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Inhibition of RANTES expression by indirubin in influenza virus-infected human bronchial epithelial cells

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Abstract

The human bronchial epithelial cells are the primary sites of influenza virus infection. In this study, the effect of indirubin on the expression of the chemokine regulated on activation, normal T cell expressed and secreted (RANTES) by the influenza virus-infected H292 human epithelial cell line was examined. The expression of RANTES mRNA was analyzed using reverse transcription polymerase chain reaction and the concentration of RANTES production was determined by the enzyme-linked immunosorbent assay. At the non-cytotoxic concentrations, indirubin was found to reduce both the expression and production of RANTES in influenza A/NWS/33-infected H292 cells. Inhibition was also observed in influenza virus B/Lee-infected cells. Significant reduction of the expression of IL-8 was not observed after the infection. Indirubin-3′-oxime, a recently developed derivative with kinase inhibitory activity, also mediates a potent inhibitory effect on the expression of RANTES. The influenza virus infection-induced phosphorylation of the nuclear transcription NF-kB regulatory molecule IkBα and the p38 MAP kinase were also found to be inhibited by indirubin-3′-oxime. This finding suggests that indirubin is one of the components in the Chinese medicinal herbs *Isatis indigotica* and *Strobilanthes cusia* with immunomodulatory activity on the expression of RANTES.

Keywords: Indirubin; Indirubin-3′-oxime; RANTES; p38 MAP kinase; IkB; Influenza

1. Introduction

Influenza A virus causes the infection of upper respiratory tract. The virus-infected epithelial cells and macrophages have been shown to produce various biologically active cytokines and chemokines. These include the chemotactic cytokines (RANTES, MCP-1, MCP-3, MIP-alpha, and IP-10), pro-inflammatory cytokines (IL-1β, IL-6, IL-18, and TNF-α), and antiviral cytokines (IFN-α/β) [1]. RANTES, a potent chemoattractant for monocytes, basophils and T cells, was found in nasal secretions from patients suffering from upper respiratory tract infection with influenza virus, parainfluenza virus, adenovirus, and RSV [2]. RANTES has been shown to play an important role in the epithelial cell-mediated inflammatory process during viral infection [3]. It plays a role in the production of allergic inflammation through its chemotactic effects. RANTES is produced by various cell types. These include epithelial cells, $CD8^+$ T cells, $CD4^+$ T cells, as well as monocytes/macrophages.

Indirubin, a naturally occurring pigment, is found in many indigo-producing plants, such as *Isatis indigotica*, *Strobilanthes cusia*, and *Polygonum tinctorium*. The plants have been used as Chinese medicine to treat respiratory viral infection and some other diseases with inflammatory nature for a long time. Recent studies showed that indirubin inhibits 2,4,6-trinitro-l-chlorobenzene (TNBC)-induced inflammatory reactions in mice [4]. In influenza virus infection, airway epithelial cells are the initial sites of

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Abbreviations: RANTES, regulated on activation, normal T cell expressed and secreted; RT–PCR, reverse transcription polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

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infection. The infected cells are suggested to participate in airway inflammatory response by expressing various cytokines, including RANTES. Results from the *in vitro* studies also demonstrated the expression and secretion of RANTES by normal human bronchial and nasal epithelial cells [5]. Because the chemokine RANTES plays an important role in both activating and recruiting leukocytes into inflammatory sites, inhibition of RANTES production has been implicated to produce a beneficial effect on controlling bronchial asthma exacerbation caused by influenza virus infection [6]. Since infected epithelial cells produce a limited number of cytokines [1], we focused our studies to examine the effects of indirubin on the infection-induced expression and production of RANTES. Using a human bronchial epithelial H292 cell line, here we show that indirubin inhibits the expression and secretion of RANTES by both influenza A and B virus-infected cells.

2. Materials and methods

2.1. Cell culture

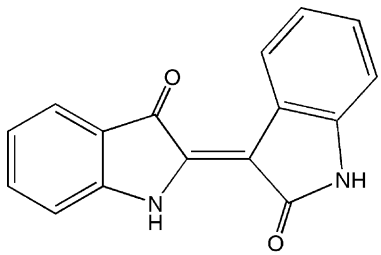
The human H292 cell line (NCI-H292 from ATCC) was grown in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and antibiotics (50 units/mL penicillin, 50 μg/mL streptomycin, and 10 μg/mL neomycin). The cells were incubated at 37° in a 5% humidified CO_2 incubator.

