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Short Communication

Antiviral effect of a selective COX-2 inhibitor on H5N1 infection in vitro

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ABSTRACT

A selective cyclooxygenase-2 (COX-2) inhibitor has been previously shown to suppress the hyper-induced pro-inflammatory responses in H5N1 infected primary human cells. Here, we demonstrate that COX-2 inhibitors suppress H5N1 virus replication in human macrophages suggesting that H5N1 virus replication (more so than seasonal H1N1 virus) is dependent on activation of COX-2 dependent signaling pathways in host cells. COX-2 and its downstream signaling pathways deserve detailed investigation as a novel therapeutic target for treatment of H5N1 disease.

Selective COX-2 inhibitors are non-steroidal anti-inflammatory drugs that target COX-2, an inducible enzyme responsible for inflammatory processes and immune responses (Claria, 2003; Futagami et al., 2003; Khanapure et al., 2007). We previously demonstrated that COX-2 plays a regulatory role in induction of H5N1-mediated pro-inflammatory responses in vitro (Lee et al., 2008). Such cytokine dysregulation is proposed to be a major contributor to the pathogenesis of H5N1 disease in humans (Peiris et al., 2009). Here we report the novel finding that a selective COX-2 inhibitor has a direct antiviral effect in H5N1 infected human primary macrophages.

Human primary monocyte-derived macrophages used in this study were prepared as reported previously (Lee et al., 2008). H5N1 viruses employed for this study included A/Hong Kong/483/97 (483/97), a clade 0 H5N1 virus and A/Vietnam/3212/04 (3212/04), a clade 1 virus, isolated from patients with H5N1 disease in Hong Kong and Vietnam, respectively. These viruses were isolated and passaged in Madin–Darby canine kidney (MDCK) cells and titrated to determine tissue culture infection dose 50% ($TCID_{50}$) in MDCK cells.

Cells were treated with nimesulide (Cayman chemical, Michigan, USA), a selective COX-2 inhibitor or drug-vehicle, dimethyl sulfoxide (DMSO) for 1 h before infection or as otherwise specified. Cells were infected with H5N1 viruses at multiplicity of infection (MOI) of 2 or 0.001 as indicated. Following virus adsorption for 30 min, the virus inoculum was removed, the cells were washed and incubated in corresponding medium with nimesulide or drug-vehicle as controls. At 8 h post-infection, cells were fixed with 4% paraformaldehyde for immunofluorescent staining using antibodies against influenza A virus antigens for the detection of influenza virus matrix (M) protein and nucleoprotein (NP) (Imagen™, Oxoid, UK). mRNA was extracted from cells at 6 h post infection using RNeasy Mini kits (Qiagen) to quantify viral NP and polymerase gene expression. Cell culture supernatants were collected at 6, 24 and 48 h after infection for quantitating infectious virus yield by $TCID_{50}$ titration and for quantifying viral M gene expression by RT-PCR using viral RNA isolated with the Viral RNA Mini kit (Qiagen) (Geeraedts et al., 2008).

In order to determine if the concentrations of nimesulide used in the study are toxic to cells, a cytotoxicity analysis using a lactate dehydrogenase (LDH) assay (Roche) was performed according to manufacturer's protocol. Serum free medium (SFM) for macrophages containing different concentrations of nimesulide (0, 20, 100, 200 and 600 μM) in 0.1% DMSO as vehicle were added to the cells. Cells treated with assay medium (SFM) only and 2% Triton X-100 only were regarded as low (0% cytotoxicity) and high (100% cytotoxicity) controls, respectively. The percentage of cytotoxicity was calculated using the absorbance values measured at 490 nm (after subtraction from the absorbance value obtained in the assay medium) of each samples and controls as follow: Cytotoxicity (%) = (experimental value − low control)/(high control − low control) × 100. The concentrations of nimesulide treatment used ranging from 0 to 600 μM were not toxic to human macrophages (Fig. 1A).

