mice were treated with Bindarit during H7N9 disease. Viral titers were measured by RT-qPCR from 5 ng of total RNA from homogenized lung samples using primers and probe specific to the viral M gene. Mice orally treated with Bindarit (70 mg kg$^{-1}$) had higher lung virus titers compared to mock-treated control mice at day 8 pi (Figure 5b). The virus titer was ~ 1 log higher in the Bindarit-treated group.

Bindarit treatment did not alter pro-inflammatory cytokines gene expression during sub-lethal H7N9 infection

To corroborate the RT-qPCR results, a Luminex enzyme-linked immunosorbent assay platform was used to measure the pro-inflammatory cytokines IL-6, IL-15, CCL2, RANTES and TNF in the BAL at day 8 pi (Figures 7a–e). The IL-15 protein level was significantly ($P<0.05$) increased when H7N9-infected mice were treated with Bindarit. Bindarit treatment did not appreciably change the level of other pro-inflammatory cytokines. Bindarit oral treatment also had minimal effect on cytokine expression levels in the lungs. Interestingly, CCL2 protein level was not reduced, but appeared enhanced after treatment with Bindarit.

Bindarit treatment did not affect the pulmonary cellular influx during sub-lethal Avian AIV H7N9 infection

To determine a mechanism for cellular infiltration into the BAL, the was investigated using flow cytometry. Total numbers of leukocytes as well as macrophages, eosinophils, and T cells were not significantly ($P<0.01$) increased after BINDARIT treatment, but were slightly higher than those in mock-treated mice (Figure 8). The effect of BINDARIT treatment on cellular infiltration appeared to be weaker after sub-lethal infection than after lethal infection. Interestingly, the number of alveolar macrophages did not change after treatment with

**Table 1 Lung scores and observations of lesions after lethal H7N9 infection in mice**

| Group          | Lung score | Bronchitis/bronchiolitis | Peribronchial/perivascular infiltration | Alveolitis | Additional observation |
|----------------|------------|--------------------------|----------------------------------------|------------|------------------------|
| H7N9+vehicle   | 3          | Severe necrosis          | Moderate                               | Mild       | Moderate               |
|                | 3          | Moderate necrosis        | Mild                                   | Moderate   | Severe Hemorrhage      |
|                | 3          | Moderate necrosis        | Mild                                   | Moderate   | Hemorrhage             |
| H7N9+Bindarit  | 3          | Moderate necrosis        | Moderate                               | Moderate   | Moderate               |
|                | 3          | Moderate necrosis        | Moderate                               | Moderate   | Moderate               |
|                | 3          | Moderate necrosis        | Moderate                               | Moderate   | Moderate               |

**Figure 4** Effect of Bindarit on cellular infiltration following avian IAV H7N9 infection. Mice were infected i.n. with a lethal dose (10 × LD$_{50}$) of A/Anhui (H7N9) or PBS. Mice were then treated with either Bindarit or vehicle starting at day 1 pi. At day 5 pi, mice were killed and BAL fluids were collected for analysis with flow cytometry. (a) The total number of BAL leukocytes, (b) the number of macrophages, (c) the number of eosinophils, (d) the number of CD3$^+$ T-cells, (e) the number of CD4$^+$ T cells, and (f) the number of CD8$^+$ T cells were determined. Data are presented as the number of specific type of cells per million total cells. Data are from five mice per group ± s.e.m. *$P<0.05$, **$P<0.01$, ***$P<0.001$. 

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Bindarit, despite its known ability to reduce production of monocyte attractant MCP-1/CCL2 in other models.18

