Inhibition of H1N1 influenza A virus growth and induction of inflammatory mediators by the isoquinoline alkaloid berberine and extracts of goldenseal (*Hydrastis canadensis*)

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Abstract:

In this study we tested whether the isoquinoline alkaloid berberine can inhibit the growth of influenza A. Our experiments showed strong inhibition of the growth of H1N1 influenza A strains PR/8/34 or WS/33 in RAW 264.7 macrophage-like cells, A549 human lung epithelial-derived cells and murine bone marrow derived macrophages, but not MDCK canine kidney cells. Studies of the mechanism underlying this effect suggest that berberine acts post-translationally to inhibit virus protein trafficking/maturation which in turn inhibits virus growth. Berberine was also evaluated for its ability to inhibit production of TNF-α and PGE_2 from A/PR/8/34 infected-RAW 264.7 cells. Our studies revealed strong inhibition of production of both mediators and suggest that this effect is distinct from the anti-viral effect. Finally, we asked whether berberine-containing ethanol extracts of goldenseal also inhibit the growth of influenza A and production of inflammatory mediators. We found strong effectiveness at high concentrations, although upon dilution extracts were somewhat less effective than purified berberine. Taken together, our results suggest that berberine may indeed be useful for the treatment of infections with influenza A.

Keywords: Goldenseal | Berberine | Cytokines | Lipid mediators | H1N1 influenza A | Anti-viral

Article:

1. Introduction

Influenza A viruses are negative-sense, single stranded RNA viruses which belong to the Orthomyxoviridae family [1] and [2]. Each year, seasonal strains of influenza A cause significant morbidity and economic losses worldwide. The Centers for Disease Control estimates that approximately 23,000 people die annually in the United States from flu-related complications [3]. Typically, influenza A infects the tracheal and bronchial epithelial cells as well as alveolar macrophages resulting in localized cell damage and the induction of an acute host inflammatory response [4], which has been characterized as a "cytokine storm". This response is the cause of the symptoms associated with influenza A infections [5], [6], [7], [8], [9],[10] and [11] and, in addition, can lead to destruction of healthy tissue [12]. As a result, infected individuals display heightened susceptibility to additional bacterial and viral infections, which are generally the cause of morbidity and mortality associated with this virus [4].

Current treatments for influenza A infection include both pharmacological and non-pharmacological approaches. Two types of influenza A specific anti-viral drugs are currently available; M2 pump inhibitors such as amantadine and rimantidine, and neuraminidase inhibitors including zanamivir and oseltamivir. However, due to side effects and the emergence of drug-resistant strains [13], vaccination has become the dominant approach for control of this disease. Vaccines generally work effectively, but they are contraindicated in neonates and the elderly. Also, vaccine production is a complex process based on the predicted emergence of new seasonal variants.

Goldenseal (*Hydrastis canadensis*) is a plant that has been used for centuries in traditional medicine to treat a variety of conditions, including; skin and eye infections, upper respiratory disorders, diarrhea, and cancer [14]. Many of the effects of goldenseal have been attributed to the isoquinoline alkaloid berberine [15] which is also found in other plants such as barberry (*Berberis vulgaris*), coptis (*Coptis chinensis*), and Oregon grape (*Mahonia aquifolium*). Studies with berberine have revealed effects on a variety of cellular processes. Berberine has been shown to inhibit the growth of certain tumor-derived cell lines [16], [17], [18], [19] and [20] and to prevent the growth of certain bacteria [21], [22], [23], [24], [25] and [26]. Berberine can effectively inhibit the growth of several viruses [27], [28] and [29] including human cytomegalovirus and herpes simplex virus. Berberine has also been shown to inhibit production of cytokines, inflammatory lipids, and nitric oxide from macrophages treated with LPS [3] and [30]. Inhibition of cytokine production also occurs *in vivo* in mice treated with LPS, suggesting berberine has potential for the treatment of endotoxemia [30] and [31].

In this report we have investigated the effects of berberine on infections with influenza A *in vitro*. An abstract published 20 years ago [32], showed that berberine could inhibit the growth of influenza A on chicken allantoic membranes, but its effects with mammalian cells had not been tested. Therefore, we evaluated berberine for its anti-viral activity against several strains of H1N1 influenza A with a number of different murine and human cell types. We also tested

berberine for its ability to suppress production of TNF-α and PGE_2 from infected macrophages. Our experiments show strong inhibition of influenza A growth. We also found strong inhibition of TNF-α and PGE_2 production from infected macrophages. Similar results were seen with berberine-containing extracts of goldenseal. Taken together, these results suggest the alkaloid berberine and extracts of plants containing berberine may be useful for the treatment of influenza A.

2. Materials and methods

2.1. Cell lines, media and reagents

All cell lines were obtained from the American Type Culture Collection (Manassas, VA). RAW 264.7 cells were cultured in Dulbecco's modification of minimal essential medium (DMEM) with 4 mM l-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate with 10% fetal calf serum (FCS). A549 cells were cultured in Ham's F-12 nutrient medium with 4 mM l-glutamine and 1.5 g/L sodium bicarbonate with 10% FCS. Madin–Darby canine kidney (MDCK) cells were cultured in DMEM with 4 mM l-glutamine, 4.5 g/L glucose, and 3.0 g/L sodium bicarbonate and supplemented with 10% FCS, 0.2% BSA and 25 mM HEPES buffer. Media, berberine chloride, amantadine, and supplements were obtained from Sigma-Aldrich (St. Louis, MO) and Cellgro (Manassas, VA). FCS was obtained from Atlanta Biologicals (Atlanta, GA) and Gemini Bio-products (West Sacramento, CA). Cells were cultured at 37 °C and 5% CO_2.

