

# Methadone enhances human influenza A virus replication

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## ABSTRACT

Growing evidence has indicated that opioids enhance replication of human immunodeficiency virus and hepatitis C virus in target cells. However, it is unknown whether opioids can enhance replication of other clinically important viral pathogens. In this study, the interaction of opioid agonists and human influenza A/WSN/33 (H1N1) virus was examined in human lung epithelial A549 cells. Cells were exposed to morphine, methadone or buprenorphine followed by human H1N1 viral infection. Exposure to methadone differentially enhanced viral propagation, consistent with an increase in virus adsorption, susceptibility to virus infection and viral protein synthesis. In contrast, morphine or buprenorphine did not alter H1N1 replication. Because A549 cells do not express opioid receptors, methadone-enhanced H1N1 replication in human lung cells may not be mediated through these receptors. The interaction of methadone and H1N1 virus was also examined in adult mice. Treatment with methadone significantly increased H1N1 viral replication in lungs. Our data suggest that use of methadone facilitates influenza A viral infection in lungs and might raise concerns regarding the possible consequence of an increased risk of serious influenza A virus infection in people who receive treatment in methadone maintenance programs.

**Keywords** buprenorphine, cytokine, influenza virus, methadone, morphine.

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## INTRODUCTION

Morphine is the principal component of heroin and is a widely abused opioid. Chronic use of heroin leads to drug craving and dependence, associated with health and social problems causing global concerns (Veilleux *et al.* 2010). Methadone, a slow-acting opioid agonist, has been commonly and effectively used for treatment in opioid addicts. Epidemiological studies have demonstrated that methadone maintenance treatment reduces transmission of immunodeficiency virus (HIV) or hepatitis C virus (HCV) through less needle sharing (Caplehorn & Ross 1995; Veilleux *et al.* 2010). However, the effectiveness of methadone in reducing HIV incidence among the injecting drug users is confounded by its ability to enhance HIV replication *in vitro* (Li *et al.* 2002). Other opioid agonists (i.e. morphine or heroin) also increase

susceptibility to HIV or HCV (Roy *et al.* 2006; Tennant & Moll 1995), suggesting that a common mechanism may be involved. Furthermore, morphine up-regulates HIV entry coreceptors and down-regulates the expression of antiviral factors in human cells, which may increase HIV and HCV replication (Guo *et al.* 2002; Li *et al.* 2007; Peterson *et al.* 1990). Taken together, these data suggest that methadone and other opioids facilitate infectivity of HIV and HCV. The interaction of methadone with other commonly seen viruses has not been well characterized.

Influenza A virus infection is the most common cause of respiratory illness in man, affecting about 10 percent of the population annually. Three influenza pandemics have resulted in mortality of millions of people in the last century (Kitler *et al.* 2002; Potter 2001). Several reports have also indicated human lethality after zoonotic

transmissions of animal influenza viruses. Unlike HIV and HCV, influenza A virus is mainly transmitted through airborne droplets to respiratory tract. The prevalence of influenza infection in injecting drug users is thus not subject to the reduction of needle sharing during methadone treatment.

This study was undertaken to examine the replication of influenza A virus in human lung epithelial cells and adult mice after methadone treatment. We report that methadone selectively enhances influenza A virus replication. Our data support the assumption that the use of methadone may facilitate influenza A viral infection in lungs, which may be critical for patients receiving methadone maintenance therapy.

## MATERIALS AND METHODS

### Cell lines and virus

Human lung epithelial A549 cells (BCRC-60074, Bioresource Collection and Research Center, Taiwan), Madin–Darby canine kidney (MDCK) cells and human neuroblastoma SH-SY5Y cells (CRL-2266, ATCC) were grown as monolayers in Dulbecco's modified eagle's medium (DMEM) supplemented with 10 percent (v/v; 15 percent for SH-SY5Y cells) heat-inactivated fetal bovine serum, antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), and a non-essential amino acid mixture (0.1 mM). All cell lines were maintained at 37°C in a humidified incubator with 5 percent CO<sub>2</sub>. The human influenza A/WSN/33 (H1N1) wild-type virus and MDCK cell line (Hale *et al.* 2009) used in this study were kindly provided by Prof. Richard E. Randall (University of St. Andrews, UK). To propagate influenza A virus, confluent MDCK cells ( $7 \times 10^6$  cells per 75 T flask) were inoculated with the virus ( $7 \times 10^3$  plaque-forming units, PFU) at a multiplicity of infection (MOI = the ratio of the virus number to the target cell number) of 0.001 PFU/cell in 10 ml serum-free DMEM containing N-acetyl tryptin (2 µg/ml; T6763, Sigma-Aldrich, St. Louis, MO, USA) at 37°C (Chen *et al.* 2012; Jackson *et al.* 2008). The culture medium was harvested at 48 hours post-infection. Cell debris was removed by centrifugation at  $1000 \times g$  for 5 minutes at room temperature, and virus progenies in the supernatant were titrated by plaque assay on MDCK cells, as described in the section on 'Plaque assay' of Materials and Methods. Unless otherwise indicated, all reagents were purchased from Invitrogen Corporation.

### Chemicals

Buprenorphine hydrochloride (Siegfried Ltd., Switzerland), methadone hydrochloride (US Pharmacopeia) and morphine hydrochloride (National Bureau of Controlled Drugs, Department of Health, Taiwan) were obtained as

lyophilized powders. Each of these drugs was dissolved in phosphate-buffered saline (PBS) at appropriate stock concentrations (buprenorphine, 0.01 M; morphine, 0.1 M; methadone, 0.1 M), sterilized by filtering through membrane filters with a pore size of 0.2 µm, and stored at –20°C until use.

### Plaque assay

Confluent MDCK cells grown in six-well plates were washed once with Dulbecco's phosphate-buffered saline (DPBS) and then inoculated with 10-fold serially diluted viral suspensions in 1 ml of DMEM at 37°C, with gentle agitation for 1 hour. After replacing the inoculation medium with 2 ml of the warm overlay medium [DMEM supplemented with 1% (w/v) agarose (50080, Lonza, Allendale, NJ, USA) and 2 µg/ml N-acetyl tryptin] for each well, plates were left at room temperature for 30 minutes to solidify the overlay medium, and cells were then incubated at 37°C for 3 days, with plates inverted. To visualize the plaques, cells were fixed with 4 percent (v/v) formaldehyde (1.04003.1000, Merck, Kenilworth, NJ, USA) in PBS for 2 hours and then incubated with staining buffer [composed of 0.05 percent (w/v) crystal violet (C3886, Sigma-Aldrich, St. Louis, MO, USA), 20 percent (v/v) ethanol and 1 percent (v/v) methanol in distilled water] at room temperature for 30 minutes, followed by a brief wash in running tap water (Chen *et al.* 2012). Plaques were photographed and counted, and the virus titer was expressed as plaque forming units per milliliter (PFU/ml). In the current study, this standard plaque assay was applied to determine the titer of influenza A virus used for inoculation and collected from infected cells or animals.

### Influenza virus attachment assay

A549 cells were seeded ( $2.5 \times 10^4$  cells/cm<sup>2</sup>) in six-well plates and grown overnight before being treated with methadone (10 µM) or left untreated at 37°C for 24 hours, followed by inoculation with influenza A/WSN/33 virus ( $3 \times 10^6$  PFU/well) in serum-free DMEM supplemented with or without methadone (10 µM), with gentle agitation at 37°C. The unattached virus was collected from the inoculation medium at different time points (15, 30, 60, 120 and 180 minutes post-inoculation) and then subjected to plaque assay in MDCK cells as described earlier. The results are given as means ± standard deviation of three replicates and expressed as percentages of unattached virus titer relative to initial input virus titer; the reduced percentage reflects less unattached virus left in the inoculation medium and indicates increased virus attachment.

### Viral growth analysis

A549 cells were seeded ( $2 \times 10^4$  cells/cm<sup>2</sup>) in 25-T flasks and grown at 37°C for 24 hours, followed by the treatment with morphine (0.1, 1, 10 μM), methadone (0.1, 1, 10 μM) or buprenorphine (1, 10, 100 nM) in the growth medium at 37°C for 24 hours. The doses of opioids for cell culture were chosen based on the serum concentrations found in heroin addicts (Aderjan *et al.* 1995; Piekoszewski *et al.* 2001) and clinical patients receiving long-term maintenance treatment (Eap *et al.* 2000; Greenwald *et al.* 2003; Hallinan *et al.* 2006). Cells were washed twice with DPBS and then infected with influenza A/WSN/33 (H1N1) virus at an MOI of 0.001 in serum-free DMEM containing N-acetyl trypsin (2 μg/ml) and the corresponding drug at indicated concentrations. The viral progenies were harvested from culture media at 48 hours post-infection and stored at -80°C until virus titers were determined by plaque assay as described earlier.