DISCUSSION

The severe disease in mice infected with avian IAV H7N9 is associated with a ‘cytokine storm’ characterized by upregulation of pro-inflammatory cytokines and chemokines such as CCL2, IL-6, IL-8, IFN-α, IFNγ, IP-10, MIG and macrophage inflammatory protein-1β. A similar cytokine response is thought to contribute to lung injury and severe pneumonia in H7N9-infected patients.2 Mouse studies suggested that TNFα and IFNγ may help to suppress viral gene expression and increase viral clearance, while CCL2 and IL-6 may contribute to lung injury during H7N9 disease.17 Therefore, this study aimed to investigate the role of CCL2 in the context of avian IAV H7N9 disease by using the potent CCL2 synthesis inhibitor Bindarit. Treatment of H7N9-infected mice with Bindarit may have enhanced some aspects of disease including increased virus titers, weight loss and cellular infiltration in the BAL. These results suggest that CCL2 has an antiviral role against H7N9 replication; thus, therapeutic approaches targeting CCL2 may be cautioned advised for that CCL2 has an antiviral role against H7N9 replication; thus, evaluating the effects of CCL2 inhibitors for the treatment of H7N9 infection. This is the first study evaluating the effects of CCL2 inhibitors for the treatment of influenza-induced disease and the results suggest that this class of drugs may not be suitable for treatment of severe influenza infections.

CCL2 is upregulated in several viral diseases in humans, such as HIV, hepatitis C virus, several herpes viruses, Japanese encephalitis virus and respiratory syncytial virus, and has been considered as a biomarker linked to disease severity in HIV.33 Furthermore, CCL2 has been linked to inflammation and tissue damage in human disease.33–35 In animal studies of various inflammatory diseases, Bindarit was effective in reducing CCL2 production in vitro and in vivo and successfully alleviated CCL2-driven diseases such as arthritis, encephalomyelitis and prostate and breast cancers.36–38 In mouse models of arthritogenic alphavirus disease, Bindarit reduced disease symptoms such as clinical score, cellular infiltration of muscle tissues and bone loss but had no effect on virus clearance.19–21 These studies indicate that Bindarit may be potentially used in the treatment of virus-induced inflammation. Furthermore, Bindarit reduces inflammation and ameliorates disease in a mouse model of autoimmune encephalomyelitis,37 which mimics many aspects of Guillain–Barre syndrome,39 indicating that CCL2 inhibitors could potentially be beneficial in preventing exacerbated Guillain–Barre syndrome. However, CCL2 may have dual roles in antiviral defense, mediating both protective and pathogenic functions. For example, in a study evaluating the role of CCL2 using an animal model of HIV, CCL2 receptor (CCR2) knockout mice showed increased virus titers and disease.35 In a different study on CHIKV a similar importance for the CCR2 was found. CCR2 deficiency promoted exacerbated chronic erosive neutrophil dominated CHIKV-induced arthritis in mice.40 CCL2 is highly upregulated in patients suffering from H7N9 influenza infection, and has been linked to lung injury in mouse models.2 However, in this study, when H7N9-infected mice were treated with Bindarit, mice exhibited heightened disease signs as demonstrated by an increase in weight loss, pro-inflammatory factors, cellular infiltration and virus titers. Thus, blocking CCL2 dampened viral clearance and was associated with upregulation of pro-inflammatory cytokines and cellular infiltration. In earlier studies examining the effects of anti-CCL2 antibodies on IAV disease, mice...
exhibited enhanced pneumonitis compared to non-treated animals, despite reduced numbers of cellular infiltrates such as leukocytes, macrophages and neutrophils in the lungs.41 Furthermore, infection of CCL2 knockout mice with a non-lethal dose of a mouse-adapted strain of IAV resulted in a profound increase in weight loss, elevated viral loads and pro-inflammatory cytokines, and enhanced leukocyte recruitment into the infected lungs compared to wild-type mice.42 Interestingly, in that study, pro-inflammatory cytokines such as TNFα, IL-6 and IFNγ were enhanced, but cellular infiltrates into the lungs were reduced.42 However, one limitation of that study was the analysis of the cellular infiltrate in full lung homogenate as opposed to BAL, which may have influenced the outcome of the study. In the current study, we observed an increase in pro-inflammatory cytokine expression and cellular infiltration in the BAL.

