Future Target Molecules for Influenza Treatment

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Abstract: Induction of apoptosis and pro-inflammatory cytokine gene expression in influenza virus-infected cells activates production of toxic superoxide by macrophages. Pyrrolidine dithiocarbamate and nordihydroguaiaretic acid inhibit influenza virus proliferation and scavenge superoxide. These results suggest that they can be potential candidates for a drug of choice for influenza chemotherapy.

Key Words: Influenza virus, chemotherapy, antioxidant, apoptosis, cytokines, superoxide, macrophages.

INTRODUCTION

Influenza is a serious debilitating respiratory illness accompanying with complications that lead to hospitalization and death [1]. Currently, two classes of anti-influenza drugs have been used for chemoprophylaxis and treatment of influenza [2, 3]; that is, viral membrane protein 2 (M2) inhibitors as the first-generation (amantadine and rimantadine), and viral neuraminidase (NA) inhibitors as the second-generation (oseltamivir and zanamivir). The use of anti-influenza drugs has been receiving much greater attention as a first-line defense against a new pandemic of influenza virus infection [2, 4]. However, influenza viruses resistant to the M2 and NA inhibitors frequently emerge during treatments of patients with the drugs [2, 5]. This suggests the need for development of the third-generation anti-influenza drugs based on alternative antiviral mechanisms that have not been considered for the therapy.

MOLECULAR PATHOGENESIS OF INFLUENZA VIRUS INFECTION

Various types of pathological manifestations are observed after influenza virus infection as a result of complex biological consequences, such as apoptosis induction in the infected cells, macrophage activation by cytokines derived from the infected cells and tissue injury by superoxide in the infected organs [6-8]. Apoptosis induction has been defined as a process for eliminating dying cells without inducing an inflammatory response [9]. However, this conventional definition may not be fit in a certain situation, such as pathogen invasion that induces an inflammatory response, resulting in the activation of an immune response [10]. Influenza virus infection induces not only apoptosis but also the expression of pro-inflammatory and monocyte chemoattractive cytokine genes in certain types of cells, such as monocytes/macrophages, bronchial epithelial cells and fetal membrane chorion cells, as listed in Table **1** [8, 11-18]. Ribavirin (1-β-D-ribofranosyl-1, 2, 4-triazole-3-carboxamide) inhibits influenza virus RNA-dependent RNA polymerase (RDRP) activity [19], resulting in the inhibition of both replication and transcription of the virus genes [20]. Furthermore, ribavirin inhibits the induction of apoptosis [11] and pro-inflammatory cytokine gene expression [21] in the virus-infected chorion cells. Consequently, influenza virus gene replication and transcription by viral RDRP are prerequisite for the induction of apoptosis and pro-inflammatory cytokine gene expression in the infected cells.

The release of virus particles is directly proportional to the expression of viral hemagglutinin (HA) virion RNA before initiating apoptotic cellular degradation, thereafter no virus particle release is observed even HA vRNA synthesis still continues [20]. Our unpublished data show that influenza virus infection induces the cleavage of pro-caspase-3 protein into an active form, oligonucleosomal DNA fragmentation and the accumulation of immunoglobulin heavy-chain binding protein (BiP), a major molecular chaperon in the lumen of the endoplasmic reticulum (ER), in the cultured chorion cells. Significant levels of the uncleaved form of viral HA polypeptide (HA0) are detected in trypsinized cells after the virus infection. A general caspase inhibitor, *N-t*-Boc-Asp(OMe)-fluoromethyl ketone, inhibits the cleavage of pro-caspase-3 protein and DNA fragmentation in the virus-infected cells, but BiP accumulation and virus replication are not inhibited. On the basis of these results, we hypothesize that HA protein is retained in the ER of the virus-infected cells, resulting in the defective viral component assembly into virions, the accumulation of BiP in the ER and subsequent caspase-3 activation [8].