2.2. Viruses

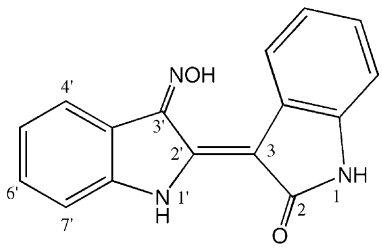
The influenza virus A/NWS/33 (ATCC VR-219) and influenza B/Lee/40 (ATCC VR-101) were grown in the allantoic sac of 10-day-old chick embryo for 44 hr. Infectious allantoic fluid was harvested and distributed into small ampoules and stored at −70° until use. Virus titers of A/NWS/33 are expressed as plaque forming units (PFU). Since the B/Lee used in this study does not form a well-defined plaque on MDCK cells, the titers are expressed as hemagglutinating units (HAU).

2.3. Chemicals

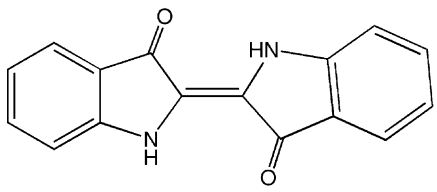
Indirubin was obtained from National Institute for the Control of Pharmaceutical & Biological Products, China. Indirubin-3′-oxime was synthesized as previously described [7]. The synthesized indirubin-3′-oxime has the following properties: deep red crystals; ^{1}H NMR (400 MHz, DMSO-d_6): 13.47 (1H, s, NOH), 11.72 and 10.71 (each 1H, s, H-1 and H-1′), 8.64 (1H, d, $J = 8.0$ Hz, H-4), 8.22 (1H, d, $J = 8.0$ Hz, H-4′), 7.40 (2H, m, H-6′ and H-7′), 7.12 (1H, t, $J = 8.0$ Hz, H-6), 7.02 (1H, m, H-5′), 6.96 (1H, t, $J = 8.0$ Hz, H-5), 6.89 (1H, d, $J = 8.0$ Hz, H-7); negative ESI-MS (%): 276 $[M - H]^-$ (40), 246 $[M - H - NO]^-$ (100). ^{1}H NMR spectrum was measured on Bruker DRX-400 with DMSO-d_6 as solvent and TMS as an internal reference. ESI-MS was taken in negative ion mode on an API 2000 LC/MS/MS instrument.



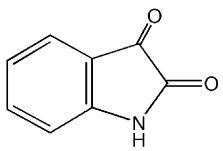
Indirubin



Indirubin-3'-oxime



Indigo



Isatin

2.4. Reverse transcription polymerase chain reaction (RT–PCR)

Guanidinium thiocyanate–cesium chloride ultracentrifugation method was used to isolate total RNA [8]. RNA samples with the $OD_{260\ nm}$:$OD_{280\ nm}$ ratio of 1.90–2.10 were stored at −70° until use. Three micrograms of total RNA in 30 μL of DEPC-treated double-distilled water was incubated at 65° for 5 min and then chilled on ice

immediately. The heat denatured total RNA was used to perform the reverse transcription reaction in a 60 μL reaction, containing 200 U of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Life Technology), 0.2 mM of each dNTP, 0.3 μg oligo $(dT)_{12–18}$, 1 × first-strand buffer, 10 mM dithiothreitol, and 40 U RNaseOUT (Invitrogen). The reaction mixture was incubated at 37° for 1 hr. The RT sample was then diluted 10-fold and boiled for 5 min and chilled on ice before use for PCR. The reverse transcribed sample was stored at −70° until use. In PCR, 30 μL of master mix containing 1 × reaction buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1.25 U Thermoprime^{PLUS} DNA polymerase (ABgene), and 50 pmol of upper and lower primers was added into 20 μL of boiled RT sample containing 0.1 μg of total RNA. PCR was performed by initial denaturation of the template at 94° for 2 min and followed by thermal cycle: 94° for 30 s, annealing at 56° for 30 s, and at 72° for 1 min. The house keeping gene GAPDH was amplified for 20 cycles, while the other genes were amplified for 30 or 33 cycles as indicated. Twenty microliters of amplified product was analyzed by electrophoresis on a 2% agarose gel. GAPDH was used for the normalization of the RNA samples. All of the analyses were performed for two to three times. The sequences of the primers and the PCR conditions were shown in Tables 1 and 2.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The levels of RANTES produced by infected H292 cells were measured using the h-RANTES ELISA system (Amersham Pharmacia Biotech). Briefly, overnight cultured H292 cells (4×10^6) in 75 cm^2 tissue culture flasks were infected with influenza virus for 1 hr at 37°. The infected cell monolayer was then washed and incubated with various concentrations of indirubin. The cell free culture supernatant was harvested at 24 hr after incubation. The concentration of RANTES in the supernatant was determined according to the procedures provided by the manufacturer. All samples were determined in triplicate.