The effective concentration $(EC)_{50}$ value (the concentration of nimesulide required to reduce the viral titer to 50%) in the cell

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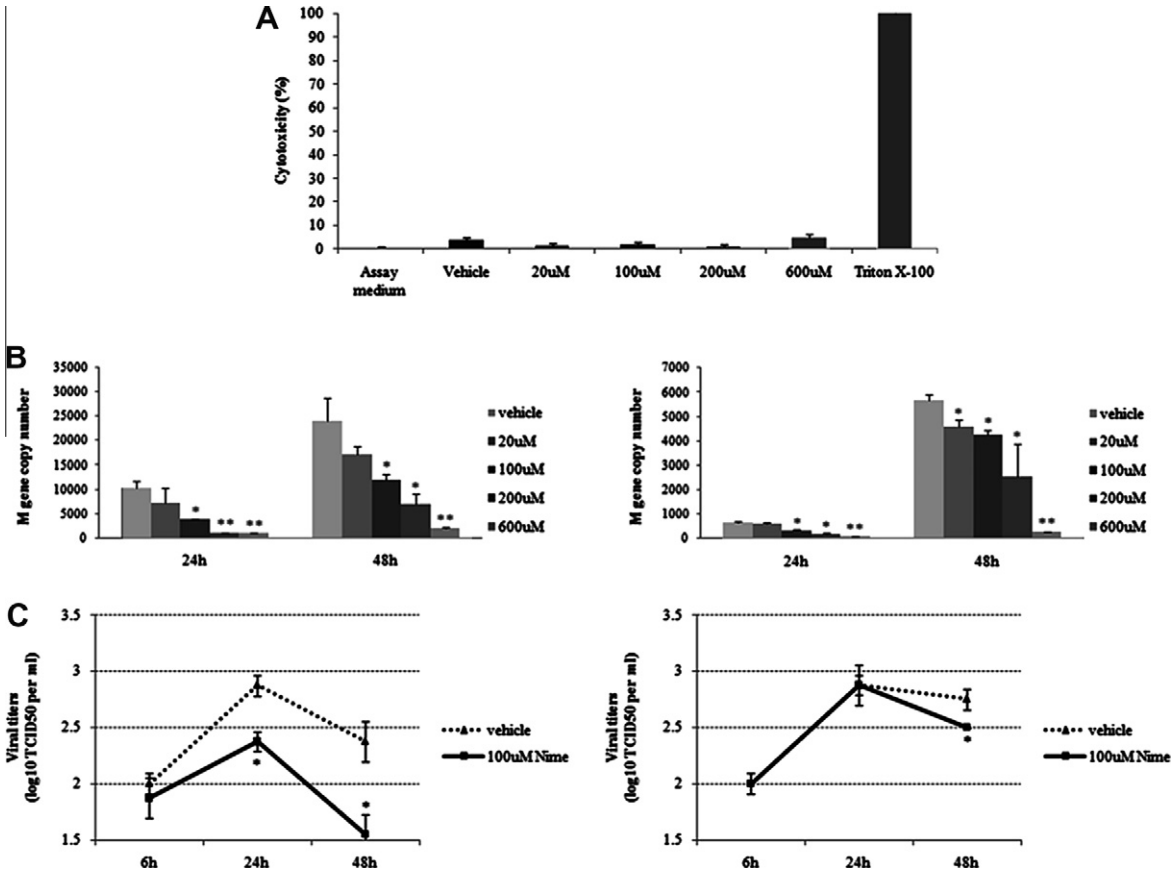