Class A scavenger receptor (SR-A) on the cell surface of macrophages is one of adhesion and apoptotic cell-recognizing molecules [22]. Membrane-integrated protein $gp91^{phox}$, existing as a heterodimer with $p22^{phox}$, functions as the catalytic core of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex in phagocytes [23]. Certain types of cells secrete pro-inflammatory cytokines, such as interleukin (IL)-6, tumor necrosis factor (TNF)-α and interferon (IFN)-β, in response to influenza virus infection prior to apoptosis induction [12-15, 17]. The cytokines bind to their cell surface receptors on monocytes and induce the expression of SR-A [24] and $gp91^{phox}$, not $p22^{phox}$,

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Table 1. Induction of Cytokine Gene Expression by Influenza Virus Infection in Cells Undergoing Apoptosis

Cell Types	Cytokines	Ref.
Chorion cells	Pro-inflammatory cytokines: IL-6, TNF-α, IFN-β, IFN-β	[11-13]
Monocytes or macrophages	Pro-inflammatory cytokines: IL-1, IL-6, TNF-α, IFN-α/β C-C chemokines: MIP-1α, MIP-1β, MCP-1, IP-10, RANTES	[14-16]
Bronchial epithelial cells	Pro-inflammatory cytokine: IL-6 C-C chemokine: RANTES C-X-C chemokine: IL-8	[17, 18]

genes [our unpublished data]. As a result of the inducible gene expression, monocytes are differentiate to well-matured macrophages capable of adhering, phagocytosing and producing superoxide through NADPH oxidase [24, 25]. The well-matured macrophages phagocytose apoptotic cell debris resulting from the virus infection [8]. The phagocytosis of cells undergoing apoptosis due to influenza virus infection by macrophages results in the presentation of viral antigen to T lymphocytes [26], the abortion of virus growth [27], the prevention of virus dissemination [28] and the decrease of virus particles [29] in the infected organs. Thus the phagocytotic reaction plays a beneficial role in the construction of the host innate immune mechanisms against influenza virus infection. Therefore, certain types of cells secrete pro-inflammatory cytokines in order to differentiate monocytes to well-matured macrophages capable of phagocytosing apoptotic cell debris resulting from the virus infection.

Subsequent to phagocytosis of apoptotic cells by macrophages, there is an abrupt increase in superoxide production known as the oxidative burst, which is catalyzed by NADPH oxidase enzyme complex [30]. The production of superoxide is necessary for remodeling tissues damaged by infectious agents [31]. However, an excessive amount of superoxide produced by macrophages is implicated in the pathogenesis of influenza virus infection [32-36]. As illustrated in Fig. (**1**), these pathways represent part of the pathogenesis of influenza virus infection.

POTENTIAL OF SELECTED ANTIOXIDANTS FOR INFLUENZA CHEMOTHERAPY

The administration of superoxide dismutase (SOD) decreases the lethal or toxic effect of influenza virus infection in mouse models [32-34]. Peptide inhibitors of caspases block the execution of apoptosis in the virus-infected cells [37]. Inhibitors of p38 mitogen-activated protein (MAP) kinase, SB203580 and SB202190, inhibit the expression of pro-inflammatory cytokine genes, such as TNF-α, in the virus-infected chorion cells at a post-transcriptional level [21]. However, the virus proliferation is not inhibited by the treatment with these agents [21, 32, 34, 37]. These results suggest that SOD and inhibitors of caspases and p38 MAP kinase are not suitable for the treatment of influenza virus infection.

Pyrrolidine dithiocarbamate ammonium salt (PDTC ammonium salt) (Fig. (**2**), compound 1) possessing superoxide-scavenging activity [38] inhibits the proliferation of influenza viruses [39]. Furthermore, PDTC ammonium salt inhibits the induction of apoptosis [39] and the expression of IL-6 [our unpublished data] and TNF-α genes [21] in the virus-infected chorion cells through its antiviral activity. Although 6-hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid (trolox) possesses superoxide-scavenging activity [40], it does not inhibit the proliferation of influenza viruses or apoptosis induction [39]. Accordingly, PDTC ammonium salt with antiviral and antioxidant activities is a candidate for a drug of choice for influenza chemotherapy [39, 41].

PDTC, dithiocarbamate and bathocuproine are divalent metal ion chelators. Dithiocarbamate, an analogue of PDTC, can chelate various divalent metal ions, leading to the formation of a lipophilic dithiocarbamate-metal complex [42], suggesting that PDTC recruits copper and zinc ions from extracellular medium to intracellular space rapidly *via* lipophilic complex formation. Copper and zinc ions inhibit influenza virus RDRP activity, and the inhibitory effect of bathocuproine-copper and bathocuproine-zinc complexes is greater than that of bathocuproine itself [43]. Moreover, PDTC-copper and PDTC-zinc complexes (Fig. (**2**), compounds 2 and 3, respectively) also inhibit the replication of rhinovirus [44] and coxsackievirus [45]. Therefore, PDTC may inhibit influenza virus gene replication and transcription through the suppression of viral RDRP activity by increasing the concentrations of intracellular copper and zinc ions or intracellular PDTC-copper and PDTC-zinc complexes. Since PDTC ammonium salt is well tolerated *in vivo* at doses by 100 mg/kg (intraperitoneal injection) and exhibits the therapeutic effect on inflammation and tissue injury in animal models [46-49], further studies for investigating the therapeutic effect of PDTC ammonium salt on animal models are warranted.