Table 1
The sequence of PCR primers and the relative size (base pairs) of the amplified fragments

Genes	Primer sequence	Base pairs
RANTES	5′-ATG AAG GTC TCC GCG GCA CGC CT-3′ 5′-CTA GCT CAT CTC CAA AGA GTT G-3′	276
IL-8	5′-CGA TGT CAG TGC ATA AAG ACA-3′ 5′-TGA ATT CTC AGC CCT CTT CAA AAA-3′	200
GAPDH	5′-ACC ACA GTC CAT GCC ATC AC-3′ 5′-TCC ACC ACC CTG TTG CTG TA-3′	452

Table 2
The PCR conditions for the amplification of the genes

Primers	Annealing temperature (°)	Concentration of $MgCl_2$ (mM)	Amplification cycles
RANTES	60	1.5	30
IL-8	60	1.5	30
GADPH	56	1.5	20

2.6. Western blot analysis of IkBα and p38 MAP kinase

H292 cells were infected with various concentrations of influenza virus A/NWS/33 (0.5–2 PFU/cell) for 6–24 hr. The treated cells were washed with two to three changes of cold PBS before cell lysis with the lysis buffer (250 mM Tris–HCL, pH 8, 1% NP-40, and 150 mM NaCl). The cell lysate containing 20 μg of total cellular protein was subject to electrophoresis in 10% SDS–PAGE. The separated proteins were electrophoretically blotted onto membrane. Western blot was conducted using the ECL™ Western Blotting Detection Reagents (Amersham Biosciences, RPN2209). The primary antibodies were rabbit antibodies specific for IkBα and phospho-IkBα (Calbiochem). The procedures were followed according to the recommended protocol for Western blotting provided by the manufacturer. Rabbit anti-phospho-p38 MAP kinase (Thr^{180}/Tyr^{182}) affinity purified polyclonal antibody was obtained from Chemicon.

3. Results

3.1. Effect of indirubin on the expression of IL-8 and RANTES mRNA in influenza A virus-infected cells

It has been previously demonstrated that influenza virus infection stimulates the production of chemotactic cytokines IL-8 and RANTES from human bronchial epithelial cells [9]. To determine whether indirubin could modulate the production of cytokines during influenza virus infection, the influenza A/NWS/33-infected H292 bronchial epithelial cells were treated with indirubin for 24 hr. The expression of RANTES was not observed in the uninfected H292 cells (Fig. 1). Infection of H292 cells with A/NWS/33 significantly up-regulated the expression of RANTES. A 4-fold reduction of expression of RANTES was observed when the infected cells were treated with 100 μM indirubin. In contrast, a low level of expression of IL-8 was detected in the uninfected H292 cells. A 6-fold increase in IL-8 expression was seen after infection. Treatment of the infected cells with indirubin had no significant effect on the expression of IL-8. These results suggest that indirubin exerts a selective inhibition on the expression of RANTES.

To ascertain the inhibitory effect of indirubin during the virus infection, influenza B/Lee-infected H292 cells were treated with indirubin. A dose-dependent (50–200 μM) inhibition of RANTES expression was also observed in the infected H292 cells (Fig. 2).

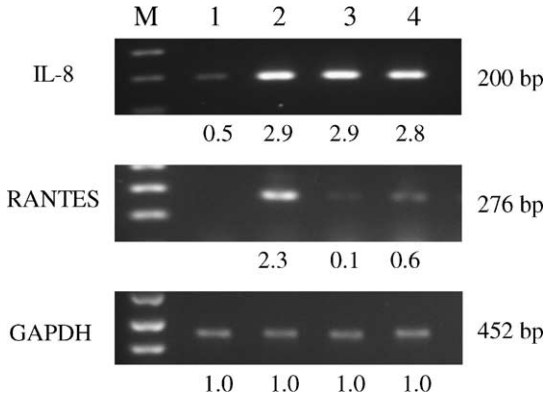


Fig. 1. Effect of indirubin on the expression of IL-8, and RANTES in influenza virus-infected H292 cells. A/NWS/33 virus (0.5 PFU/cell)-infected H292 cells were treated with indirubin (100–200 μM) for 24 hr. Total RNA samples were used for RT–PCR analysis and the amplified products were analyzed on 1% agarose gel as described. The intensity of the DNA bands was estimated by the gel documentation system. M, 1 kb plus DNA ladders. Lane 1, uninfected H292 cells; lane 2, H292 infected with A/NWS/33; lane 3, infected H292 + indirubin (200 μM); and lane 4, infected H292 + indirubin (100 μM).