2.2. Virus propagation and focus forming assays

A/PR/8/34 and A/WS/33 viruses were originally obtained from the American Type Culture Collection (Manassas, VA). Virus stocks were prepared by infecting MDCK cells at a multiplicity of infection (moi) of 0.001 (1 infectious particle per thousand cells). The virus was added to cells for 30 min in a small volume of serum free virus growth media (DMEM with 4 mM l-glutamine, 4.5 g/L glucose, and 3.0 g/L sodium bicarbonate and supplemented with 0.2% BSA, 2 μg/ml Trypsin-TPCK and 25 mM HEPES buffer) followed by the addition of fresh virus growth media and incubating for 36–48 h or until cells displayed 90+% cytopathic effect (CPE). Cell supernatants were collected, cell debris was removed by centrifugation (1000 rpm for 10 min), aliquots prepared, and stored at − 80 °C. For production of experimental supernatants, viruses were added to cells at either low (0.002) or high (5) moi following the same protocol. Berberine and/or extracts were added with the virus and supernatants were collected at the indicated time points.

Virus titers were determined using a focus forming assay (FFA). MDCK cells were seeded in 24 or 48 well plates and incubated overnight at 37 °C and 5% CO_2. Virus containing cell supernatants were added for 30 min followed by the addition of an overlay containing 1.2% tragacanth. Plates were incubated for 24 h; cell monolayers were washed with 1× PBS (Sigma, St. Louis, MO) and fixed with 1:1 acetone/methanol at − 20 °C overnight. The acetone/methanol was removed and plates were allowed to fully dry before blocking in 1% normal horse

serum/PBS for approximately 1 h. Primary mouse anti-HA monoclonal antibody (Fitzgerald, Acton, MA) was diluted 1:3000 in blocking buffer and incubated for 1 h (2.3 μg/ml final Ab concentration). Cell monolayers were washed three times with 1× PBS and incubated with 1:1000 diluted goat anti-mouse IgG-HRP conjugated secondary Ab (Sigma St. Louis, MO) for 30–45 min (0.8 μg/ml final Ab concentration). Foci were visualized with Vector VIP peroxidase substrate kit (Vector Burlingame, CA). Foci were enumerated using GelDoc XR (BioRad Hercules, CA) imaging software to determine focus forming units/ml. Titers derived from FFAs which are expressed as focus forming units (FFU) are equivalent to plaque forming units (PFU).

2.3. Preparation of bone marrow derived macrophages

C57BL/6 murine bone marrow-derived macrophages (BMDM) were obtained from the laboratory of Dr. Frank Scholle, PhD, Department of Microbiology, North Carolina State University. Cells were cultured in DMEM with 10% FCS, 30% L929 conditioned media, 1× l-glutamine and 1× non-essential amino acids. Virus inoculations to generate experimental supernatants were performed as in Section 2.2 using serum free medium supplemented with 2 μg/ml Trypsin-TPCK.

2.4. Immunoblot analysis

RAW 264.7 cells were plated at a cell density of 5×10^5 cells/60 mm tissue culture dish (Corning, Corning, NY) for 24 h, then infected at either low (0.002) or high (5) moi with the A/PR/8/34 virus in the absence or presence of 25 μM berberine as described above. At indicated times, cell monolayers were washed twice with cold phosphate buffered saline (PBS) (Gibco, Carlsbad, CA), lysis buffer added (50 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 0.5% SDS), lysates collected by scraping, and centrifuged for 30 min at 14,000 rpm. The protein concentration for each sample lysate was determined using the Pierce BCA system (Pierce, Rockford, IL). Equal protein samples (10 μg) were loaded on 4–12% Bis–Tris gels (Invitrogen, Carlsbad, CA) and subjected to electrophoresis using the Novex Mini-Cell System (Invitrogen). Following transfer to PVDF membranes, (Millipore, Billerica, MA) membranes were blocked for 24 h with 5% powdered milk in TBS/0.1% Tween-20 and probed with a primary goat anti-H1N1 polyclonal antibody (Fitzgerald, Acton, MA) for 1 h and secondary rabbit anti-goat HRP for 45 min (Southern Biotech, Birmingham, AL) diluted in 1% powdered milk in TBS/0.1% Tween-20. Bands were visualized using ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ).

2.5. Immunofluorescence

RAW 264.7 cells were plated in 8-well chamber slides (Lab-Tek II, Chamber Slide System, NUNC, Rochester, NY), incubated for 24 h, and then infected and/or treated with berberine as indicated above. Infections were allowed to proceed for indicated times then processed for immunofluorescence detection of viral hemagglutinin (HA) protein. Media was removed and

monolayers were rinsed with PBS 1× and then fixed with 10% formaldehyde for 20 min at room temperature. Fixative was removed and monolayers were rinsed with 1× with PBS. Cells were then permeabilized with 0.5% Triton X 100 (Sigma, St. Louis, MO) in PBS for 10 min at room temperature. Blocking buffer was added (2% BSA, 5% NHS, 10 mM glycine) for 1 h at room temperature followed by a 1 h incubation with a viral HA specific monoclonal antibody (mAb) (Fitzgerald Acton, MA). The primary mAb was removed, 2 additional PBS washes were performed, followed by addition of the secondary rabbit anti-mouse tetramethyl rhodamine isothiocyanate (TRITC) conjugated IgG secondary antibody. Staining was visualized using a Zeiss Axioscop 2 plus (Carl-Zeiss Oberkochen, Germany) microscope equipped with a SPOT camera (Diagnostic Imaging, Sterling Heights, MI). Images were captured using SPOT software and analyzed with Photoshop (Adobe, San Jose, CA) software.

2.6. ELISA

TNF-α and PGE_2 ELISA kits were purchased from eBioscience (San Diego, CA) and Enzo Life Sciences (Plymouth Meeting, PA). Assays were performed according to the manufacturer's recommendations. In each case, sample values were interpolated from standard curves. Optical density was determined using a PolarStar microplate reader (BMG Labtechnologies, Durham, NC).

2.7. Extract preparation and analysis of berberine content

Goldenseal (*H. canadensis* L. Ranunculaceae) roots were cultivated in a hardwood forest in western North Carolina, as described elsewhere [26], and a voucher was deposited at the Herbarium of the University of North Carolina (NCU583414). Three individual extracts were used in these studies; one prepared from a pooled sample of goldenseal roots, one from a pooled sample of goldenseal leaves, and one prepared from a single root sample. Extracts were prepared in a solvent of 50:50 ethanol:nanopure water at a ratio of 1 ml solvent: 5 g plant material. The extracts were analyzed for alkaloid content using LC-MS, as described in detail previously [26], and diluted to contain the indicated concentrations of berberine.