### Immunofluorescence staining

The following procedures were performed at room temperature unless otherwise stated. At 24 hours post-infection, human lung epithelial A549 cells grown on 12-mm glass coverslips were washed twice with PBS, fixed with 4 percent formaldehyde (33220, Sigma-Aldrich, St. Louis, MO, USA) in PBS for 10 minutes and permeabilized with 0.3 percent Triton X-100 in PBS for 10 minutes. The cells were incubated with 4 percent bovine serum albumin (BSA; 10857, USB Corp., Cleveland, OH, USA) in PBS to block non-specific binding of the antibodies and stained with a mouse monoclonal antibody (1:1000; sc-101352, Santa Cruz, Dallas, TX, USA) against the nucleoprotein (NP) of human influenza A virus for 1 hour. After washing with PBS three times for 5 minutes each, cells were incubated with an Alexa-fluor-488-conjugated goat polyclonal antibody (1:1000; A11029, Invitrogen, Carlsbad, CA, USA) against mouse IgG for 30 minutes, followed by washing with PBS three times for 5 minutes each. The cells were fixed again with 4 percent formaldehyde for 10 minutes and washed with distilled water once. The coverslips were then mounted on glass slides by using a mounting medium (H-1200, Vector Laboratories, Burlingame, CA, USA), which contains 4', 6-diamidino-2-phenylindole (1.5 μg/ml) for localizing the nucleus; cells were then examined and fluorescence images were obtained by using an Axiovert 200M microscope system (Carl Zeiss, Oberkochen, Germany). NP-positive cells were counted from six microscopic fields with >90 percent cell confluence, as described previously (Chen *et al.* 2012). As a control for immunocytochemistry, the secondary antibody was used to

stain influenza A virus infected and naive A549 cells with omission of the primary antibody, and no obvious fluorescence signal was detected under microscopic examination.

To determine whether A549 and SH-SY5Y cells express mu-opioid receptors, cells were grown on 12-mm glass coverslips at 37°C for 24 hours. For SH-SY5Y cells, glass coverslips were pre-coated with both poly-L-lysine (12.5 μg/ml; 354210, BD Biosciences, San Jose, CA, USA) and laminin (12.5 μg/ml; 354232, BD Biosciences, San Jose, CA, USA) in 400 μl DMEM for 1 hour. The cells were fixed with 4 percent paraformaldehyde (15710, EMS) in PBS and then stained with a rabbit polyclonal antibody (1:50; sc-15310, Santa Cruz, Dallas, TX, USA) against mu-opioid receptor and a DyLight-488-conjugated goat polyclonal antibody (1:1000; GTX76756, GenTex, Hsinchu, Taiwan) against rabbit IgG, following the staining procedures described previously. As a control for immunocytochemistry, the secondary antibody was used to stain A549 and SH-SY5Y cells with omission of the primary antibody, and no obvious fluorescence signal was detected under fluorescence microscopic examination.

### Western blotting

Unless otherwise indicated, the following procedures were performed at room temperature as reported previously (Chen *et al.* 2012). The infected and uninfected A549 cells were washed twice with PBS, and lysed in 2× Laemmli buffer (containing 4 percent SDS, 125 mM Tris-HCl pH 6.8, 10 percent β-mercaptoethanol, 20 percent glycerol and 0.004 percent bromophenol blue in distilled water). Whole cell lysates were passed through a 25-gauge needle several times to reduce the viscosity, heated at 90°C for 10 minutes and then separated by electrophoresis on 12 percent SDS-polyacrylamide gels. The separated proteins were electrophoretically transferred to polyvinylidene fluoride membranes (RPN303F, GE Healthcare, Pittsburgh, PA, USA) and then subjected to immunoblotting by using appropriate antibodies. The membranes were incubated with blocking buffer [containing 5 percent skim milk (70166, Sigma-Aldrich, St. Louis, MO, USA) and 0.1 percent Tween-20 in PBS] for 1 hour and then incubated with the target-specific primary antibody at an appropriate dilution in 10 ml blocking buffer for 2 hours with gentle agitation. After washing with 0.1 percent Tween-20 (in PBS) three times for 10 minutes each, the membranes were incubated with the secondary antibody (specific to the immunoglobulin isotype of the primary antibody) at an appropriate dilution in 10 ml blocking buffer for 1 hour, followed by the washing procedure as described earlier. Note, for the primary antibody specific to tyr-701 phospho-STAT1,

the skim milk content of blocking buffer was replaced with BSA, and the incubation with primary antibody was performed at 4°C overnight. The target proteins on the membrane were detected by using an enhanced chemiluminescence reagent (RPN2106, GE Healthcare, Pittsburgh, PA, USA), and the intensity of the generated images on X-film (NEF596, Kodak, Boston, MA, USA) was quantified by ImageQuant TL software (GE Healthcare, Pittsburgh, PA, USA).

The primary antibodies used here include three mouse monoclonal antibodies against cellular actin (1:20 000; MAB1501, Millipore, Billerica, MA, USA), influenza A virus NS1 protein (1:2000; sc-130568; Santa Cruz, Dallas, TX, USA), influenza A virus M1 protein (1:2000; GTX76107, clone GA2B, GeneTex, Hsinchu, Taiwan), two rabbit monoclonal antibodies against STAT1 (1:2000; EPYR2154, Epitomics, Burlingame, CA, USA), tyr-701phospho-STAT1 (1:2000; 9167, clone 58D6, Cell Signaling, Danvers, MA, USA) and one rabbit polyclonal antibody against cellular MxA protein (1:4000; GTX110256, GeneTex, Hsinchu, Taiwan). Two horseradish peroxidase-conjugated goat polyclonal antibodies against mouse IgG (GTX213111-01, GeneTex, Hsinchu, Taiwan) and rabbit IgG (GTX213110-01, GeneTex, Hsinchu, Taiwan) were used as secondary antibodies.

#### Cytotoxicity test

The effects of morphine, methadone and buprenorphine on the viability and proliferation activity of human lung epithelial A549 cells were determined by trypan blue dye exclusion and MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assays. For the trypan blue dye exclusion assay, A549 cells were seeded ( $1 \times 10^4$  cells/cm<sup>2</sup>) in 24-well plates and grown at 37°C for 24 hours, followed by replacing the culture medium with fresh medium supplemented without (as control) or with the tested drugs at various concentrations (morphine: 0.1 μM ~ 5 mM; methadone: 0.1 μM ~ 5 mM; buprenorphine: 1 nM ~ 50 μM) at 37°C every 24 hours for 72 hours; after that, cells were harvested by trypsinization and stained with 0.4 percent trypan blue dye (T8154, Sigma-Aldrich, St. Louis, MO, USA). Each drug treatment had three replicates, and the assay was performed three times separately. The positively stained and negatively stained cells were counted three times as dead and viable cells, respectively, by using a hemocytometer under a light microscope. The relative cell survival rate was calculated by using the formula: [(number of viable counts in the drug-treated group) / (number of viable counts in the control group)] × 100 percent (Chen *et al.* 2012).

For the MTS assay, cells were seeded ( $1 \times 10^4$  cells/cm<sup>2</sup>) in 96-well plates and grown at 37°C for 24 hours before the treatment with tested drugs as described

earlier. Each drug treatment had three replicates, and the assay was performed three times separately. The activity of dehydrogenases in metabolic active cells was measured by using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (G3580, Promega, Madison, WI, USA), according to the manufacturer's instruction. Briefly, 20 μl of the MTS reagent was added to each well (containing 100 μl of culture medium) of the assay plate; cells were then incubated at 37°C for 2 hours, and the absorbance at 490 nm was measured using a microplate reader (SpectraMax M2e, Molecular Devices, Sunnyvale, CA, USA). In principle, the conversion of MTS salts into colored formazan products is accomplished by NADPH or NADH produced by cellular dehydrogenases; therefore, the quantity of formazan is directly proportional to the proliferation activity of viable cells and indirectly reflects viable cell numbers.