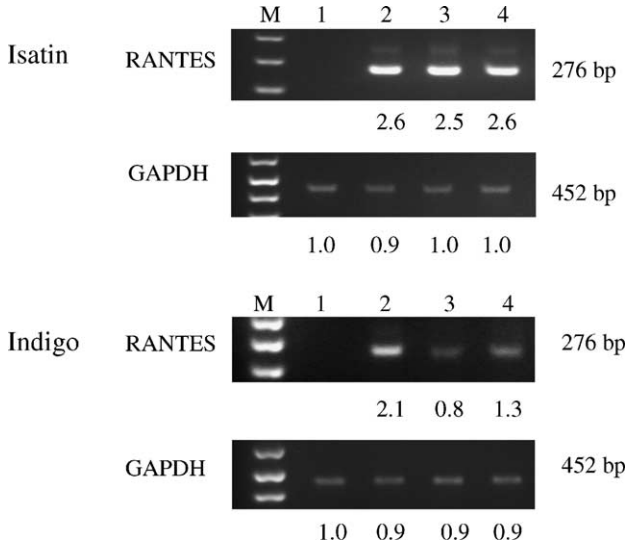


Fig. 3. Failure of inhibition of expression of RANTES by isatin. A/NWS/33-infected H292 cells were treated with indigo or isatin (100–200 μM) for 24 hr. The expression of RANTES mRNA was analyzed. M, 1 kb plus DNA ladders. Lane 1, uninfected H292; lane 2, A/NWS/33 infected H292, lane 3, isatin or indigo (200 μM), and lane 4, isatin or indigo (100 μM).

3.2. Effects of indirubin, indigo, and isatin on the expression of RANTES mRNA

To further examine the inhibitory effect of indirubin, we have compared the activity of indirubin with its isomer indigo and the precursor molecule isatin on the production of RANTES. A similar dose-dependent inhibition of RANTES mRNA expression was observed in indigo-treated A/NWS/33 influenza virus-infected cells (Fig. 3). However, the precursor molecule isatin was ineffective in reducing the expression of RANTES mRNA.

3.3. Effects of indirubin analogue on mRNA and protein expression of RANTES in infected cells

We next studied the effect of indirubin-3′-oxime, a synthetic indirubin analogue, on the expression of RANTES. This compound has an improved solubility than indirubin [7]. H292 cells were infected with influenza A/NSW/33, and the infected cells were then treated with indirubin-3′-oxime (1 nM–10 μM). A dose-dependent (10 nM–10 μM) inhibition of expression of both RANTES mRNA and the protein was observed in the infected cell culture (Fig. 4).

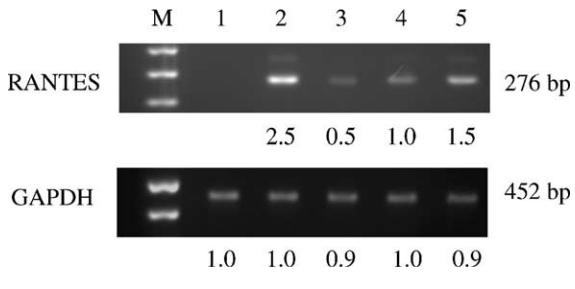


Fig. 2. Effect of indirubin on the expression of RANTES mRNA in influenza B/Lee-infected cells. H292 cells were exposed to B/Lee (20 HAU/10^6 cells) for 1 hr. The infected cells were washed and treated with indirubin for 24 hr. PCR was performed as described in Section 2. M, 1 kb plus DNA ladders. Lane 1, uninfected H292; lane 2, H292 infected with B/Lee; lanes 3–5, infected H292 + indirubin (200, 100, and 50 μM, respectively).

3.4. Production of RANTES by infected cells

To ascertain the inhibition on the expression of RANTES is correlated with the reduced production of RANTES, ELISA was performed to measure the level of RANTES in the infected cell culture supernatant. Table 3 shows the results on the effect of indirubin on the production of RANTES by influenza A and B virus-infected cells. RANTES was not detected in the uninfected cell culture supernatant. Infection of the H292 cells with influenza A/NWS/33 or B/Lee virus resulted in the production and

Table 3
Inhibition of RANTES production by indirubin

	Infection (A/NWS/33)	Treatment indirubin (μM)	RANTES (pg/mL)
A/NWS/33	−	−	0.2 ± 0.4
	+	−	217 ± 2.1*
	+	100	158 ± 13.4#
	+	200	152 ± 9.3#
B/Lee	−	−	0.09 ± 0.01
	+	−	180.7 ± 9.5*
	+	100	102.7 ± 6.4#
	+	200	69.3 ± 4.2#

H292 cells were infected with influenza A/NWS/33 (0.5 PFU/cell) or B/Lee (20 HAU/10^6 cells) in 75 cm^2 culture flasks. Cell culture supernatant was collected at 24 hr after infection. Results were expressed as mean ± SD from three replicates. (#), (*): $P = 0.05$.