2.8. Cell viability assays

RAW 264.7 cells (1.5×10^5/well) were plated in 24-well tissue culture dishes in 1 ml DMEM and incubated for 24 h. Berberine (25 μM) or a goldenseal extract containing an equivalent berberine concentration was added and incubated for an additional 12 or 24 h. Cells were harvested with 0.25% Trypsin–EDTA (Sigma, St. Louis, MO) and counted by hemocytometer with a 0.04% solution of trypan blue. Cell viability was determined by calculation of the ratio of trypan blue stained cells to the number of total cells.

For DNA cleavage experiments, RAW 264.7 cells (5×10^6/dish) were plated in 100 mm tissue culture dishes and incubated for 24 h. Cells were treated with berberine or goldenseal extract as above and, in addition, a treatment with cycloheximide (100 μg/ml, Sigma, St. Louis, MO) was

used as a positive control for the induction of apoptosis. Following 24 h incubation, cells were harvested in lysis buffer (50 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 0.5% SDS) and digested with proteinase K overnight at 56 °C. Lysates were then extracted using phenol and chloroform and DNA precipitated with isopropyl alcohol. Resulting DNA was resuspended in TE buffer (10 mM Tris–Cl, pH 8.0. 1 mM EDTA) and treated with 1 μl of RNAse A (Sigma, St. Louis, MO) for 30 min at room temperature. DNA (5 μg) was separated by electrophoreses on a 2% agarose gel containing ethidium bromide and visualized using BioRad XR Gel documentation system (Bio-Rad Hercules, CA).

3. Results

3.1. Berberine inhibits the growth of H1N1 influenza A in RAW 264.7 cells and bone marrow derived macrophages

The effect of berberine on the growth of influenza A was first tested with the murine macrophage-like cell line RAW 264.7 and the influenza A strain PR/8/34, a mouse-adapted strain of influenza A that has been used extensively in studies of influenza A pathogenesis and vaccine production. Typically, the replication time for A/PR/8/34 is 6–8 h depending on cell type [33] and [34]. Fig. 1A shows the results of a typical time course experiment for the growth of this virus in RAW 264.7 cells where infections were performed at a low moi (0.002) to approximate conditions of infection *in vivo*. A total of 10^3 FFU of virus was added to 5×10^5 cells in a 1 ml culture. As shown in Fig. 1A, six h after the infection was initiated, levels of infectious virus in the culture supernatant had not increased; in fact only small amounts of virus were detectable likely representing residual inoculating virus. However, by 12 h we found that infectious virus reappeared in the culture supernatant and by 24 h levels of infectious virus had increased by 2–3 log units. Our experiments revealed that a concentration of 25 μM berberine strongly inhibited virus growth under these conditions (Fig. 1A). The increase in virus titer noted at 12 h was blocked completely and while at 24 h the level of inhibition was 90%. Based on these experiments, a series of dose–response curves was performed at the 24 h time point. As shown in Fig. 1B, near complete inhibition of the growth of A/PR/8/34 in RAW 264.7 cells by berberine occurred at concentrations above 1 μM, and the IC_{50} was 0.01 μM. For this virus, berberine was more effective than amantadine, a known anti-influenza compound that targets the M2 protein of influenza. Amantadine displayed an IC_{50} of 27 μM in these experiments, which is comparable to the IC_{50} reported previously (33 μM) [35]. The inhibitory effect of berberine on the growth of influenza A was also seen with a second H1N1 virus (WS/33) (IC_{50} = 0.44 μM) (Fig. 1B).