Interestingly, Ccl2 gene expression was not reduced in the lungs after oral treatment with Bindarit, despite its known capability in reducing CCL2 synthesis in vitro and in vivo from earlier studies by other groups.18 Oral administration of Bindarit may be a limitation in the treatment of pneumonia, as there are difficulties in reaching therapeutic concentrations of drugs in the lungs when administered...
by this route. It remains a possibility that oral delivery of Bindarit was not completely effective or led to a systemic reduction of CCL2 production that contributed to disease enhancement. In future studies this limitation will be addressed by i.n. administration of Bindarit, which may increase the concentration of the drug in the lungs. In addition, IL-15 was highly upregulated in the BAL of Bindarit-treated H7N9-infected mice. IL-15 has recently been described as a critical factor in the pathogenesis of IAV in mice with virus-induced acute lung injury. Whether there is a link between increased IL-15 production in the lung and a systemic inhibition of CCL2 synthesis remains a subject of further studies.

Various approaches have been investigated for the treatment of H7N9 infection in the recent years. Treatment with corticosteroids was evaluated, but it led to increased mortality in patients suffering from acute H7N9 infection. Due to increased drug resistance among circulating and novel IAV strains and the lack of specific vaccines against H7N9, there remains an imminent need for drug repurposing because of the availability for clinical use.

METHODS

Cell cultures and influenza virus stock
All in vivo experiments were performed under the guidance of the Institutional Animal Care and Use Committee (IACUC) and Animal Resources at the Animal Health Research Center (AHRC), which has approved biosafety level 3 laboratories. Avian IAV A/Anhui/1/2013 (A/Anhui; H7N9) was propagated in embryonated chicken eggs. Influenza A/California/04/09 (A/Ca; H1N1) was propagated in Madin-Darby canine kidney cells (ATCC CCL-34). Viruses were titrated on Madin-Darby canine kidney cells as described previously. The human type II respiratory epithelial cell line A549 (ATCC CCL-185) was maintained in Dulbecco’s modified eagle’s medium (HyClone, Logan, UT, USA) supplemented with 5% heat-inactivated fetal bovine serum. Viruses were propagated in embryonated eggs and stored at −80 °C until assayed for titer. Titers were determined by serial dilutions of infectious virus in embryonated eggs and by endpoint titration as described previously. At the time of infection, cell lines were regularly tested for mycoplasma contamination. Virus propagation in embryonated eggs was carried out in strict accordance with the recommendations by the University of Georgia IACUC. The protocol was approved by the University of Georgia IACUC.

In vitro influenza infection and Bindarit treatment
Bindarit (2-Methyl-2-[[1-(phenylmethyl)-1H-indazol-3-yl]methoxy]propanoic acid) was synthesized by Chemlin (Nanjing, China). Bindarit was dissolved in ultrapure water (Thermo Fisher Scientific, Waltham, MA, USA). A549 cells were grown to 80% confluency in a 48-well plate and infected with A/Ca (H1N1) at multiplicity of infection (MOI) of 0.1. After infection, Bindarit was added to the wells at a concentration of 100 μM. After 24 h, cells were collected in TRIzol (Thermo Fisher) for total RNA purification.

RNA isolation and RT-qPCR
Total RNA was isolated using TRIzol as previously described. In brief, the concentration of total RNA was measured using a microplate spectrophotometer (Epoch; BioTek, Winooski, VT, USA). RT-qPCR was used to validate mRNA expression changes and virus load using the Strategene Mx3005P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA). The reverse transcription reactions were performed using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) and 1000 ng total RNA for each reaction. qPCR was performed using the GoTaq Green Master Mix (Promega, Madison, WI, USA) to determine mRNA levels, and data were normalized to 18S expression using the 2−ΔΔCt method. Primer sequences for CCL2 were: 5′-GAAACACATCCACGCAGTA-3′ (forward primer) and 5′-CACCCACCCTCCTCTTGATTAC-3′ (reverse primer). The virus load was determined using 5 ng of total RNA with a TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific). The standard curve was produced using an M gene plasmid.

Histopathological evaluation
Lungs from infected mice were harvested at 4 days (pi), perfused with 10% buffered formalin, and fixed in 10% buffered formalin. The sections were embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin. The sections were evaluated using light microscopy. A histological score for each lung was determined according to the following criteria: 0 = no lung abnormality; 1 = <10% of airways inflamed; 2 = 10–30% of airways inflamed; 3 = 30–50% of airways inflamed and 4 = >50% of airways inflamed. The slides were evaluated by a pathologist without prior knowledge of the infection and treatment status.