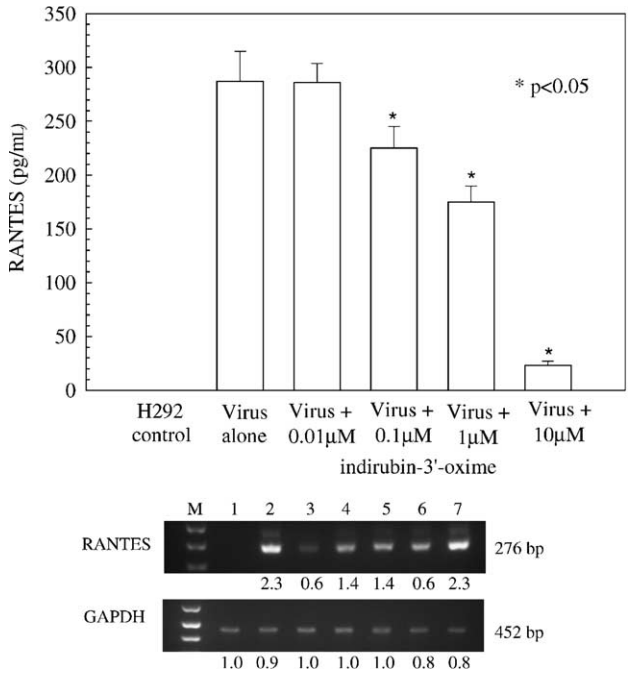


Fig. 4. Effects of indirubin-3′-oxime on the expression and production of RANTES in infected cells. H292 cells were infected with influenza A virus as described in Figs. 2 and 3. The infected cells were treated with various concentrations of indirubin-3′-oxime (0.01–10 μM). RANTES concentrations in cell culture supernatants were determined by ELISA (mean ± SD from three replicates). M, 1 kb plus DNA ladders. Lane 1, uninfected H292; lane 2, H292 infected with A/NWS/33; lanes 3–7, infected H292 + indirubin-3′-oxime (10, 1, and 0.1 μM, and 10 and 1 nM, respectively). $^{*}P < 0.05$, significantly different from the control group (virus + indirubin-3′-oxime).

secretion of RANTES into the culture medium. Significant reduction of production of RANTES was observed in indirubin-treated infected cell cultures. The reduction of the RANTES protein is correlated with the reduced expression of the RANTES mRNA in the infected cells.

3.5. Effect of indirubin-3′-oxime on influenza virus infection-induced expression of phospho-IkB and p38 MAP kinase

It has been previously shown that the gene expression of many inflammatory mediators is influenced by the nuclear transcription activities, such as the nuclear transcription factor NF-kB. The activity of NF-kB is regulated by the phosphorylation state of the regulatory molecule IkB. During influenza virus infection, both of the NF-kB pathway and the stress-induced MAP kinase pathway have been shown to be involved in the regulation of RANTES expression. Inhibition of the transcription and production of RANTES may be due to the inhibition of the expression of the phospho-IkB, and p38 MAP kinase. To test this possibility, the H292 cells were infected with influenza virus A/NWS/33 and the infected cells were then treated with a more soluble derivative indirubin-3′oxime. Influenza virus infection-induced phosphorylation of p38 MAP kinase and IkBα was measured at 6 and 24 hr after infection, respectively. Results in Figs. 5 and 6 showed that the levels of phosphorylated IkBα and p38 MAP kinase were increased after influenza virus infection. Treatment of the infected cell culture with indirubin-3′-oxime (10 μM) resulted in the reduction of phosphorylation of the IkB and p38 MAP kinase.

4. Discussion

Bronchial epithelial cell-derived cytokines and chemokines have been shown to play an important role in regulating the inflammatory response during respiratory infection. Infection of airway epithelial cells and leukocytes with influenza virus results in the production of cytokines and chemokines that promote airway inflammation [1,5,10–12]. RANTES is a C–C chemokine. It is a potent chemoattractant for eosinophils, monocytes, and T lymphocytes. RANTES has been shown to be induced and secreted in the bronchial mucosa of patients infected with influenza virus. During influenza virus infection, monocytes/macrophages and lymphocytes were found selectively infiltrated into the infected tissues [13]. The production of RANTES is implicated in the pathogenesis of airway that is caused by the infiltration of inflammatory cells during influenza virus infection [5,14].

The bronchial epithelial cell line H292, derived from human pulmonary mucoepidermoid carcinoma, has been

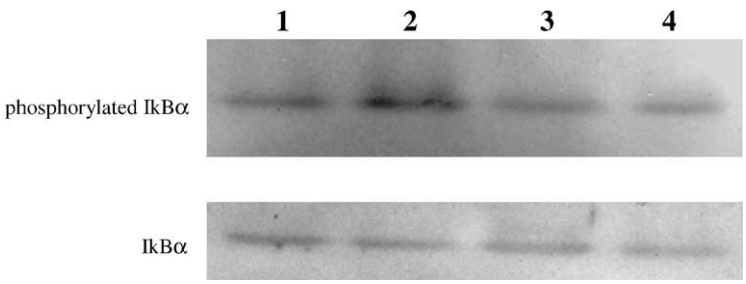


Fig. 5. Phosphorylation of IkBα in influenza virus-infected cells. Influenza virus A/NWS/33-infected H292 cells (0.5 PFU/cell) were treated with indirubin-3′-oxime (10 μM) for 24 hr. The cell lysates were subjected to PAGE and Western blotting with anti-IkBα or anti-phospho-IkBα antibody. Each lane was loaded with an equal amount (20 μg) of total cellular protein. Lane 1, H292 cell alone; lane 2, A/NWS/33-infected H292 cells; lane 3, H292 cells treated with indirubin-3′-oxime (10 μM); and lane 4, A/NWS/33-infected H292 cells + indirubin-3′-oxime (10 μM).