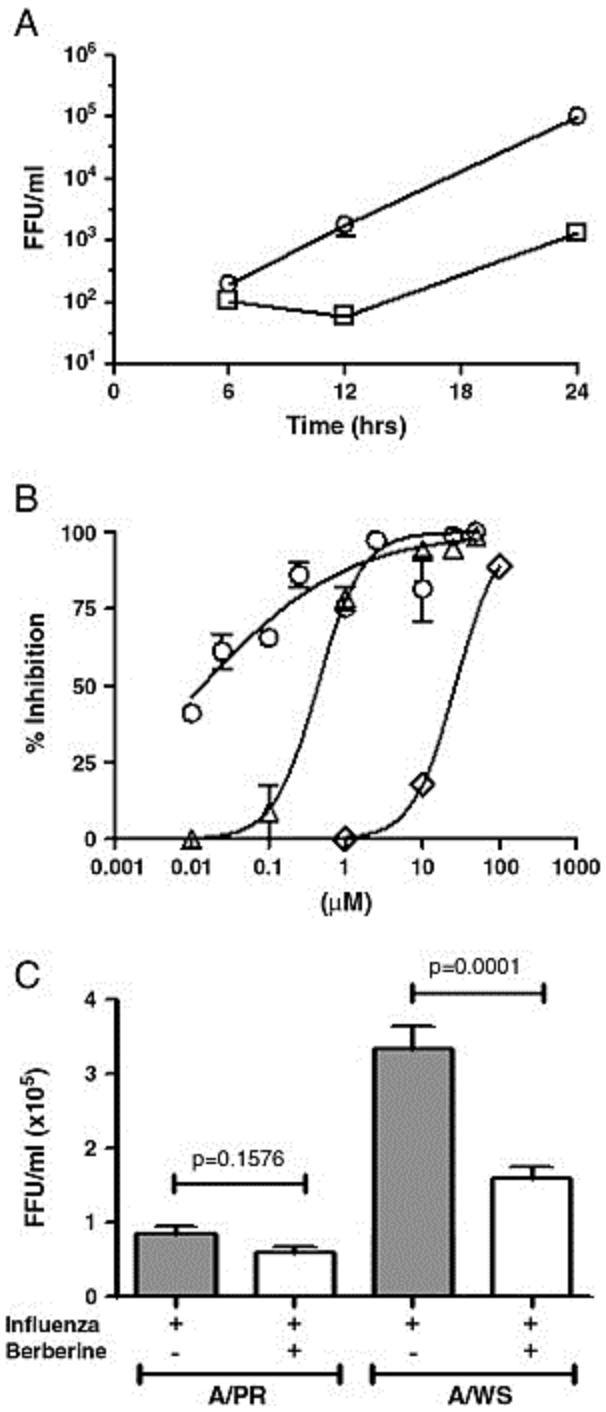

Fig. 1. Effects of berberine on the growth of influenza A in macrophage-type cells. (A) RAW 264.7 cells were infected with A/PR/8/34 (moi = 0.002, 1000 FFU) for varying times in the absence (○) or presence (□) of 25 μM berberine. Supernatants were collected and virus titers determined using a focus forming assay with MDCK cells. Values shown are means +/− S.E.M. of triplicate measurements of virus titers from a single representative experiment. (B) RAW 264.7 cells were infected with A/PR/8/34 (○) or A/WS/33 (Δ) at moi = 0.002 for 24 h in the presence or absence of varying doses of berberine. Titers were determined as described above

with % inhibition calculated by comparison with controls. Values shown are from three independent experiments with virus titers determined in duplicate in each experiment. Growth of strain A/PR/8/34 in the presence of amantadine (◊) is also shown. (C) Bone marrow derived macrophages infected with either A/PR/8/34 or A/WS/33 virus (moi = 0.002, 1000 FFU) in the absence or presence of 25 μM berberine for 48 h or 24 h, respectively. Values shown are means +/− S.E.M. from three independent experiments with virus titers determined in duplicate in each experiment.

The effect of berberine on the growth of influenza A was also tested with cultures of primary macrophages. Bone marrow derived murine macrophages (BMDM) were infected with strains A/PR/8/34 or A/WS/33 in the absence or presence of 25 μM berberine. Supernatants were harvested after 24–48 h and titers determined using the FFA. As shown in Fig. 1C, berberine inhibited the growth of both viruses, but that the effect was variable. With strain A/PR/8/34, which produced only 10^2 FFU after 48 h of growth (after subtracting inoculating dose); we measured 30% inhibition by berberine (not statistically significant). On the other hand, we found that strain A/WS/33 grew faster, and to higher titers in BMDM. This growth was inhibited 53% by berberine at 24 h, which was statistically significant ($p = 0.0001$).

3.2. Berberine inhibits the growth of influenza in A549 human lung epithelial cells but not MDCK cells

The effects of berberine were also tested with A549 human lung epithelial cells. These experiments were of interest because epithelial cells are a key host target cell for influenza A *in vivo*. As shown in Fig. 2A, berberine completely blocked the growth of strain A/PR8/34 with these cells (98% inhibition at 48 h), although we did note that this mouse-adapted viral strain grew poorly in this human cell type. Finally, we tested whether berberine could inhibit the growth of influenza A in the MDCK canine kidney cell line. This cell line is noted for its highly efficient replication of influenza A viruses (note the scale on the y-axis) [36],[37] and [38] and is used routinely in plaque and focus forming assays. As shown in Fig. 2B, although we did measure 39 and 32% growth inhibition with A/WS/33 and A/PR/8/34 strains, respectively, these values were not statistically significant.

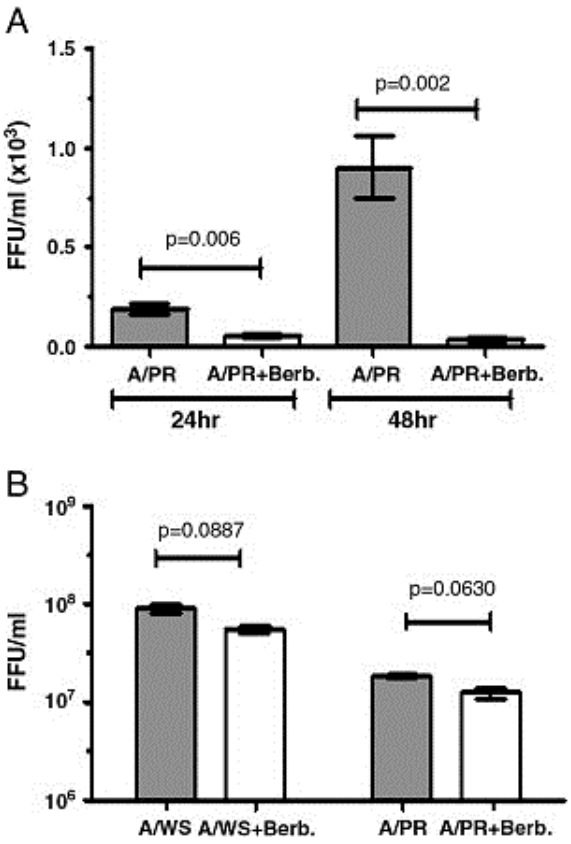

Fig. 2. Effects of berberine on the growth of influenza A in additional cell types. A) A549 human epithelial cells were infected with A/PR/8/34 (moi=0.002, 300 FFU) for 24 or 48 h in the absence or presence of 25 μM berberine. Supernatants were collected and viral titers determined using a focus forming assay with MDCK cells. B) MDCK cells were infected with influenza strains WS/33 or PR/8/34 (moi=0.002, 400 FFU) for 24 h in the presence or absence of 25 μM berberine. Values shown are means +/- S.E.M. from three independent experiments with virus titers determined in duplicate in each experiment.

3.3. Studies on the mechanism of viral growth inhibition by berberine

To gain insight into the mechanism of growth inhibition by berberine, we investigated its effects on the expression of several influenza A proteins using a polyclonal goat anti-H1N1 antiserum. Four proteins are typically recognized by Abs during influenza A infections, including HA (75 kDa), NA (58 kDa), NP (60 kDa), and M (25 kDa)[1]. As shown in Fig. 3A, in proteins prepared from cells infected at a low moi (0.002), this antiserum revealed three bands at the 12 and 24 h time points. Based on predicted molecular weights, the bands running at approximately 70 and 25 kDa are HA and M proteins, respectively, while the identity of the protein(s) running at approximately 60 kDa is not as clear. This band may represent either NA or NP proteins since under these SDS-PAGE conditions, their mass differences cannot be resolved. Fig. 3A also shows that treatment of infected cells with berberine strongly inhibited production of these proteins.