Fig. 1. (A) Evaluation of potential cytotoxic effect of nimesulide on human macrophages. The effect of different concentrations of nimesulide (ranging from 0 to 600 μM) on cytotoxicity was determined. All the tested concentrations of nimesulide treatment did not cause cytotoxic to human macrophages. Cells treated with assay medium (SFM) and 2% Triton X-100 were negative and positive controls and regarded as 0% and 100% cytotoxicity. (B) Effect of nimesulide on release of infectious virus from H5N1-infected human macrophages. Human monocyte-derived macrophages were treated with different concentrations of nimesulide or drug-vehicle control for 1 h before infection with H5N1 viruses at MOI = 2. After infection, cells were incubated in the presence of nimesulide or drug-vehicle control throughout the experiment. Viral RNA was extracted from the supernatants collected from cells infected with H5N1 viruses at 24 and 48 h post infection. After cDNA synthesis, influenza M gene was assayed using real-time PCR. M gene expression in supernatants collected from 483/97 (left) and 3212/04 (right) H5N1-infected macrophages was significantly suppressed by nimesulide in a dose-dependent manner. (C) Viral titers of 483/97 (left) and 3212/04 (right) in culture supernatants collected from infected cells treated with 100 μM nimesulide or drug-vehicle were determined in a $TCID_{50}$ assay. Representative data of duplicate experiments with means of triplicate assays are shown. $^{*}p < 0.05$; $^{**}p < 0.005$.

culture supernatant was determined by plotting the percentage of viral titer against nimesulide concentration. The EC_{50} value for nimesulide against H5N1(483/97) was found to be 170 μM.

Nimesulide treatment reduced viral M gene expression in supernatants collected from 483/97 and 3212/04 H5N1-infected human macrophages in a dose-dependent manner and statistical significance ($p < 0.05$) was observed starting from 100 μM nimesulide treatment at 24 and 48 h post infection with both H5N1 viruses compared with vehicle treatment (Fig. 1B). Similarly, the infectious virus titer ($TCID_{50}$) was significantly suppressed ($p < 0.05$) in 100 μM nimesulide treated compared with vehicle treated-cells at 48 h after infection with both 483/97 and 3212/04 H5N1 viruses (Fig. 1C).

Compared with the vehicle-treated cells (Fig. 2A), nimesulide suppressed influenza viral protein expression in H5N1-infected macrophages at a dose of 200 μM (Fig. 2B) and 600 μM (Fig. 2C). The expression of viral NP, PB1, PB2 and PA in H5N1 infected cells was significantly reduced by nimesulide treatment compared to treatment with vehicle ($p < 0.05$) (Fig. 2D).

In order to understand whether nimesulide exerts its effect before, during or after the early steps of virus entry and uncoating, we compared the M gene expression in H5N1-infected macrophages treated with nimesulide from 1 h before and throughout the infection and cells with nimesulide treatment commencing 30 min after infection at MOI of 2. We found comparable suppression of M gene expression when nimesulide treatment was commenced 1 h before or 30 min after infection (data not shown) suggesting that the drug exerts its effect after the entry and uncoating steps of the virus.

We next investigated the effect of nimesulide on infectious virus yield in vitro over multiple rounds of virus replication following infection of cells at low MOI (MOI = 0.001) with a H5N1 virus (483/97), an avian influenza H9N2 virus A/Quail/Hong Kong/G1/97 (H9N2/G1) which shares six viral "internal genes" with 483/97 (H5N1) and a human seasonal influenza H1N1 virus A/Hong Kong/54/98 (54/98). Nimesulide reduced the viral yield of H5N1 and H9N2 virus replication in a dose-dependent manner (Fig. 3A and B) but had a less pronounced effect on human seasonal H1N1 virus replication (Fig. 3C). A similar effect was also observed using another selective COX-2 inhibitor, NS-398 (data not shown). This suggests that virus replication of H5N1 and H9N2 virus may be more dependent on COX-2 signaling compared with seasonal H1N1 virus. Interestingly, both H5N1 and H9N2 viruses induced much higher COX-2 expression in infected cells than seasonal H1N1 did (Fig. 3D). Our previous study showed that COX-2 expression was dramatically upregulated in H5N1-infected macrophages in vitro and in alveolar epithelial cells of autopsy lung tissue samples of patients with fatal H5N1 disease (Lee et al., 2008). This