#### Infection-reduction assay

A549 cells were seeded ( $2 \times 10^4$  cells/cm<sup>2</sup>) in 25-T flasks and grown at 37°C for 24 hours, followed by the treatment with different opioids at various concentrations (morphine: 0.1, 1, 10 μM; methadone: 0.1, 1, 10 μM; buprenorphine: 1, 10, 100 nM) in the growth medium at 37°C for 24 hours. After that, cells were washed twice with DPBS and then infected with influenza A/WSN/33 (H1N1) virus at an MOI of 0.001 in serum-free DMEM supplemented with different opioids at corresponding concentrations. At 16 hours post-infection, the collected condition media were centrifuged at 1000 × g for 10 minutes, and the supernatants were exposed to ultraviolet light for 20 minutes (to inactivate infectious virus particles) and then stored at -80°C until use. A549 cells were seeded at a density of  $4 \times 10^4$  cells/cm<sup>2</sup> and grown on 12-mm glass coverslips in 24-well plates at 37°C for 24 hours. The cells were left untreated, or incubated with interferon β (IFNβ) (1000, and 100 U/ml; cat. no. 300-02BC; PeproTech) in growth medium or with the collected conditioned media (1-fold and 0.1-fold) at 37°C for 24 hours, followed by infection with influenza A virus at an MOI of 0.1 PFU/cell in the absence of trypsin (single-cycle growth). At 24 hours post-infection, cells were subjected to immunofluorescence staining for the viral NP, and NP-positive cells were counted as described earlier in the section on Immunofluorescence staining.

#### Reverse transcription polymerase chain reaction and quantitative real-time reverse transcription polymerase chain reaction analyses

The expression of opioid receptor genes in human cell lines was detected by reverse transcription polymerase chain reaction (RT-PCR) analysis. Total cellular RNA was extracted

from human lung epithelial cells (A549,  $10^6$  cells) and human neuroblastoma cells (SH-SY5Y,  $10^6$  cells) by using RNeasy mini kit (74104, Qiagen, Germantown, MD, USA). A universal human RNA extract (740000, Stratagene, Santa Clara, CA, USA) composed of total RNA from 10 human cell lines was used as control RNA. Next, 2  $\mu$ g of each RNA sample was subjected to reverse transcription using SuperScript II Reverse Transcriptase (18064-022, Invitrogen, Carlsbad, CA, USA) with random primers (48190-011, Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The resulting cDNA was used as a template for polymerase chain reaction (PCR) amplification to detect the gene expression of opioid receptors (delta, kappa and mu) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, as a control). The PCR mixture (30  $\mu$ l) contains 2  $\mu$ l of cDNA, 0.1 mM of dNTPs, 0.5  $\mu$ M each of the two primers, 1 $\times$  reaction buffer and 1.5 U of PowerTAQ DNA polymerase (GP5500, GeneTeks, Taipei, Taiwan) in distilled water. The PCR cycling program was set as follows: 95°C for 5 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and a further elongation step at 72°C for 7 minutes. To optimize PCR performance, the annealing temperature was reduced from 60°C to 55°C for the delta-opioid receptor, and the cycle number was reduced to 30 cycles for GAPDH. The primer pair sequences were designed by the Primer-3 program to span one mRNA splice site and are listed as follows: delta-opioid receptor (561 bp), 5'-gagtgttgaccgctacatcg-3' (forward), 5'-aagcagcgttggaagtctc-3' (backward); kappa-opioid receptor (581 bp), 5'-atcatcagcggcttactc-3' (forward), 5'-ttgagacgcaggatca tcag-3' (backward); mu-opioid receptor (549 bp), 5'-tgcaactggatcctctctcagccattggt-3' (forward), 5'-ggctctagtgttctgacgaattcgagtgg-3' (backward); GAPDH (130 bp), 5'-gggtgctcctctgactcaaca-3' (forward), 5'-gtgctgtagccaaattcgtgt-3' (backward). The amplified PCR products were subjected to electrophoresis on 2 percent agarose gels and then visualized under UV light excitation after staining with ethidium bromide.

The influenza-induced expression of IFN $\beta$  mRNA was detected and quantified by quantitative real-time RT-PCR analysis performed on an ABI StepOnePlus system. A549 cells were treated with methadone at various concentrations (0, 0.1, 1, 10  $\mu$ M) and infected with or without influenza virus at an MOI of 0.001 for 48 hours. Total intracellular RNA was extracted using TRIzol Reagent (15596-026, Invitrogen, Carlsbad, CA, USA), and residual genomic DNA was digested with DNase I (M0303, New England BioLabs, Ipswich, MA, USA), as per the manufacturer's instructions. After the extraction with chloroform, the purified RNA was quantified by spectrophotometry and subjected to reverse transcription for cDNA synthesis as described earlier. Human IFN $\beta$  primers (target length 254 bp) were listed as follows: 5'-tgctctggcacaacagtag-3'

(forward), 5'-gctgcagctgcttaattcc-3' (backward). Each 15- $\mu$ l reaction mixture contains 1  $\mu$ l of 40-fold diluted cDNA, 7.5  $\mu$ l of 2 $\times$  SYBR green PCR master mix (4367659, ABI, Austin, TX, USA) and 0.3  $\mu$ M of each primer. The PCR cycling program was set as follows: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. The 18S rRNA expression levels were determined in parallel using a TaqMan Endogenous Control Kit (4319413E, ABI, Austin, TX, USA). Each 15- $\mu$ l reaction mixture contained 1  $\mu$ l of 400-fold diluted cDNA, 0.75  $\mu$ l of 20 $\times$  primers/probe mix (43194131E, ABI, Austin, TX, USA) and 7.5  $\mu$ l of 2 $\times$  universal PCR master mix (4304437, ABI, Austin, TX, USA). The PCR cycling program was set as follows: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The expression level of IFN $\beta$  mRNA was normalized to that of 18S rRNA, and data were expressed as fold changes relative to control (methadone-untreated infected group) using the  $2^{-\Delta\Delta Ct}$  method (Livak & Schmittgen 2001).

#### *In vivo study*

Male C57BL/6 and ICR mice were purchased (BioLASCO Co., Ltd. Taiwan) at 8 weeks of age and group housed under environmentally controlled conditions for 1 week prior to the beginning of experiments. Mice were intraperitoneally injected with methadone (10 mg/kg/day) or PBS for 7 days; on day eight, mice were anesthetized by intraperitoneally administering 2 percent Avertin (20  $\mu$ l/g of body weight) and infected with human influenza A/WSN/33 virus ( $10^4$  PFU in 50  $\mu$ l PBS per animal) via the intranasal route. Avertin was prepared by fully dissolving 2,2,2-tribromoethyl alcohol (T48402, Sigma-Aldrich, St. Louis, MO, USA) in tert-amyl alcohol (152463, Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1 g/ml, and then diluted to 2 percent (v/v) in PBS, followed by sterilizing through a 0.2- $\mu$ m filter before use.

On day four post-infection, mice were sacrificed; lung tissues and serum samples were collected and stored at  $-80^\circ\text{C}$  until use. Frozen tissues were thawed on ice, homogenized in 500  $\mu$ l cold PBS by a homogenizer (POLYTRON PT2100, Kinematic, Luzern, Switzerland) and clarified by centrifugation at 2000  $\times$  g at 4°C for 5 minutes. The clarified lung homogenates and thawed serum samples were analyzed by plaque assays on MDCK cells to determine virus titers, as described previously. No influenza virus was detected in all collected serum samples. All procedures were reviewed and approved by the Animal Research Ethics Board at National Health Research Institutes, Taiwan.

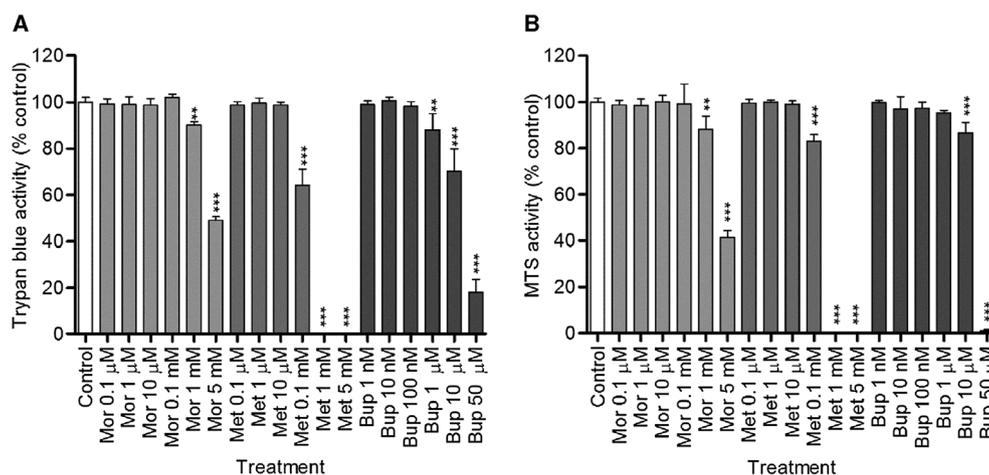
## Statistical analysis

Data were collected from three replicates in three independent experiments in the *in vitro* studies. Values were expressed as means  $\pm$  standard deviation. Statistical differences between multiple groups were evaluated by one-way analysis of variance (ANOVA) or two-way ANOVA followed by Bonferroni's *post hoc* test. Student's *t*-test was applied to assess the difference between two groups. A statistically significant difference was defined as  $p < 0.05$ .