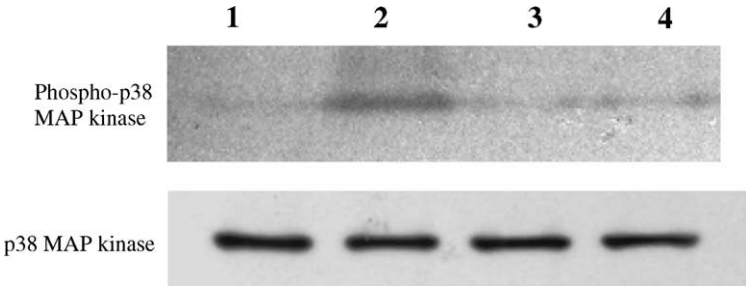


Fig. 6. Phosphorylation of p38 MAP kinase in influenza virus-infected H292 cells. Influenza virus A/NWS/33-infected H292 cells (2 PFU/cell) were treated with indirubin-3′-oxime (10 μM) for 6 hr. Total cellular proteins were prepared from the treated cells. Twenty micrograms of total cellular protein from each sample was subjected to PAGE and Western blotting analysis using anti-p38 MAP kinase or anti-phospho-p38 MAP kinase antibody. Lane 1, H292 cell alone; lane 2, A/NWS/33-infected H292 cells; lane 3, H292 cells treated with indirubin-3′-oxime (10 μM); and lane 4, A/NWS/33-infected H292 cells + indirubin-3′-oxime (10 μM).

shown to exhibit many characteristics common to human bronchial epithelial cells [15,16]. The H292 cells are sensitive to many viruses, including paramyxovirus and certain strains of influenza A viruses. Similar to normal human epithelial cells, influenza virus-infected H292 cells also produce the chemoattractant RANTES [6]. Indirubin from *Indigofera tinctoria* is useful for the treatment of chronic myelocytic leukemia [17]. Recent study shows that indirubin is a compound with anti-inflammatory effects [4]. It is also one of the components in the Chinese medicinal herbs Banlangen, a common name refers to the medicinal herbs *S. cusia* and *I. indigotica*. Banlangen is commonly used in mainland China for the treatment of various viral diseases, including influenza and mumps. Since the effect of indirubin on the expression of cytokines has not been studied, we focused our study to examine the effect of indirubin during viral infection. We infected the H292 epithelial cells with influenza virus and examined the effect of indirubin on the expression of the chemokine RANTES. Since the indirubin analogue (indirubin-3′-oxime) has recently been shown to have a more potent activity than indirubin [7], this compound is also used for a more detailed study. We found that both of the indirubin, the isomer indigo, and indirubin-3′-oxime reduce the expression and secretion of RANTES in influenza A and influenza B virus-infected epithelial cells. The effective concentration of indirubin-3′-oxime is lower than indirubin. The results indicate that inhibition of transcription and production of the C–C chemokine RANTES is one of the anti-inflammatory mechanisms of indirubins.

During influenza virus infection, the viral RNA and the viral proteins have been shown to activate the nuclear transcription factor NF-kB [18]. Under normal condition, translocation of cytoplasmic NF-kB to the nucleus is prevented by the formation of complex with the inhibitor IkB. Phosphorylation of IkB by IkB kinase (IKK) results in the release of NF-kB from the complex, and the subsequent translocation of NF-kB to the nucleus for the gene activation. It has also been demonstrated that the phosphorylation of IkBα, a member of IkB family, is increased in cells expressing influenza virus proteins, such as hemagglutinin and matrix [19] since the expression of chemokine gene RANTES is enhanced by NF-kB [20]. Inhibition of the kinase activity of IKK might prevent the phosphorylation of IkB and the subsequent activation of RANTES expression. Results from this study clearly showed that the level of phospho-IkBα was reduced in indirubin-3′-oxime-treated and influenza virus-infected epithelial cells. Recent studies also show that indirubin and its derivatives (e.g. indirubin-3′-oxime) are inhibitors of certain protein kinases. The crystal structure of cyclin-dependent kinases CDK2 in complex with indirubin derivatives shows that indirubin interacts with the kinase's ATP-binding site through van der Waals interactions and three hydrogen bonds [7]. Inhibition of kinase activity in human tumor cells is a major mechanism by which indirubin derivatives exert their potent anti-tumor efficacy [21]. In addition, the kinase inhibitory activity of indirubin and its derivatives was also observed for the enzyme glycogen synthase kinase-3beta (GSK-3β) [22]. Thus, if the inhibition of RANTES expression is due to the kinase inhibitory activity of indirubin, it is logic that indirubin may also reduce the phosphorylated form of kinases involved in the RANTES production pathways. Recently, the intracellular signals involved in the regulation of RANTES production by influenza virus-infected airway epithelial cells have been studied [23]. Among the three well-characterized molecularly stress-induced MAP kinase signaling pathways (p38 MAP kinase, c-*jun*-NH_2-terminal kinase (JNK), and extracellular signal-regulated kinase (Erk)), influenza virus infection was found to increase both of the phosphorylation and activities of p38 MAP kinase and JNK. Each of these MAP kinase subfamilies is activated by specific upstream MAP kinase kinases (MKKs), which dually phosphorylate MAP kinases on a threonine and tyrosine residue separated by an intervening amino acid characteristic for each MAP kinase subfamily. In the case of influenza virus infection, both p38 MAP kinase and JNK were found to regulate, at least in part, RANTES production in influenza virus-infected bronchial epithelial cells [23]. In the present study, influenza virus was also found to increase the cytoplasmic phospho-p38 MAP kinase in the infected H292 cells. The reduced transcription