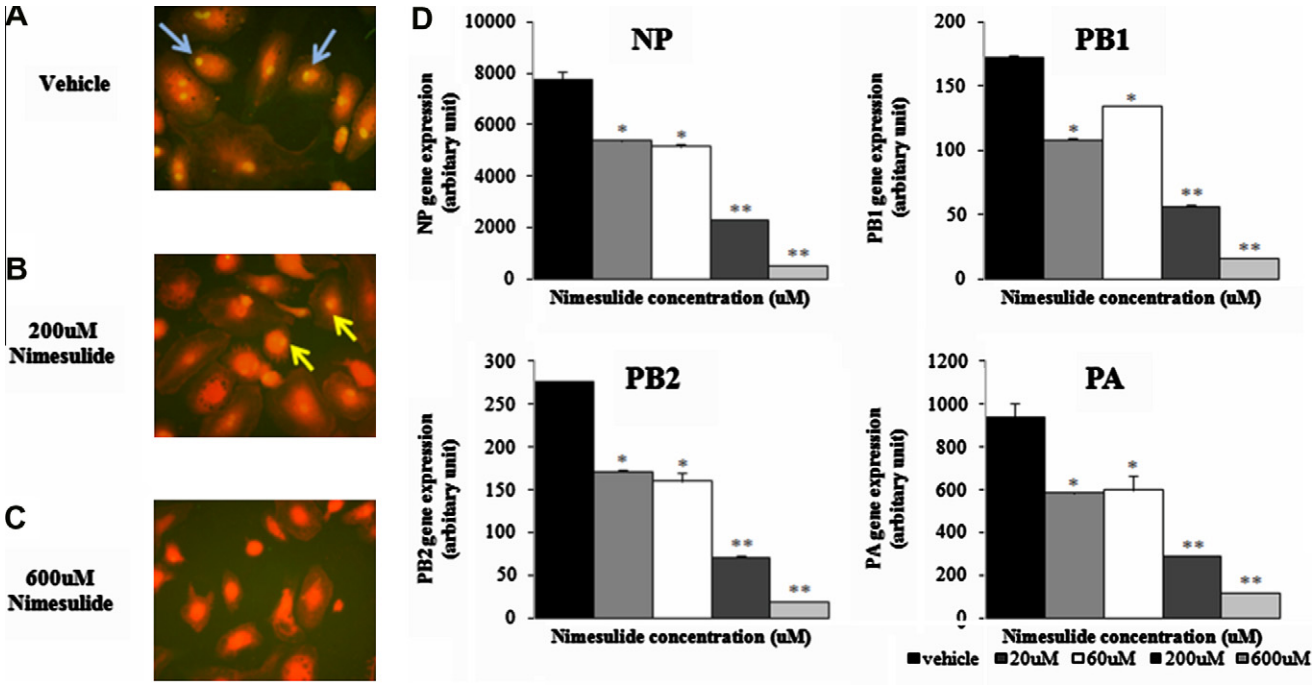

Fig. 2. Nimesulide reduces the viral protein and viral gene expression in H5N1 (483/97) infected cells. Immunofluorescent staining for influenza viral proteins M/NP was performed on H5N1-infected macrophages fixed at 8 h post-infection. Compared with (A) vehicle treated cells which showed strong viral protein expression (blue arrows), the viral protein expression was reduced (yellow arrows) or abolished in cells treated with (B) 200 μM nimesulide or (C) 600 μM nimesulide respectively. Evans blue counterstain produced a red background. Magnification 400×. (D) Viral gene expression analysis using real-time PCR showed that the NP and viral polymerase subunits, PA, PB1, PB2 gene transcription in H5N1-infected cells was significantly reduced by nimesulide in a dose-dependent manner. Representative data of duplicate experiments with means of triplicate assays are shown. $^{*}p < 0.05$; $^{**}p < 0.005$.