## RESULTS

### Methadone, morphine and buprenorphine did not produce apparent cytotoxicity in human lung epithelial A549 cells

Cytotoxic effects of methadone, morphine and buprenorphine were examined in human lung epithelial A549 cells. Cells were treated with these opioid agonists for 72 hours; survival and proliferation of cells were measured by trypan blue exclusion and MTS assays. At clinically compatible doses, methadone (0.1–10  $\mu$ M), morphine (0.1–100  $\mu$ M) or buprenorphine (1–100 nM) (Aderjan *et al.* 1995; Eap *et al.* 2000; Greenwald *et al.* 2003; Hallinan *et al.* 2006; Piekoszewski *et al.* 2001) did not alter cell survival and proliferation, comparing with vehicle controls (Fig. 1). At toxic doses, methadone (0.1–5 mM), morphine (1–5 mM) and buprenorphine (1–50  $\mu$ M) dose-dependently reduced cell survival and proliferation (trypan blue exclusion assay:  $p < 0.0001$ ,  $F = 753.6$ , one-way ANOVA; MTS assay:  $p < 0.0001$ ,  $F = 927$ , one-way ANOVA). Altogether, these data suggest that methadone, morphine and buprenorphine have no apparent cytotoxic effects in A549 cells at physiologically relevant doses.



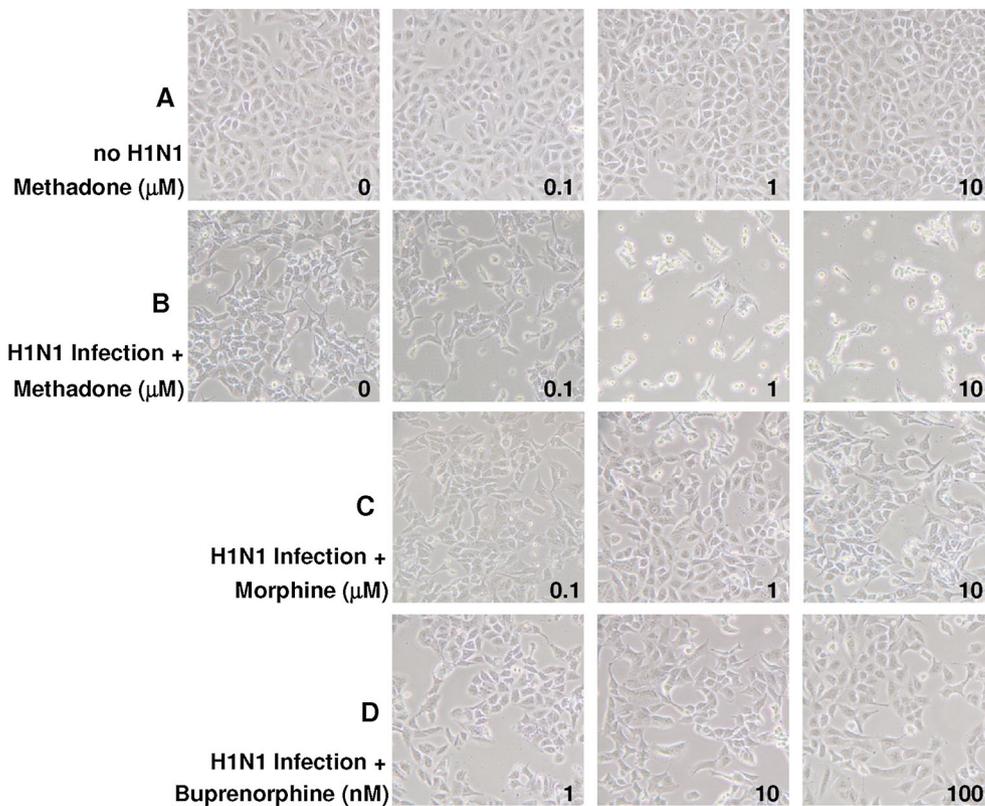
**Figure 1** Methadone, morphine and buprenorphine did not produce cytotoxicity in human lung epithelial A549 cells. A549 cells were treated with morphine (Mor), methadone (Met), buprenorphine (Bup) or vehicle (control) for 72 hours. Cytotoxicity was determined by (a) trypan blue dye exclusion and (b) MTS assays. Methadone, morphine and buprenorphine induced cytotoxicity only at non-physiological doses. All data were normalized to the mean of the control group. \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.0001$ ; one-way ANOVA (versus control)

### Low-dose methadone-enhanced H1N1-mediated cell loss in A549 cells

A549 cells were treated with low-dose methadone (0.1–10  $\mu$ M) or vehicle followed by infection with H1N1 virus (MOI = 0.001). Methadone (Fig. 2a) alone did not reduce cell density at 48 hours post drug administration. However, methadone + H1N1 significantly induced cytotoxicity (Fig. 2a versus b). Administration of morphine (0.1–10  $\mu$ M) or buprenorphine (1–100 nM) did not potentiate H1N1-mediated cell loss (Fig. 2c & d).

### Methadone selectively enhanced replication of H1N1 and viral protein production in A549 cells

A549 cells were treated with methadone, morphine, buprenorphine or vehicle followed by infection with H1N1 virus (MOI = 0.001) in the presence of trypsin (multi-cycle growth). Viral progenies were collected at 48 hours post-infection and titrated by a standard plaque assay in MDCK cells. Treatment with methadone (0.1, 1 or 10  $\mu$ M) dose-dependently increased H1N1 viral titers (5, 10 or 55-fold increase;  $p < 0.0001$ ,  $F = 20$ , one-way ANOVA; Fig. 3a). In contrast, morphine or buprenorphine did not alter virus titers (Fig. 3a). The production of M1 and NS1 viral proteins was examined in the A549 cell lysates by Western blot analysis at 48 hours post-infection. Methadone (Fig. 3b–e), but not morphine or buprenorphine (Fig. 3f & g), significantly increased the expression of M1 and NS1. Taken together, these results indicate that methadone selectively enhanced H1N1 viral replication and viral protein production in human lung epithelial A549 cells.



**Figure 2** Methadone-enhanced H1N1-mediated cytotoxicity in A549 cells. A549 cells were treated with methadone, morphine, buprenorphine or vehicle followed by infection with H1N1 virus (multiplicity of infection = 0.001). Phase-contrast images were taken at 48 hours post drug treatment of viral infection. (a) Methadone alone did not reduce cell density. (b) Methadone, in the presence of H1N1, dose-dependently increased cell loss. (c) Morphine + H1N1 or (d) buprenorphine + H1N1 did not change cell density

#### Methadone did not alter the proliferation of viral particles in the absence of A549 cells

H1N1 virus stocks were pre-treated with methadone (0.1, 1 and 10  $\mu\text{M}$ ) or vehicle in serum-free medium at 4°C for 24 hours. Viral concentration was determined by plaque assays in MDCK cells as we and others have previously described (Chen *et al.* 2012; Miyamoto *et al.* 2008). Methadone did not alter plaque number and size in the absence of A549 cells (Fig. 4), suggesting that methadone had no direct proliferative effect on the viral particles.

#### Methadone increased viral susceptibility in A549 cells

A549 cells grown on glass coverslips were treated with opioid agonists or vehicle prior to H1N1 viral infection (MOI = 1) in the absence of trypsin for single-cycle growth as trypsin is required for viral proliferation after infection (Hale *et al.* 2009). Viral susceptibility was examined for the presence of viral NP immunoreactivity at 24 hours post-infection. Treatment with methadone (0.1, 1 and 10  $\mu\text{M}$ ) significantly increased the density of viral NP (+) cells (Fig. 5). Morphine (0.1, 1 and 10  $\mu\text{M}$ ) or buprenorphine (1, 10 and 100 nM) had no effect on the susceptibility to viral infection (data not shown).