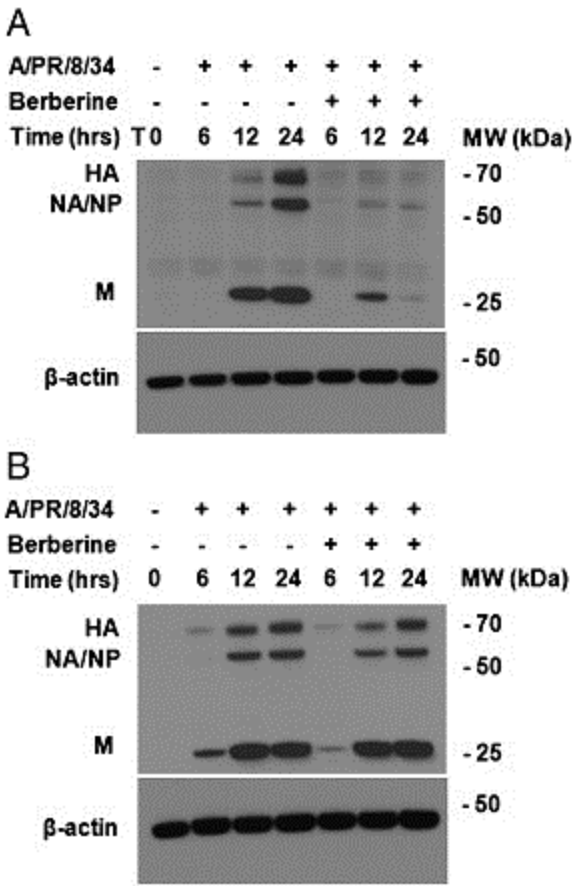

Fig. 3. Effects of berberine on expression of influenza A proteins. RAW 264.7 cells were infected with A/PR/8/34 at (A) low moi (0.002) or (B) high moi (5) for indicated times in the absence or presence of 25 μM berberine. Expression of influenza A proteins was detected by immunoblot assay using a polyclonal goat anti-H1N1 primary antibody and anti-goat-HRP secondary antibody. Identification of viral proteins based on predicted molecular weights. Blots were reprobed with mAb to β-actin to ensure equal loading of samples. Blots shown are representative of two experiments at each multiplicity of infection.

One possible interpretation of these data is that berberine is blocking virus protein production by inhibiting an early step in the virus replication pathway (i.e., entry, uncoating, transcription). Alternatively, it may be directly inhibiting the translation of viral proteins. However, since low multiplicity infections require several rounds of virus replication, release, and re-infection to achieve detectable levels of viral protein (or infectious virus) in the culture, it is also possible that berberine is inhibiting a later step in the replication pathway such as protein translocation or virus release. Inhibition of these processes would reduce the spread of the virus in culture and produce the same result as shown in Fig. 3A.

To address this question we also examined protein production in high multiplicity infections (moi = 5). Under these conditions, each cell is infected at the start of the experiment (super-infections do not occur with H1N1 influenza A [39]) and proceeds through the early stages of the

viral life cycle relatively synchronously with viral RNAs and proteins produced simultaneously by all cells in the culture. Release of progeny virus to fully infect the cells in culture is not required. As shown in Fig. 3B, under these conditions, berberine did not exert a strong effect on the production of any of proteins detected. This result indicates that the effects of berberine on viral protein production observed under low moi conditions (Fig. 3A) arose from inhibition of spread of the virus through the culture not from the inhibition of protein production by individual infected cells. The results shown in Fig. 3B also suggest that berberine is interfering with the growth of influenza A at a post-translational stage in the virus life cycle.

We addressed this hypothesis by examining intracellular trafficking of the influenza A HA protein, an important aspect of influenza A virus replication that occurs post-translationally. The influenza A HA protein follows a well defined translocation process through the endoplasmic reticulum (ER) and Golgi *en route* to the plasma membrane for the formation of virus particles [40]. To determine whether berberine interferes with this process, immunofluorescence experiments were performed using a mAb against the viral HA protein, in both low and high multiplicity infections. As shown in Fig. 4A, 20 h after low moi infections were initiated in the absence of berberine, HA displayed a pattern of cell surface expression with weak intracellular staining. As shown in Fig. 4B, we found that berberine treatment of similarly infected cells produced a change in the pattern of HA staining. Intracellular HA staining was more pronounced while surface staining was reduced. Berberine also caused enhanced intracellular HA staining in cells infected under high moi conditions (compare Fig. 4C and D). These results suggest that the effect of berberine on the growth of influenza A may stem from a post-translational effect on the intracellular movement and/or maturation of viral proteins.