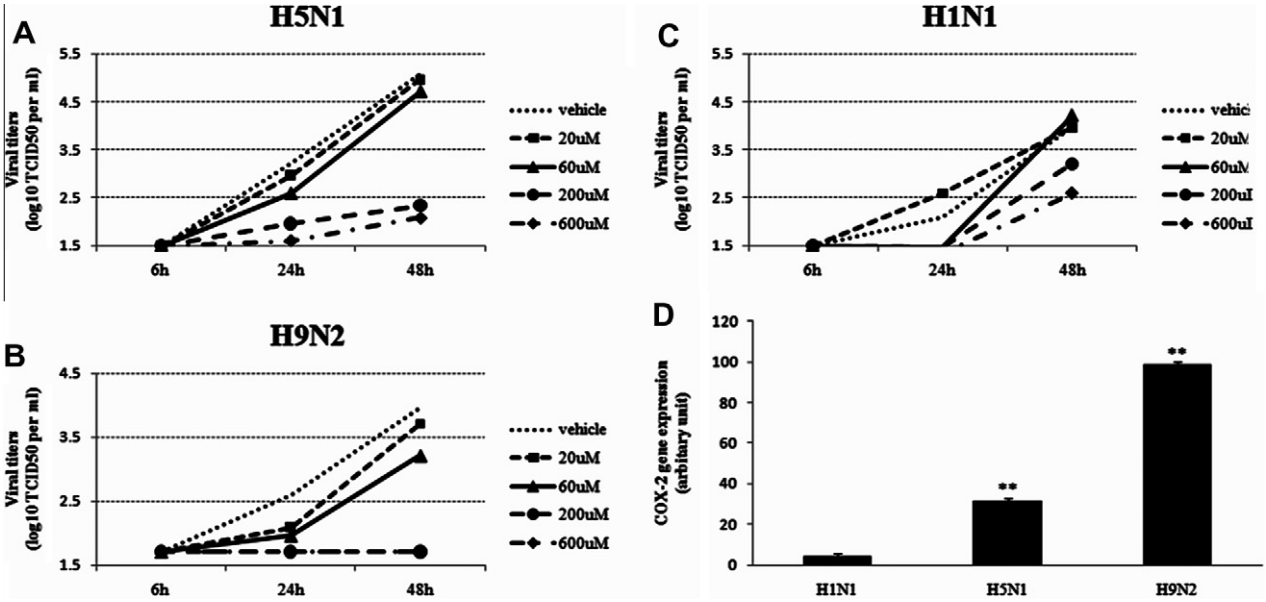

Fig. 3. Effect of nimesulide on multiple cycles of influenza virus replication in human macrophages. Human monocyte-derived macrophages were treated with different concentrations of nimesulide or drug-vehicle control for 1 h before infection with H5N1 (483/97), H1N1 (54/98) or H9N2 (G1/97) viruses at a MOI of 0.001. After infection, cells were incubated in the presence of nimesulide or drug vehicle control throughout the experiments. Viral titers in supernatants from the infected macrophages at 6, 24 and 48 h post-infection were determined using $TCID_{50}$ assay. Nimesulide suppressed (A) H5N1 and (B) H9N2 influenza virus replication in a dose-dependent manner but exerted a much less antiviral effect on (C) seasonal influenza H1N1 virus. (D) COX-2 expression in influenza A virus-infected macrophages. Both H5N1 and H9N2 viruses significantly induced COX-2 expression compared with human seasonal H1N1 virus. Representative data of duplicate experiments with means of triplicate assays are shown. $^{**}p < 0.005$.

leads one to hypothesize that the replication of avian viruses, in particular those that strongly up-regulate COX-2 (e.g. H5N1) are more dependent on host cell factors regulated by COX-2 than human seasonal H1N1 viruses. It is therefore important to further investigate the effects of COX-2 regulated downstream pathways that may be involved in promoting H5N1 or H9N2 virus replication.