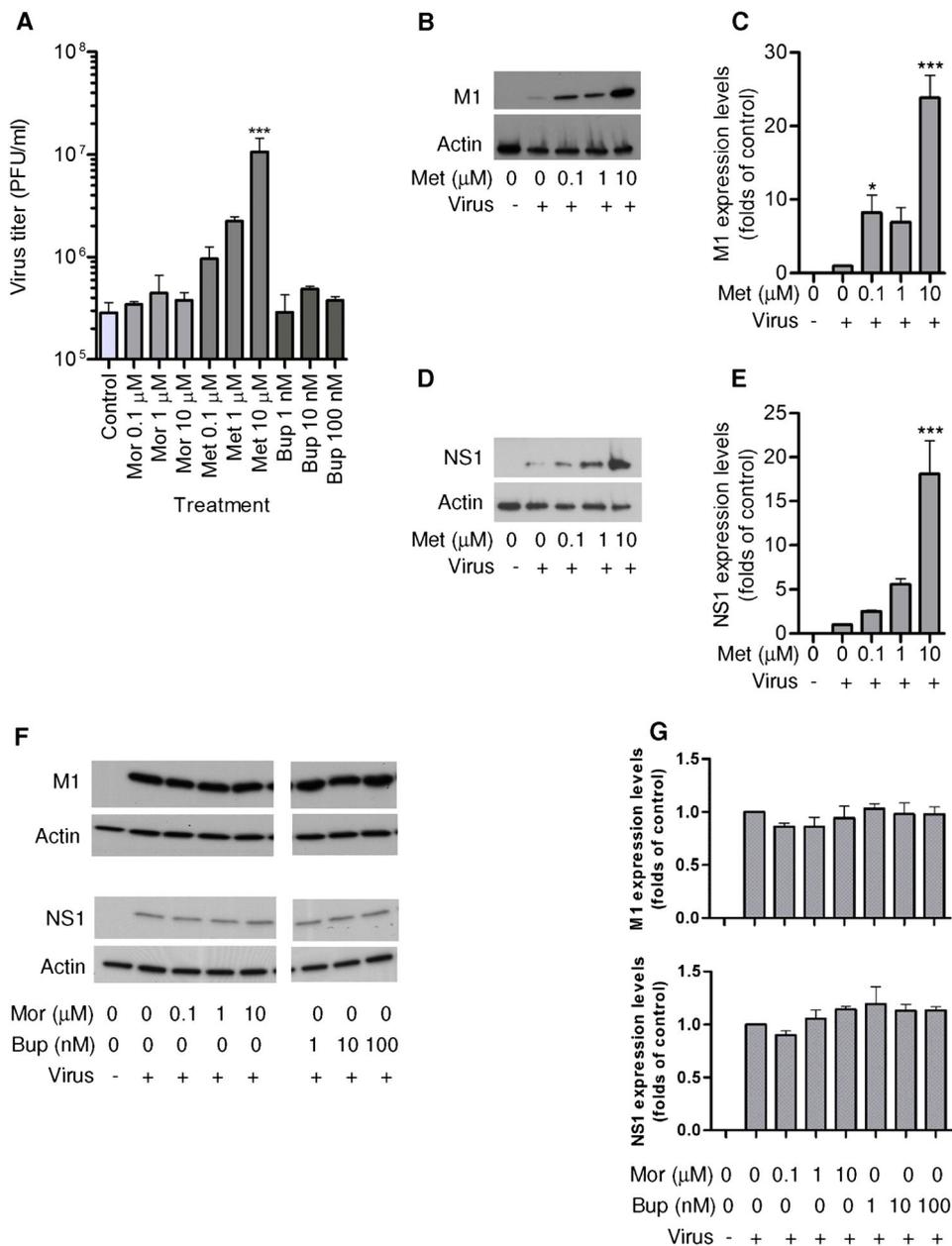
Taken together, these data suggest that methadone selectively enhanced H1N1 virus infection in human lung epithelial A549 cells.

#### Methadone-enhanced attachment of influenza A virus to A549 cells

Virion attachment to the target cells, an initial step of virus infection, was indirectly examined in culture media as previously described (Jaspers *et al.* 2005). A549 cells were pretreated with methadone (10  $\mu\text{M}$ ) or vehicle; H1N1 virus was added to the media 24 hours later. Unattached influenza virus in the media, determined by a plaque assay, was significantly and time-dependently reduced after infection (Fig. 6). This effect was significantly potentiated by methadone, suggesting that exposure to methadone facilitated the attachment of influenza virus to A549 cells (Fig. 6,  $p < 0.0001$ , two-way ANOVA).

#### Methadone-enhanced H1N1 evoked-interferon response in A549 cells

Type-I IFN is an indispensable mediator against viral injury in vertebrates. In response to viral infection, cells synthesize and secrete type-I IFN, which activates the

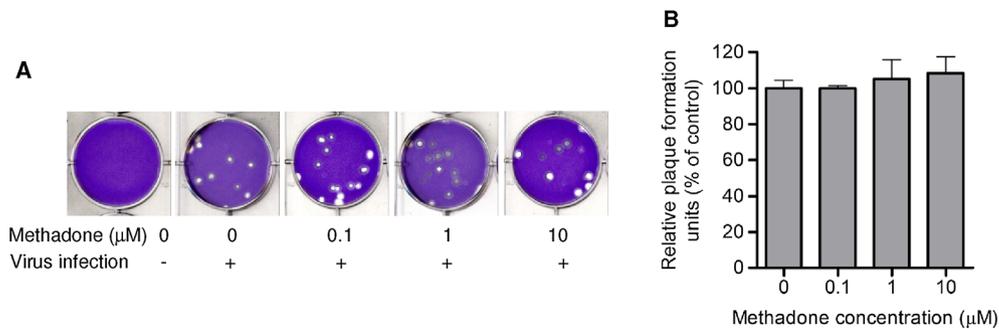


**Figure 3** Methadone-enhanced replication of H1N1 virus and viral protein production in A549 cells. A549 cells were treated with methadone (Met), morphine (Mor), buprenorphine (Bup) or vehicle (control) for 24 hours, followed by infection with H1N1 virus. Virus progenies were collected from the culture supernatants at 48 hours post-infection. (a) Treatment with methadone dose-dependently increased H1N1 viral titers. No difference was found in cells treated with morphine or buprenorphine. The expression of (b) viral matrix protein-1 (M1) and (d) non-structural protein-1 (NS1) in the A549 cell lysates was examined by Western blot analysis at 48 hours post-infection. (c, e) Methadone significantly increased M1 and NS1 viral protein levels. (f, g) Treatment with Mor or Bup did not alter M1 and NS1 expression. \* =  $p < 0.05$ ; \*\*\* =  $p < 0.0001$ ; one-way ANOVA versus control

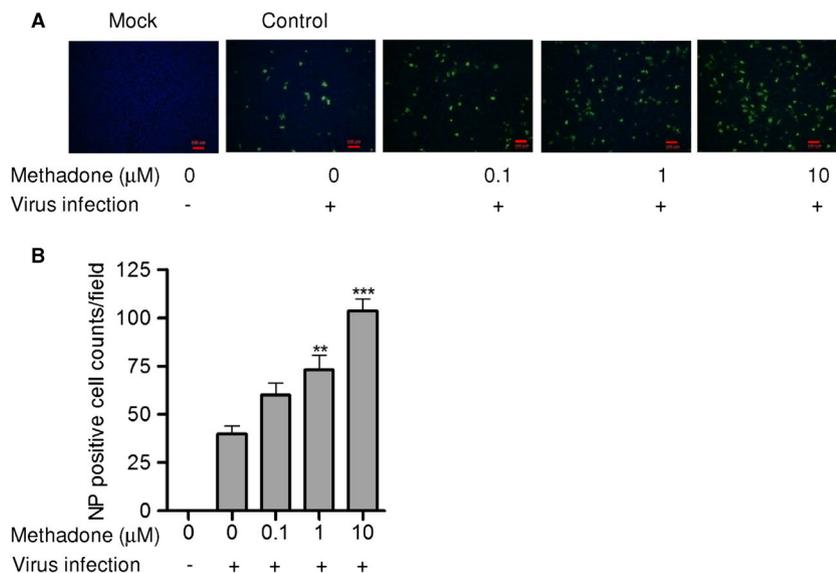
production of antiviral proteins in neighboring cells and limits further viral growth (Haller *et al.* 2006; Randall & Goodbourn 2008). We next examined IFN $\beta$  expression after methadone and H1N1 treatment. A549 cells were pretreated with vehicle or methadone for 24 hours, followed by infection with H1N1 virus (0.001 PFU/cell). H1N1 infection up-regulated IFN $\beta$  expression (Fig. 7a1). This response was greatly potentiated by methadone (0.1–10  $\mu$ M). Methadone

(0.1–10  $\mu$ M), by itself, did not increase the expression of IFN $\beta$  mRNA (Fig. 7a2).