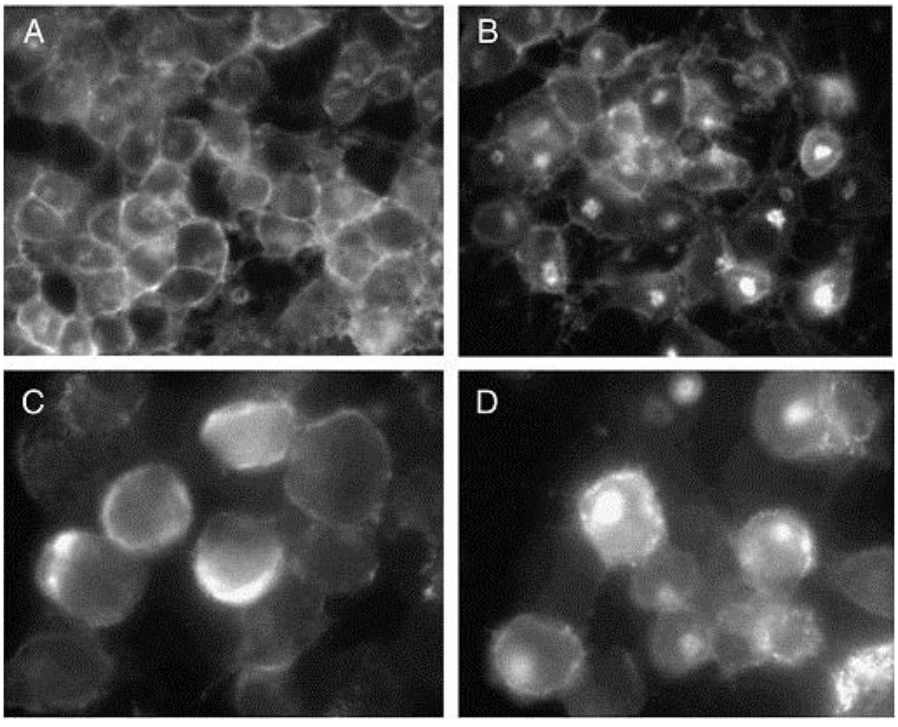

Fig. 4. Effects of berberine on HA protein localization. RAW 264.7 cells were grown on 8-well chamber slides and infected with strain A/PR/8/34 at low moi (0.002) for 20 h in the absence (A) or presence of 25 μM berberine (B). Cells were also infected with A/PR/8/34 at high moi (5) for 12 h in the absence (C) or presence of 25 μM berberine (D). Cells were fixed, permeabilized and HA protein detected using an anti-HA mAb followed by a TRITC-labeled goat anti-mouse secondary Ab. Staining was visualized using a Zeiss Axioskop 2 plus fluorescence microscope. Magnifications are 40× (A and B) and 100× (C and D). Images are representative of at least five experiments performed at each multiplicity of infection.

Finally, we considered the hypothesis that treatment with berberine is causing cell death and thereby reducing production of infectious virus or viral proteins. Previous studies with A549 cells [41] and THP-1 [42] macrophage-like cell lines have failed to find any cytotoxic effects with berberine. In agreement, as shown in Fig. 5A, we did not find any decrease in cell viability when RAW 264.7 cells were treated with 25 μM berberine or a goldenseal extract with an equivalent berberine concentration. In addition, we did not find any evidence of apoptosis, when assayed by DNA fragmentation, in RAW 264.7 cells treated by berberine or goldenseal extract with equivalent berberine concentration (Fig. 5B).

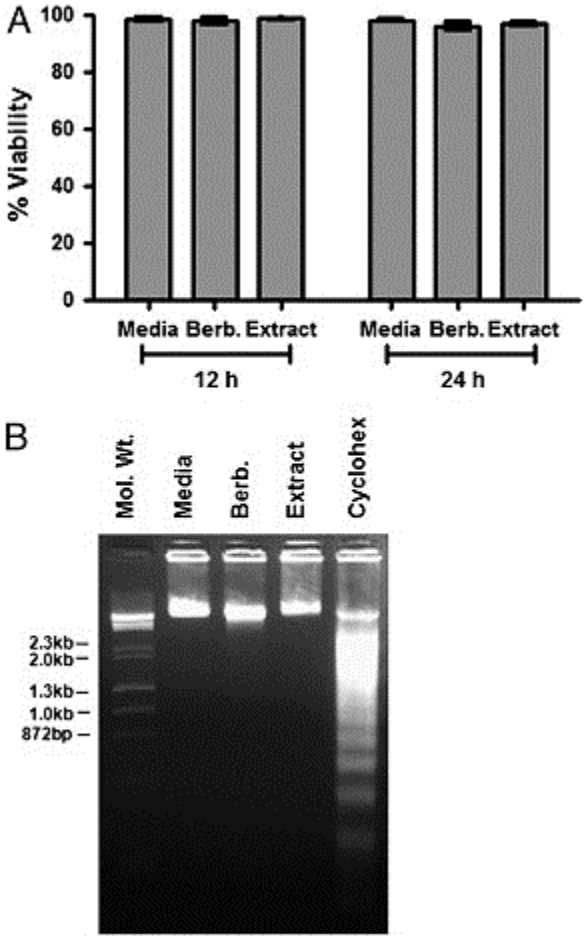

Fig. 5. Effect of berberine and goldenseal extract on cell viability. RAW 264.7 cells were treated with media, 25 μM berberine, or the goldenseal root extract containing an equivalent berberine concentration. (A) Following 12 and 24 h incubation periods cells were harvested and viable cells counted by staining with trypan blue. (B) Identically treated cultures were harvested; DNA isolated as indicated in the Materials and methods, and separated on 2% agarose gels. A treatment with 100 μg/ml cycloheximide was also included as a positive control for induction of apoptosis. Results shown are representative of four independent experiments.

3.4. Berberine inhibits influenza-induced production of TNF-α and PGE_2

In these experiments, we evaluated the effects of berberine on the ability of influenza A to induce inflammatory mediators from infected macrophages. We focused on TNF-α, one of the major pro-inflammatory cytokines associated with influenza pathogenesis; [43], [44], [45] and [46] and PGE_2, which recent reports suggest is also responsible for many of the symptoms associated with infections by influenza A [47] and [48]. RAW 264.7 cells were infected with strain A/PR/8/34 and levels of TNF-α and PGE_2 in culture supernatants were determined by ELISA. In preliminary experiments with low moi infections, we found that levels of inflammatory mediator production were low and inconsistent. Therefore, in these experiments, infections were performed at an moi of 5. As shown in Fig. 6A and B, we found that cells infected in this manner produced consistent high levels of TNF-α and PGE_2, respectively. Both mediators were readily detected at both 12 and 24 h time points. In contrast, in the presence of 25 μM berberine, we found strong, significant inhibition of both TNF-α and PGE_2 (Fig. 6A and B) ($p < 0.05$) at both 12 and 24 h time points.