BAL collection and quantification of cytokines
Eight days pi, mice were killed and tracheotomized to collect the lungs. The lungs were flushed with 1 ml of PBS, and the retained BAL was centrifuged at 400 g for 5 min at 4 °C. The recovered supernatants were collected and stored at −80 °C until assessed for cytokine concentration, and the cell pellet was resuspended in 200 μl of 10% buffered formalin. Total cell numbers were counted using a hemocytometer. Cytokines in BAL supernatants were quantified with the Luminex xMAP system using a MILLIPLEX MAP mouse cytokine immunoassay (MCYTOMAG-70K; Millipore, Billerica, CA, USA) according to the manufacturer protocol. In brief, beads coupled with anti-CCL2, anti-IL-6, anti-interferon-γ, anti-RANTES, anti-IL-15 and anti-TNF monoclonal antibodies were sonicated, mixed and diluted 1:50 in cytokine immunoassay (MCYTOMAG-70K; Millipore, Billerica, CA, USA) according to the manufacturer protocol. In brief, beads coupled with anti-CCL2, anti-IL-6, anti-interferon-γ, anti-RANTES, anti-IL-15 and anti-TNF monoclonal antibodies were sonicated, mixed and diluted 1:50 in assay buffer. For the assay, 25 μl of beads were mixed with 25 μl of PBS, 25 μl of assay buffer, and 25 μl of BAL supernatant, and incubated overnight at 4 °C. After washing, beads were incubated with biotinylated detection antibodies for 1 h and the reaction mixture was then incubated with streptavidin-phycocerythrin (PE) conjugate for 30 min at room temperature, washed and resuspended in PBS. The assay was analyzed on a Luminex 200 instrument (Luminex Corporation, Austin, TX, USA) using the Luminex xPONENT 3.1 software.

Flow cytometry
For flow cytometry analysis, cell suspensions were incubated in FACS staining buffer (PBS containing 1% BSA) and subsequently stained for 30 min at 4 °C with an optimized concentration of antibodies (BD Bioscience, Franklin Lakes, NJ, USA): PE-conjugated anti-CD3, PerCP/Cy5.5-conjugated anti-CD8, PE Cy7-conjugated anti-CD4, PerCP/Cy5.5-conjugated anti-CD45, APC-conjugated anti-CD11c and PE-conjugated anti-SiglecF to determine cell types in the BAL. Cells were acquired on an LSRII flow cytometer (BD Bioscience) and the data were analyzed using the FlowJo software (v 7.6.5; Ashland, OR, USA). Based on surface marker expression, six different
cell types were identified: CD45+ (total leukocytes), CD45+SiglecF+CD11Clox (eosinophils), CD45+SiglecF+CD11chih (alveolar macrophages), CD45+CD3+ (total T cells), CD4 T cells (CD45+CD3+CD4+), and CD8 T cells (CD45+CD3+CD8+).

**Statistical analysis**

All experiments were performed in minimum in triplicate to ensure adequate power and the experiment independently repeated at least twice. Using power analysis, 12 animals in each group are required to give a 90% probability of detecting a treatment difference at a 5% significance level if the true difference between the treatments is one s.d. of the variation with each treatment group. However, decreasing the number of mice to five per group, gives a 90% probability of detecting a treatment difference if the true difference between the treatments is 1.63 s.d.s of the variation with each treatment group. Further decreasing the treatment group size to three mice per group, gives a 90% probability of detecting a treatment difference only if the true difference between the treatments is 2.36 s.d.s of the variation with each treatment group. Data are expressed as mean ± s.e.m. Differences between groups were determined by one-way analysis of variance. Individual differences between groups were tested by multiple comparison and analysis using the Tukey post-test. P-values of <0.05 were considered significant. All analysis was performed using Graphpad Prism Software (La Jolla, CA, USA).

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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