As the production and phosphorylation of transcription factor STAT1 critically regulated type-I IFN signaling and its downstream myxovirus resistance protein (MxA) (Horisberger 1995), we further studied the IFN $\beta$ -evoked STAT1 and MxA expression. Similar to IFN $\beta$  mRNA, STAT1, phospho-STAT1 and MxA proteins were all up-regulated by methadone + H1N1



**Figure 4** Methadone had no direct effect on the biological activities of H1N1 virus. Influenza A virus was incubated with methadone at indicated concentrations in serum-free culture medium in the absence of host A549 cells at 4°C for 24 hours, and then 10-fold serially diluted for the plaque assay in Madin–Darby canine kidney cells. (a) Plaque phenotype. A representative result of three independent experiments with similar results is shown. No obvious differences in plaque size were observed between groups. (b) Relative plaque formation units (means  $\pm$  standard deviation) are expressed as percentages of plaque numbers of methadone-pretreated viruses relative to that of methadone-untreated viruses (control) at the same dilution. No significant differences in relative plaque formation units were found among groups



**Figure 5** Methadone increased susceptibility to H1N1 virus infections in A549 cells. (a) A549 cells grown on glass coverslips were treated with methadone for 24 hours, followed by infection with H1N1 virus in the absence of trypsin (for single-cycle growth). Cells were fixed at 24 hours post-infection for immunocytochemistry. Methadone treatment increased the density of viral nucleoprotein (NP) (+) cells. Viral NP (green); cellular nuclei were stained with 4', 6-diamidino-2-phenylindole (blue). Scale bar = 100  $\mu\text{m}$ . (b) Methadone dose-dependently increased the density of NP-positive cells. Significant differences from control are indicated (\*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.0001$ ; one-way ANOVA)

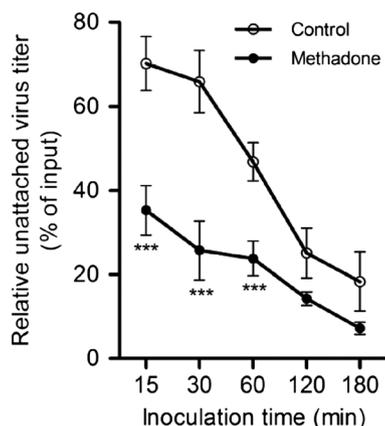
(Fig. 7b–g). Together with the increase in viral replication (Fig. 3a), these data suggest that methadone-enhanced H1N1-mediated IFN production and signaling in A549 cells.

#### Methadone did not alter antiviral function in the conditioned media

H1N1 and IFN $\beta$ -mediated antiviral function was studied in cultured A549 cells grown on glass coverslips (see timeline, Fig. 8a lower panel). Viral NP immunoreactivity was examined at 24 hours after infection. Treatment with H1N1 (0.1 PFU/cell) significantly increased the density of viral NP (+) cells (Fig. 8b-b & c),

which was attenuated by IFN $\beta$  (1000 and 100 U/ml; Fig. 8b-c, b-d & c). Similarly, condition media (1-fold and 0.1-fold) collected from the A549 cell cultures pretreated with H1N1 (0.001 PFU/cell) + vehicle (see timeline, Fig. 8a upper panel) dose-dependently reduced viral NP (+) cell density (Fig. 8b-b versus b-e & c), suggesting that antiviral factor(s) were released to the media after H1N1 viral infection (Basu *et al.* 2006; Young & Parks 2003).

We next examined if methadone altered H1N1-mediated antiviral function. H1N1 + methadone conditioned media (Fig. 8a, upper panel) was included in the A549 cells exposed to H1N1 (Fig. 8a, lower panel). Methadone did not alter conditioned media-induced viral NP reduction



**Figure 6** Methadone increased attachment of H1N1 to host A549 cells. A549 cells were pretreated with methadone (10  $\mu$ M) or vehicle. H1N1 virus was added to the media 24 hours later. In cells receiving vehicle, unattached influenza virus in the media was significantly and time-dependently reduced after infection. Treatment with methadone further reduced the titer of unattached H1N1. Data were expressed as percentages of unattached virus titer relative to the initial input virus titer ( $3 \times 10^6$  plaque-forming unit). \*\*\* =  $p < 0.0001$ ; two-way ANOVA

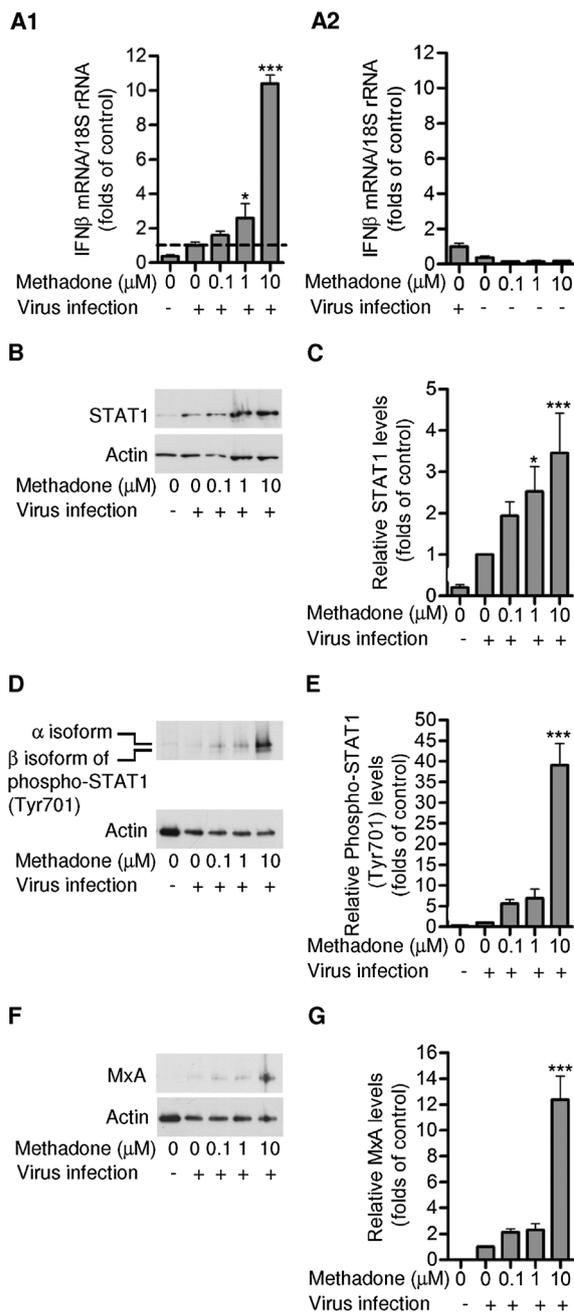
(Fig. 8b-e–b-h, 1 $\times$  fold conditioned media; Fig. 8b-i–b-l, 0.1 $\times$  fold conditioned media). Similarly, conditioned media collected from morphine + H1N1 (Fig. 8d) or buprenorphine + H1N1 (Fig. 8e) cell culture did not alter NP reduction. Taken together, these data suggest that methadone, morphine and buprenorphine had no apparent effect on the endogenous antiviral function induced by H1N1 infection.