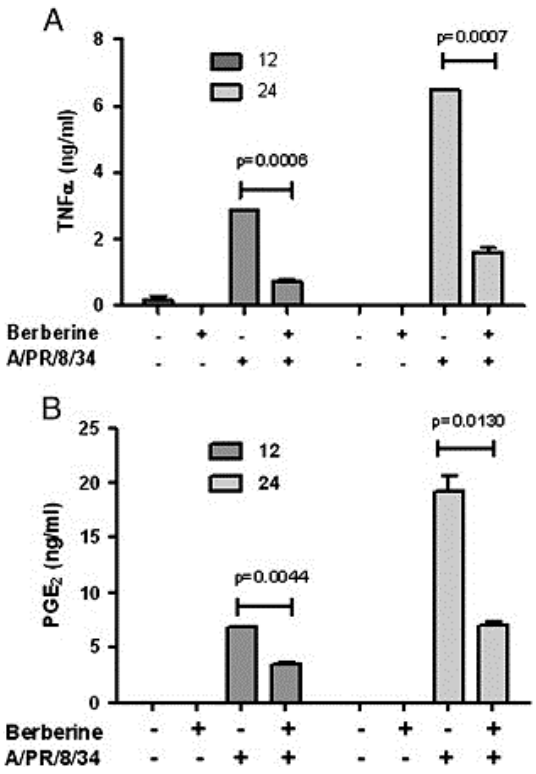

Fig. 6. Effect of berberine on the production of TNF-α and PGE_2 from RAW 264.7 macrophage-like cells. RAW 264.7 cells were infected with strain A/PR/8/34 (moi = 5) 12 or 24 h in the presence or absence of 25 μM berberine. Cell supernatants were collected and levels TNF-α (A) or PGE_2 (B) determined using commercial ELISA kits. Values shown are means +/− S.E.M. from two independent experiments with mediator determinations performed in duplicate in each ELISA assay.

3.5. Goldenseal extracts can inhibit influenza growth and block the production of TNF-α and PGE_2

Previous results from our laboratories have shown that the activity of botanical compounds may differ when tested in purified form or as components of crude extracts [49]. Therefore, we tested whether berberine-containing extracts of goldenseal also display anti-viral and anti-inflammatory activity. Samples of goldenseal were collected, extracts produced, and levels of berberine measured by LC-MS [26]. As shown in Fig. 7A, with extract dilutions containing greater than a 2.5 μM concentration of berberine, we found complete suppression of virus growth, equivalent to the effect of purified berberine. However, when extracts were diluted to contain a concentration of 0.25 μM berberine, we found that the extracts were not as effective as purified berberine. As a result, IC_{50} values for extracts were greater than for purified berberine. The *H. canadensis* root extract displayed an IC_{50} value of 0.22 μM, while an extract produced from *H. canadensis*leaves displayed an IC_{50} of 0.40 μM. Finally, a dilution of a root extract with a concentration of 25 μM berberine was tested for its ability to suppress production of inflammatory mediators. As shown

in Fig. 7B and C, the root extract strongly inhibited production of TNF-α and PGE_2, at levels similar to those seen with purified berberine (see Fig. 6).

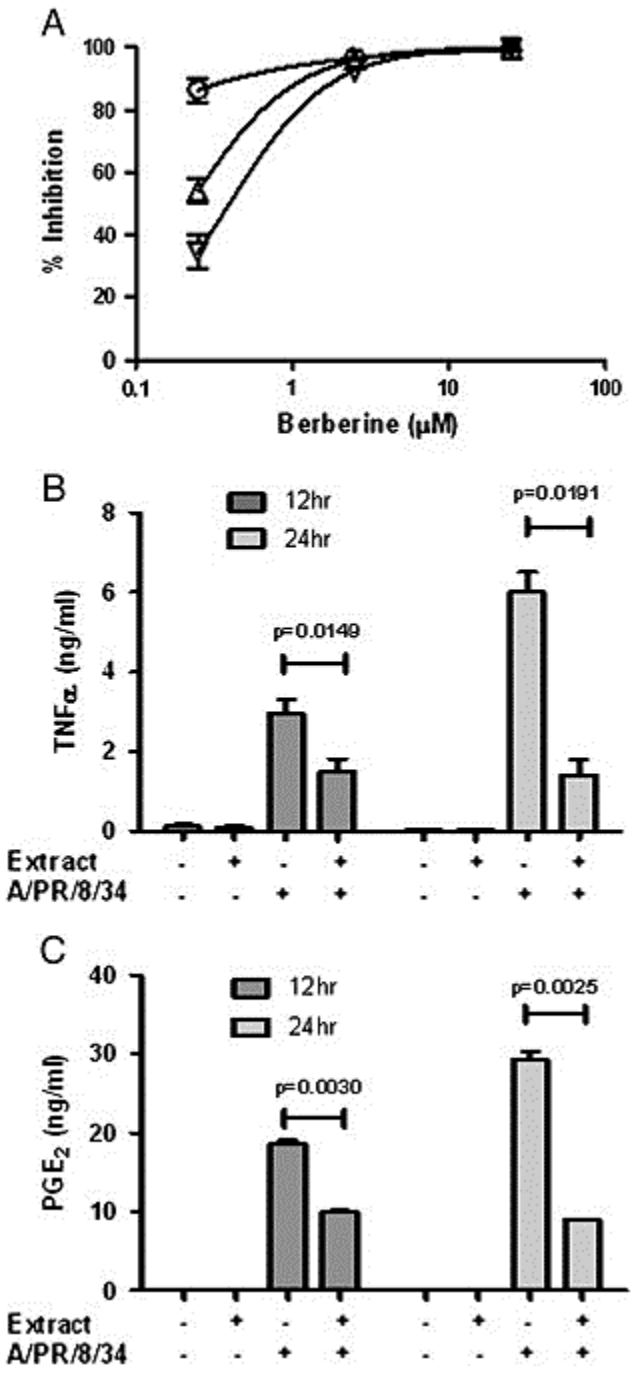

Fig. 7. Effect of goldenseal extracts on the growth of influenza A and the influenza A-induced production of TNF-α and PGE_2. (A) RAW 264.7 cells were infected with strain A/PR/8/34 (moi = 0.002) for 24 h in the absence or presence of 25 μM berberine (○) or a dilution of pooled root (Δ) or leaf (∇) extracts containing equivalent berberine concentrations. Culture supernatants were collected and virus titers determined using a focus forming assay with MDCK cells.