of RANTES mRNA and production of RANTES is correlated with the reduced level of phospho-p38 MAP kinase in indirubin-3′-oxime-treated and influenza virus-infected H292 cells. Collectively, this observation further supports our hypothesis that the inhibitory effect of indirubins is due to the kinase inhibitory activity of indirubins.

During influenza virus infection, infected epithelial cell has been shown to express a wide variety of cytokines, including the chemotactic peptide IL-8. In this study, we found that influenza virus-induced expression of IL-8 was not affected by the treatment of indirubin. The indirubin analogue indirubin-3′-oxime has also no inhibitory activity on the expression and production of IL-8 by the infected H292 (data not shown). Although the NF-kB signaling pathway has been shown to be involved in respiratory virus-induced expression of RANTES and IL-8, these two cytokines appear to be differentially regulated during the infection [20,24]. Differential expression of IL-8 and RANTES was also observed in eotaxin-stimulated H292 cells [25]. In addition, IL-8 gene expression has also been shown to require the coordinate action of at least three different signal transduction pathways which cooperate to induce mRNA synthesis [26]. The involvement of other signaling pathways and the differential sensitivity of the various protein kinases, such as IKK-α and IKK-β, to the inhibitory effect of indirubin may explain the ineffective inhibitory action of indirubin on the expression of IL-8 [7,27].

In conclusion, indirubin inhibits the transcription and production of RANTES by influenza virus-infected human bronchial epithelial cells. The intervention of virus-induced p38 MAP kinase activation and NF-kB translocation by indirubin and its analogues may be used as a strategy in controlling virus-induced bronchial asthma.

Acknowledgments

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References

[1] Julkunen I, Sareneva T, Pirhonen J, Ronni T, Melen K, Matikainen S. Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. Cytokine Growth Factor Rev 2001;12:171–80.

[2] Bonville CA, Rosenberg HF, Domachowske JB. Macrophage inflammatory protein-1alpha and RANTES are present in nasal secretions during ongoing upper respiratory tract infection. Pediatr Allergy Immunol 1999;10:39–44.

[3] Becker S, Reed W, Henderson FW, Noah TL. RSV infection of human airway epithelial cells causes production of the beta-chemokine RANTES. Am J Physiol 1997;272:L512–20.

[4] Kunikata T, Tatefuji T, Aga H, Iwaki K, Ikeda M, Kurimoto M. Indirubin inhibits inflammatory reactions in delayed-type hypersensitivity. Eur J Pharmacol 2000;410:93–100.

[5] Matsukura S, Kokubu F, Kubo H, Tomita T, Tokunaga H, Kadokura M, Yamamoto T, Kuroiwa Y, Ohno T, Suzaki H, Adachi M. Expression of RANTES by normal airway epithelial cells after influenza virus A infection. Am J Respir Cell Mol Biol 1998;18:255–64.

[6] Asai Y, Hashimoto S, Kujime K, Gon Y, Mizumura K, Shimizu K, Horie T. Amantadine inhibits RANTES production by influenzavirus-infected human bronchial epithelial cells. Br J Pharmacol 2001;132:918–24.

[7] Hoessel R, Leclerc S, Endicott JA, Nobel ME, Lawrie A, Tunnah P, Leost M, Damiens E, Marie D, Marko D, Niederberger E, Tang W, Eisenbrand G, Meijer L. Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. Nat Cell Biol 1999;1:60–7.

[8] Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 1979;18:5294–9.