#### A549 cells do not express opioid receptors

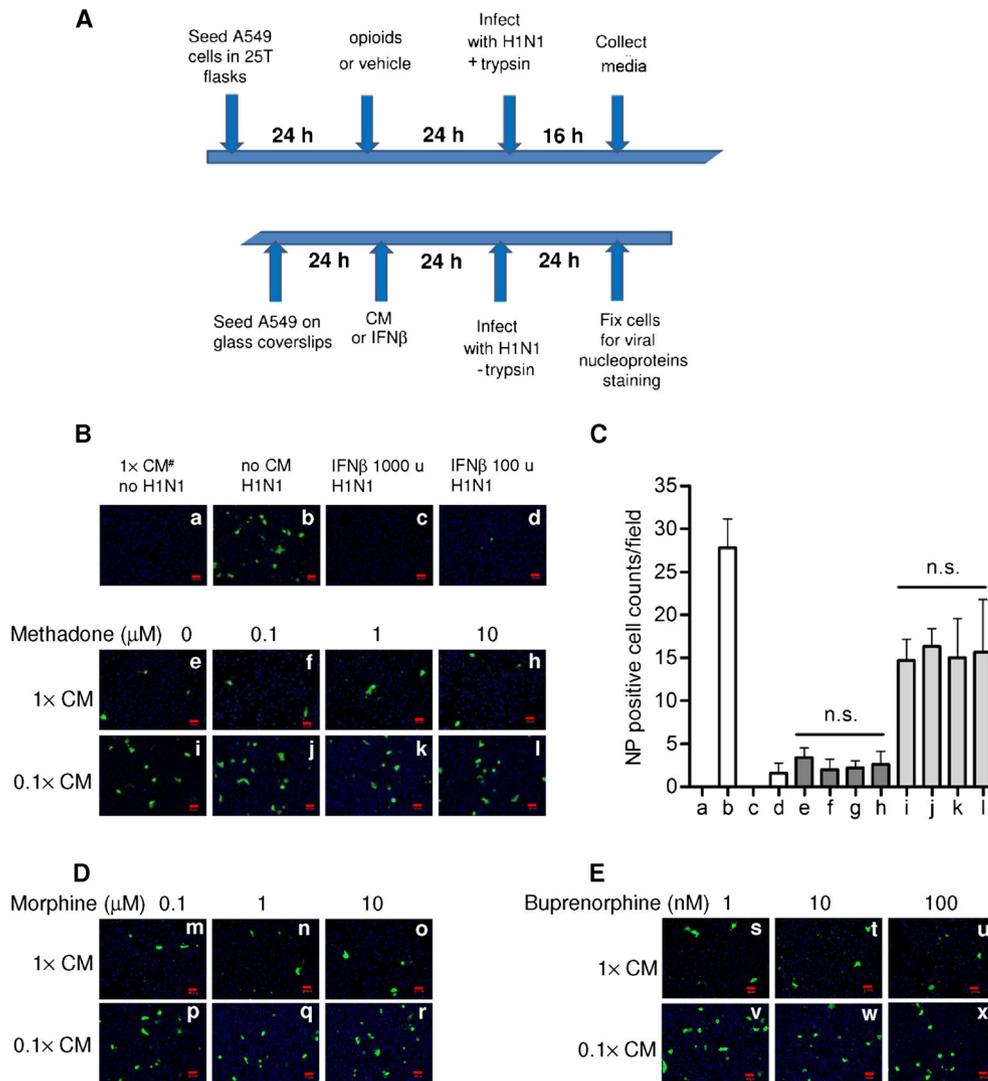
The expression of delta-opioid, mu-opioid and kappa-opioid receptors was examined in A549 cells by RT-PCR and immunocytochemistry. In order to avoid amplifying the target gene fragments from contaminating chromosomal DNA, the gene-specific primer pairs were designed to span one mRNA splicing site. None of these three opioid receptor mRNAs was detected in A549 cells (Fig. 9a). For positive controls, mu-opioid and delta-opioid receptor mRNAs were identified in SH-SY5Y cells. Delta-opioid and kappa-opioid receptor mRNAs were detected in the universal human reference RNA extract (Fig. 9a). Furthermore, the expression of mu-opioid receptor proteins was detected in the SH-SY5Y, but not A549, cells using a selective antibody (Fig. 9b), supporting that A549 cells did not express mu-opioid receptor.

#### Methadone exposure enhances influenza A virus replication in mouse lungs

Two strains of mice (C57BL/6 and ICR) were used to examine H1N1 replication *in vivo*. Animals (9-week-old, male,  $n = 23$ ) received daily injections of methadone



**Figure 7** Methadone-enhanced H1N1-induced interferon  $\beta$  (IFN $\beta$ ) responses in A549 cells. A549 cells were treated with methadone for 24 hours followed by administration of (a1) H1N1 virus or (a2) vehicle in the presence of trypsin (for multi-cycle growth). IFN $\beta$  mRNA, examined by quantitative reverse transcription polymerase chain reaction, was normalized to control (methadone 0  $\mu$ M + virus). (a1) Methadone significantly potentiated H1N1-mediated IFN $\beta$  expression. (a2) Methadone itself (in the absence of H1N1) did not increase the expression of IFN $\beta$  mRNA. Total cellular lysates were analyzed for the expression of cellular (b, c) STAT1, (d, e) phospho-STAT1 (Tyr701) and (f, g) MxA by the Western blot and were normalized to the control (methadone 0  $\mu$ M + virus). Methadone significantly potentiated H1N1-mediated STAT1, phospho-STAT1 and MxA protein levels. \* =  $p < 0.05$ ; \*\*\* =  $p < 0.0001$ ; versus control; one-way ANOVA



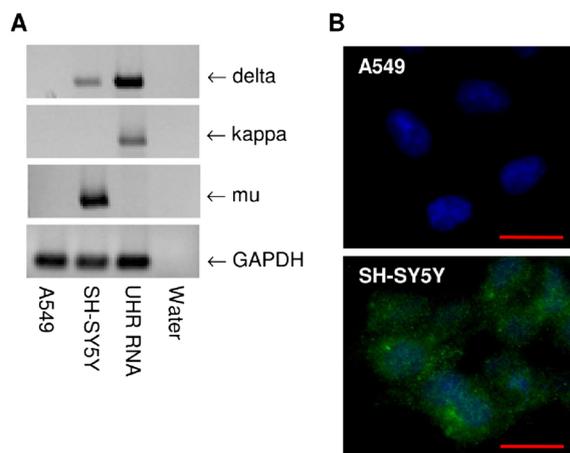
**Figure 8** Methadone did not alter antiviral function of secreted proteins in H1N1-infected A549 cells. (a) Experimental timeline for infection-reduction assay. Viral nucleoprotein (NP) immunoreactivity was examined in cultured A549 cells grown on glass coverslips. H1N1 infection increased the density of NP (+) cells (b-a versus b-b). Co-treatment with IFNβ significantly attenuated viral NP expression (b-c, b-d versus b-b); c). Co-treatment with conditioned media (CM) collected from the opioid-exposed infected A549 cell cultures dose-dependently reduced NP (+) cell density (b-b versus b-e–b-l; c). Methadone did not alter the antiviral function induced by CM (b-e–b-h, 1× CM; b-i–b-l, 0.1× CM; c). CM-induced reduction in viral NP expression after H1N1 infection was not affected by morphine (d) or buprenorphine (e). Viral NP (green); 4', 6-diamidino-2-phenylindole staining (blue); scale bar = 50 μm; # = the conditioned medium was collected from methadone (10 μM)-exposed H1N1-infected A549 cell culture

(10 mg/kg/day, i.p.) or PBS for 7 days and were treated with H1N1 virus ( $10^4$  PFU/50 μl/mouse) or vehicle intranasally on the eighth day. We did not see obvious respiratory distress in mice receiving methadone and H1N1 infection. However, other signs of respiratory illness, such as sniffing, sneezing, cough, wheeze and discharge from nose were not intensively examined. Detailed pulmonary functional tests may be useful to distinguish respiratory illness between the control and treated animals. These functional tests may be included in our future studies. Lung tissues were collected 4 days after viral infection for plaque assay. Methadone treatment significantly increased virus titers in lungs by  $3.3 \pm 1.6$

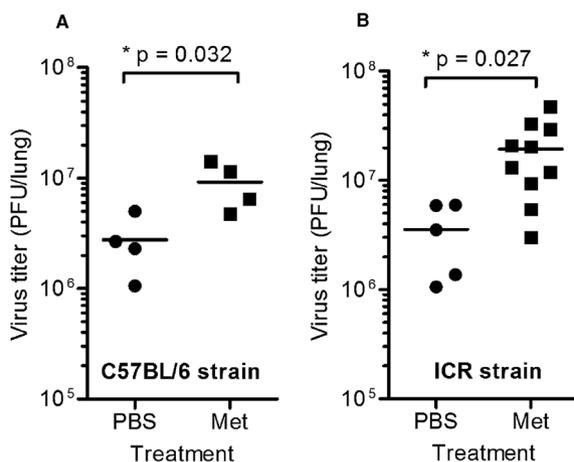
fold and  $5.5 \pm 3.9$  fold in C57BL/6 and ICR mice, respectively (Fig. 10,  $p < 0.05$ , *t*-test). These results indicated that methadone exposure increased influenza A virus replication *in vivo*.