Percent inhibition of viral growth was calculated as in Fig. 1. Values shown are means +/− S.E.M. from three independent experiments with virus titers determined in duplicate in each experiment. (B and C) RAW 264.7 cells were infected with strain A/PR/8/34 (moi = 5) for 24 h in the absence or presence of a dilution of a single root goldenseal extract containing a 25 μM concentration of berberine. Levels of TNF-α (B) or PGE_2 (C) were determined by ELISA. Values shown are means +/− S.E.M. from 5 (12 h) or 2 (24 h) independent experiments with mediator determinations performed in duplicate in each ELISA assay.

4. Discussion

Our investigations have revealed that berberine can inhibit the growth of influenza A *in vitro* with two different H1N1 strains of influenza A. This effect was observed with two murine macrophage type cells (RAW 264.7 macrophage-like cells and normal bone marrow derived macrophages) and the A549 human lung epithelial-derived cell line. Dose response curves with the A/PR/8/34 virus growing on RAW 264.7 cells revealed several orders of magnitude more inhibition by berberine than with amantadine, a well characterized M2 inhibitor. The A/PR/8/34 virus is known to be relatively resistant to amantadine [50] and its susceptibility to berberine may indicate that berberine is working through a distinct mechanism. In addition, we showed that berberine can inhibit the influenza A-induced production of TNF-α and PGE_2 from RAW 264.7 macrophages.

Berberine has been shown to exert a large number of effects on cellular machinery; including the reduction of F-actin polymerization [51], moderation of lipid and glucose metabolism [52], direct binding to polyadenylic acid [53], and modulation of MAP family kinase activity [54]. To gain insight into the effect of berberine on the growth of influenza A, we focused our studies on the production of viral proteins. We reasoned that finding inhibitory effects on protein production in high multiplicity infections would allow us to concentrate future studies on early events in the viral replication cycle, such as entry or transcription of viral genes. Conversely, a lack of effect of berberine on protein production would allow us to shift our focus to later events in the replication cycle. Our experiments with high moi infections did not reveal an effect of berberine on the expression of several different viral proteins, suggesting that berberine is not affecting the entry and uncoating of the virus or transcription and translation of viral mRNAs. Berberine also likely does not interfere with production of viral genomic RNAs (vRNA, cRNA), since these molecules are produced prior to mRNA synthesis. On the other hand, we did detect a change in the intracellular position of HA in infected cells that were treated with berberine. The HA protein did not display its normal pattern of cell surface staining. Instead, a more punctate intracellular pattern was observed. It is possible, therefore, that berberine is acting to block the intracellular translocation as HA as it progresses toward the cell surface. Alternatively, berberine may be causing HA to misfold, and the punctate intracellular staining we observed arises from accumulations of HA targeted for degradation. Finally, we did not observe any evidence of berberine-induced cell death.

Interestingly, we did not find a significant inhibitory effect of berberine on growth of influenza A with the MDCK cell line. This cell line is used extensively *in vitro* for the growth of influenza A, and produces many orders of magnitude more virus than any of the other cells we have tested (Fig. 2B). It is possible, therefore, that the changes in this cell that allow for highly efficient production of influenza A also enable it to be resistant to the effects of berberine. MDCK cells are, for example, more efficient in the folding, maturation, and subsequent transport of HA and NA proteins, and it is these changes that may endow them with resistance to berberine [55]. The inability of berberine to inhibit influenza A growth in MDCK cells may also support a role for the host interferon response in the effect of berberine. Seitz et al. have shown that the canine interferon response is ineffective against influenza A [56]. Finally, it is also possible that these cells are resistant to all effects of berberine because they have elevated levels of an MDR protein responsible for berberine efflux. This type of berberine resistance is common among tumor derived cell lines [57] and [58].

In addition to the effects of berberine on the growth of the virus, we found that berberine could strongly inhibit production of TNF-α and PGE_2, two mediators linked to the symptoms and pathology associated with infections by influenza A. Activation of the host innate response to influenza A has been studied extensively [59] and [60]. These studies have shown that recognition of influenza A v- and cRNA by host (TLR)-3, -7, -8, RIG-I/MDA5, and NLRs is the key to initiating this response [61], [62], [63] and [64]. Collectively, activation of these TLR pathways leads to activation of a number of different transcription factors including NF-κB, IRF-3, IRF-7, AP-1 and IKKε [65], [66], [67], [68] and [69]. The inhibitory effects of berberine on mediator production could arise at any number of points in these pathways. Production of a viral RNA ligand could be blocked, although this is unlikely since our experiments were conducted under high moi conditions, where all cells will contain high levels of viral RNAs and proteins (which we confirmed). It is also possible that berberine is interfering with ligand recognition by one of the TLRs. As noted above, berberine has been shown to bind polyadenylic acid [53], [70] and [71], and it may be that berberine-bound influenza A RNAs do not effectively trigger signaling though one or more TLR pathways. It is also possible that berberine is acting downstream in the TLR pathways, preventing activation of key transcription factors. Berberine has been shown to block the activity of a number of kinases [54], [72], [73] and [74], several of which are known to regulate the activity of pro-inflammatory transcription factors.

The results of our experiments suggest that berberine may be useful for the treatment of influenza A. Since berberine is derived from a natural product, many individuals may prefer to use a berberine-containing plant extract rather than the purified compound. Therefore, we sought to determine whether goldenseal extracts containing berberine also exert the anti-viral and anti-inflammatory effects. The results of our investigation revealed that at high concentrations, the extracts inhibited the growth of influenza A at levels that would be predicted from their berberine concentration. However, in more dilute samples; the extracts were less effective than would have been predicted from their berberine concentration. The molecular basis for this

finding is not clear. It is possible that the extracts contain other compounds that reduce the available concentration of berberine effectively causing a shift in the dose response curve. Alternatively, the extracts may contain compounds that counteract the effects of berberine on the infected cell.

In summary, we have shown that berberine can effectively inhibit the growth of two H1N1 strains of influenza A with a number of different cell types. We have also shown that berberine can inhibit the production of TNF-α and PGE_2 from infected RAW 264.7 cells. Experiments using animal models will be necessary to determine whether these effects are also seen *in vivo*. However, given that berberine has proven effective for suppressing acute inflammation in mice treated with LPS [31], there is no *a priori* reason why it would not be effective for the treatment of influenza A infections *in vivo*.

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