A previous study demonstrated that acetylsalicylic acid (ASA), also known as aspirin, a blocker of NF-κB activation as well as the activity of COX, inhibits influenza virus replication (Mazur

et al., 2007). In that study, treatment with indomethacin, a pure COX inhibitor, which inhibits both COX-1 and COX-2 activity failed to show any effect on virus replication. It was therefore concluded that ASA blocks influenza virus replication via inhibition of NF-κB signaling (Mazur et al., 2007). Unlike the inducible COX-2, COX-1 is constitutively expressed in most normal body tissues (bronchiolar/alveolar epithelial cells, alveolar macrophages in the lung) in rats and non-human primates (Ermert et al., 1998; Khan et al., 2000; Wilborn et al., 1995) and is involved in important physiological functions such as vasodilatation, bronchodilation and surfactant synthesis (Brannon et al., 1998). Therefore, treatment with indomethacin not only blocks the COX-2 activity, but also blocking the activity of the physiologically important COX-1, and may lead to different effects on the infected cells when compared with a pure COX-2 inhibitor. In fact, an in vivo study demonstrated that defects of COX-1 or COX-2 may lead to opposite effects in influenza H3N2 infected mice. While COX-1 deficiency was detrimental to influenza infected mice, COX-2 deficiency improved survival (Carey et al., 2005). A more recent study from the same group showed that the lung viral titers were found to be similar between COX-1 inhibitor, COX-2 inhibitor and mock-treated H3N2 infected mice. While COX-1 inhibitor treatment was detrimental to the host, outcome in COX-2 inhibitor treated mice was comparable to that of controls (Carey et al., 2010). In fact, this data correlates well with our findings and suggests that influenza A viruses which are high COX-2 inducers (such as H5N1 and H9N2 viruses) are more dependent on COX-2 pathways for replication compared with the relatively low COX-2 inducer viruses (such as seasonal H1N1 and H3N2 viruses) and may explain why these low COX-2 inducer viruses are less susceptible to selective COX-2 inhibitor treatment.

Currently, antiviral drugs such as the adamantanes and neuraminidase inhibitors are the mainstay used for treating H5N1 patients. However, even when patients are treated with oseltamivir within the first 4 days of illness, survival is less than 50% (Kandun et al., 2008). Thus, there is a clear need for adjunctive treatment modalities that may synergize with conventional antiviral therapies. Selective COX-2 inhibitors which have both anti-viral and anti-inflammatory effects on H5N1 infection may be one such strategy that deserves to be explored, in combination with conventional antiviral treatments. A COX-2 inhibitor, celecoxib, in combination with mesalazine and the antiviral drug zanamivir has been shown to significantly improve survival of mice infected with H5N1 virus (Zheng et al., 2008). In that paper, the beneficial effects of celecoxib and mesalazine were assumed to be solely due to their immunomodulatory and anti-apoptotic activity rather than to an antiviral effect. Our findings imply that the antiviral effect of COX-2 inhibitors should also be considered in explaining such findings and a detailed investigation, such as a time-dependent study on drug administration in animal models will deserve further examination.

In conclusion, we demonstrate that a selective COX-2 inhibitor has a markedly suppressive effect on avian H5N1 viral replication in mammalian cells, but less so on human adapted seasonal influenza viruses. Taken in conjunction with its immuno-modulatory effects (Lee et al., 2008), the drug may be relevant as adjunctive therapy in patients with H5N1 disease. However, a number of studies have highlighted the role of COX-2 in the resolution of late stage inflammation (Chan and Moore, 2010; Fukunaga et al., 2005; Mochizuki et al., 2005) and it is possible that inhibition of COX-2 may have detrimental effects in treating acute lung injury (Lukkarinen et al., 2006). Thus, future work should aim to identify the host factors in COX-2 induced downstream pathways that are crucial for H5N1 virus replication and this may lead to a separation of the antiviral effects from those responsible for the induction of pro-resolution mediators that are important for recovery from lung injury. These observations highlights the relevance of targeting host factors that affect viral replication as potentially novel therapeutic targets since this strategy is less likely to be associated with emergence of drug resistant strains (Tan et al., 2007).

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The authors declare no competing interests.

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