## DISCUSSION

In this study, we investigated the effect of methadone on influenza A virus replication *in vitro* and *in vivo*. Our data showed that methadone enhanced influenza A virus replication in human lung epithelial A549 cells. Methadone increased adsorption of virus, susceptibility to infection and viral protein synthesis in cell culture. In



**Figure 9** A549 cells did not express opioid receptors. (a) The expression of mu, kappa and delta opioid receptors in A549, SH-SY5Y cells and a commercially available human reference RNA extracted from 10 human cell lines (UHR RNA) was examined by reverse transcription polymerase chain reaction. None of the three opioid receptor mRNAs was detected in A549 cells. For positive controls, mu-opioid and delta-opioid receptor mRNAs were found in SH-SY5Y cells. Delta-opioid and kappa-opioid receptor mRNAs were detected in the universal human reference RNA extract. (b) A549 and SH-SY5Y cells grown on glass coverslips were fixed with 4 percent paraformaldehyde and subjected to immunostaining. Mu-opioid receptor immunoreactivity (green) was found in SH-SY5Y cells but not in A549 cells (top panel); 4', 6-diamidino-2-phenylindole (blue); scale bar = 20  $\mu$ m



**Figure 10** Methadone increased H1N1 virus replication in mouse lungs. (a) Male C57BL/6 strain (methadone-treated group,  $n=4$ ; PBS-treated group,  $n=4$ ) and (b) ICR strain [methadone-treated group,  $n=10$ ; phosphate-buffered saline (PBS)-treated group,  $n=5$ ] mice were treated with methadone (10 mg/kg/day, i.p.) or PBS for 7 days, and then intranasally inoculated with H1N1 virus ( $10^4$  PFU/mouse) on day eight. Methadone treatment significantly increased viral titers in lung tissues at 4 days after viral infection. Met = methadone-treated group. \* =  $p < 0.05$

addition, exposure to methadone enhanced influenza virus replication in mouse lungs. To our knowledge, these data are the first to suggest that methadone enhances

influenza infection in a human cell line and in a mouse model.

We demonstrated that methadone at clinically relevant doses (0.1–10  $\mu$ M) had no toxic effects in human lung epithelial A549 cells but reduced cell survival at high concentrations (0.1–5 mM). These findings were consistent with previous reports that high concentrations of methadone promoted cell death in hepatocytes, leukemia cells and neuroblastoma cells (Friesen *et al.* 2008; Gomez-Lechon *et al.* 1987; Perez-Alvarez *et al.* 2010). Although methadone by itself had no toxicity for the A549 cells at low doses, it dose-dependently potentiated cell death in the presence of influenza virus. Cell toxicity induced by methadone and H1N1 virus was associated with an increase in viral protein synthesis and viral proliferation. Morphine and buprenorphine also exerted no apparent toxic effects to A549 cells at the low clinically relevant doses while inducing cytotoxicity at high concentrations, in agreement with previous reports (Polanco *et al.* 2009; Ponsoda *et al.* 1991). Unlike methadone, however, morphine and buprenorphine did not potentiate influenza-mediated cell loss and viral proliferation at these clinically relevant doses. Taken together, our data support that methadone has a differential response for enhancing influenza viral toxicity in A549 cells.

We found that methadone selectively increased H1N1 proliferation and the expression of viral protein M1 and NS1 in A549 cells. In contrast, methadone did not increase the proliferation of viral particles in the absence of the host cells. These data suggest that methadone had no direct action on the viral particles, and the synergistic action of viral infection and methadone takes place in lung epithelial cells. This assumption was further supported by the increase in viral attachment to the target cells in the presence of methadone. Previous studies have shown that endocytosis of influenza virus was mediated through N-linked sialoglycans receptors on the host cell membrane; blocking these receptors inhibits influenza virus replication in host cells (Hidari *et al.* 2013; Hoffmann *et al.* 2014). It will be of interest to further identify the interaction of methadone and N-linked sialoglycans receptors in future studies.

Although methadone is an opioid agonist, the potentiation of H1N1 replication reported here may not be mediated through the opioid receptors based on the following supporting data: (1) A549 cells used in current study did not express mu-opioid, delta-opioid and kappa-opioid receptor mRNAs. Using immunocytochemistry, we showed that A549 cells did not contain mu receptor protein. Similar findings have been reported by other laboratories (Daijo *et al.* 2011). (2) We demonstrated that the mu receptor agonist morphine and buprenorphine did not influence H1N1 viral replication. A549 cells expressed high levels of NMDA (N-methyl-D-aspartate) receptors (Choi & Viseskul 1988; Ebert *et al.* 1995); methadone can interact

with non-opioid, including NMDA, receptors (Choi & Viseskul 1988; Ebert *et al.* 1995; Gorman *et al.* 1997; Krug *et al.* 1993). It is thus possible that methadone may enhance influenza virus replication through NMDA or other non-opioid receptors in A549 cells.

After viral infection, host cells produce type-I IFNs (i.e. IFN $\alpha$  and IFN $\beta$ ), which activate the production and secretion of antiviral proteins from infected and nearby cells through the JAK-STAT pathway and limit further infection (Randall & Goodbourn 2008). This defense mechanism is further regulated by transcription factor STAT1 and myxovirus resistance protein MxA (Haller *et al.* 2006; Randall & Goodbourn 2008). In this study, we demonstrated that methadone did not alter IFN $\beta$  expression by itself; however, it greatly increased IFN $\beta$  mRNA in the presence of H1N1. Methadone also dose-dependently up-regulated STAT1, phospho-STAT1 and MxA in the presence of H1N1 infection, suggesting that the antiviral IFN response was not compromised by methadone. Furthermore, methadone did not alter antiviral function in the conditioned media collected after H1N1 infection. Taken together, our data support that methadone-induced H1N1 replication is not mediated through suppression of production or antiviral function of IFNs or other secretory factors.

Consistent with the *in vitro* results, we found that chronic administration of methadone increased H1N1 virus titers in the lungs of C57BL/6 and ICR mice. It is possible that methadone enhances influenza A virus replication *in vivo* through similar mechanisms to those seen in our *in vitro* study. It has been reported that methadone inhibits systemic immune functions (Hutchinson & Somogyi 2004; Peterson *et al.* 1989), which may exacerbate viral infection and promote viral replication *in vivo*. Furthermore, methadone is rapidly distributed to the lungs because of its high lipid solubility (Dole & Kreek 1973; Ferrari *et al.* 2004). Altogether, these data support that animals receiving methadone are more susceptible to influenza virus infection in lungs.

Previous studies have shown that morphine enhanced HIV or HCV replication in human blood mononuclear cells or hepatocytes through mu-opioid receptors (Guo *et al.* 2002; Li *et al.* 2007; Peterson *et al.* 1990). Morphine has been shown to suppress a variety of immune responses, including proliferation of lymphocytes, natural killer cell activity, production of antiviral cytokines by immune cells and induction of antibodies (Hutchinson & Somogyi 2004; Peterson *et al.* 1989; Vallejo *et al.* 2004; Wang *et al.* 2011). Several lines of evidence indicate that this immune suppression is also directly mediated via specific opioid receptors in the immune cells (Bayer *et al.* 1990; Gaveriaux-Ruff *et al.* 1998; Nair *et al.* 1997; Roy *et al.* 1998) or indirectly via the action through the hypothalamus–pituitary–adrenal axis (Hernandez *et al.*

1993; Mellon & Bayer 1998). In contrast, we found that morphine did not alter influenza A virus replication in human lung epithelial A549 cells. The lack of modulation of H1N1 virus replication by morphine may be attributed to the absence of opioid receptors in these cells.

Buprenorphine, a partial mu-opioid receptor agonist, has been used for analgesia and opioid maintenance therapy (Davis 2012; Pecoraro *et al.* 2012). We demonstrated that buprenorphine did not affect influenza virus replication in A549 cells. Similar findings have been reported in that buprenorphine did not suppress the immune reaction when given acutely or chronically (Martucci *et al.* 2004; Sacerdote 2006) and had no apparent effects on the course of disease in rabbits after myxoma viral infection (Robinson *et al.* 1999). These data suggest that buprenorphine, compared with methadone, may have less comorbidity after influenza virus infection *in vivo* and may be a drug of choice for chronic use in addiction treatment.

In conclusion, we demonstrated that methadone enhanced influenza A virus replication *in vitro* and *in vivo*. Our findings raise concerns that methadone use may be a risk factor for influenza infection. Future epidemiological studies regarding influenza infection in patients receiving methadone maintenance treatment may further support this concern.

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#### Conflict of Interest

The authors declare that they have no competing interests.

#### Authors Contribution

YHC conceived and designed the experiments. KLW, MTT and YHC performed the experiments. KLW, MLC, MTT, WHC, YHC and YW analyzed the data. YW and MLC contributed reagents, materials and analysis tools. YHC and YW wrote the manuscript. All authors read and approved the final manuscript.

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