[9] Matsukura S, Kokubu F, Noda H, Tokunaga H, Adachi M. Expression of IL-6, IL-8, and RANTES on human bronchial epithelial cells, NCI-H292, induced by influenza virus A. J Allergy Clin Immunol 1996;98:1080–7.

[10] Julkunen I, Melen K, Nyqvist M, Pirhonen J, Sareneva T, Matikainen S. Inflammatory responses in influenza A virus infection. Vaccine 2000;19(Suppl 1):S32–7.

[11] Van Reeth K. Cytokines in the pathogenesis of influenza. Vet Microbiol 2000;74:109–16.

[12] Lillard Jr JW, Boyaka PN, Taub DD, McGhee JR. RANTES potentiates antigen-specific mucosal immune responses. J Immunol 2001;166:162–9.

[13] Sprenger H, Meyer RG, Kaufmann A, Bussfeld D, Rischkowsky E, Gemsa D. Selective induction of monocyte and not neutrophil-attracting chemokines after influenza A virus infection. J Exp Med 1996;184:1191–6.

[14] Adachi M, Matsukura S, Tokunaga H, Kokubu F. Expression of cytokines on human bronchial epithelial cells induced by influenza virus A. Int Arch Allergy Immunol 1997;113:307–11.

[15] Castells E, George VG, Hierholzer JC. NCI-H292 as an alternative cell line for the isolation and propagation of the human paramyxoviruses. Arch Virol 1990;115:277–88.

[16] Hierholzer JC, Castells E, Banks GG, Bryan JA, McEwen CT. Sensitivity of NCI-H292 human lung mucoepidermoid cells for respiratory and other human viruses. J Clin Microbiol 1993;31:1504–10.

[17] Han R. Highlight on the studies of anticancer drugs derived from plants in China. Stem Cells 1994;12:53–63.

[18] Matikainen S, Pirhonen J, Miettinen M, Lehtonen A, Govenius-Vintola C, Sareneva T, Julkunen I. Influenza A and Sendai viruses induce differential chemokine gene expression and transcription factor activation in human macrophages [record supplied by publisher]. Virology 2000;276:138–47.

[19] Flory E, Kunz M, Scheller C, Jassoy C, Stauber R, Rapp UR, Ludwig S. Influenza virus-induced NF-kappaB-dependent gene expression is mediated by overexpression of viral proteins and involves oxidative radicals and activation of IkappaB kinase. J Biol Chem 2000;275:8307–14.

[20] Thomas LH, Friedland JS, Sharland M, Becker S. Respiratory syncytial virus-induced RANTES production from human bronchial epithelial cells is dependent on nuclear factor-kappa B nuclear binding and is inhibited by adenovirus-mediated expression of inhibitor of kappa B alpha. J Immunol 1998;161:1007–16.

[21] Marko D, Schatzle S, Friedel A, Genzlinger A, Zankl H, Meijer L, Eisenbrand G. Inhibition of cyclin-dependent kinase 1 (CDK1) by indirubin derivatives in human tumour cells. Br J Cancer 2001;84:283–9.

[22] Leclerc S, Garnier M, Hoessel R, Marko D, Bibb JA, Snyder GL, Greengard P, Biernat J, Wu YZ, Mandelkow EM, Eisenbrand G, Meijer L. Indirubins inhibit glycogen synthase kinase-3 beta and CDK5/p25, two protein kinases involved in abnormal tau phosphor-

ylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors? J Biol Chem 2001;276:251–60.

[23] Kujime K, Hashimoto S, Gon Y, Shimizu K, Horie T. p38 mitogen-activated protein kinase and c-jun-NH_2-terminal kinase regulate RANTES production by influenza virus-infected human bronchial epithelial cells. J Immunol 2000;164:3222–8.

[24] Gern JE, French DA, Grindle KA, Brockman-Schneider RA, Konno S, Busse WW. Double-stranded RNA induces the synthesis of specific chemokines by bronchial epithelial cells. Am J Respir Cell Mol Biol 2003;28:731–7.

[25] Cui CH, Adachi T, Oyamada H, Kamada Y, Kuwasaki T, Yamada Y, Saito N, Kayaba H, Chihara J. The role of mitogen-activated protein kinases in eotaxin-induced cytokine production from bronchial epithelial cells. Am J Respir Cell Mol Biol 2002;27:329–35.

[26] Holtmann H, Winzen R, Holland P, Eickemeier S, Hoffmann E, Wallach D, Malinin NL, Cooper JA, Resch K, Kracht M. Induction of interleukin-8 synthesis integrates effects on transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. Mol Cell Biol 1999;19:6742–53.

[27] Escotte S, Tabary O, Dusser D, Majer-Teboul C, Puchelle E, Jacquot J. Fluticasone reduces IL-6 and IL-8 production of cystic fibrosis bronchial epithelial cells via IKK-beta kinase pathway. Eur Respir J 2003;21:574–81.