Nordihydroguaiaretic acid (NDGA) (Fig. (**2**), compound 4) possesses superoxide-scavenging activity [50]. Several methylated derivatives of NDGA have an antiviral activity against herpes simplex virus, human papillomavirus and human immunodeficiency virus [52]. NDGA also inhibits influenza virus proliferation, the inhibitory activity of which is much greater than that of PDTC ammonium salt [51]. It has been speculated that NDGA could inhibit an intracellular transport of influenza virus glycoproteins, such as HA, as well as vesicular stomatitis virus glycoproteins [51, 53].

CONCLUSION

On the basis of current knowledge on influenza virus infection process described here, both PDTC ammonium salt and NDGA can be potential candidates for a drug of choice

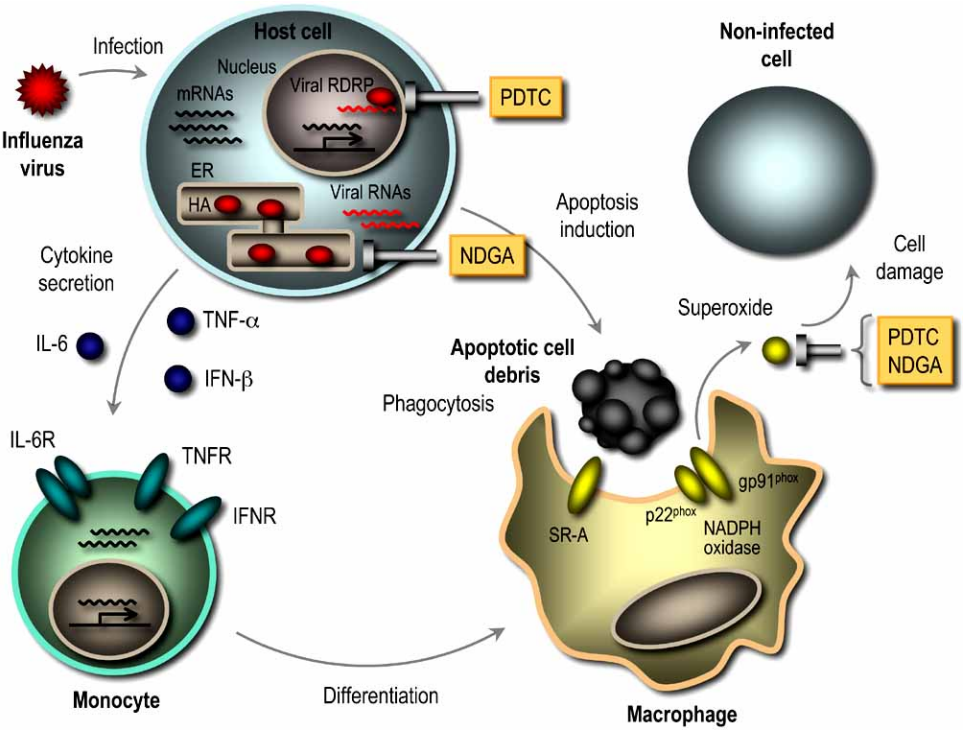

Fig. (1). Possible molecular pathogenesis of influenza virus infection.

Influenza viruses invade into cells, the genes of which are replicated and transcribed by viral RDRP in the nucleus of the cells. The virus-infected cells secrete pro-inflammatory cytokines, such as IL-6, TNF-α and IFN-β, in response to the synthesis of influenza virus macromolecules (i.e., viral RNAs and proteins) prior to undergoing apoptotic cellular degradation. Thereafter, the retaining of HA protein in the ER triggers the signals of apoptosis induction. The cytokines bind to their receptors on the cell surface of monocytes and induce the expression of SR-A and gp91^{phox} genes, resulting in the induction of monocyte differentiation to well-matured macrophages capable of phagocytosing and producing superoxide through NADPH oxidase. The well-matured macrophages phagocytose apoptotic cell debris resulting from the virus infection. An abrupt increase in superoxide production by phagocyte NADPH oxidase occurs during phagocytosis, and superoxide induces cell damage in non-infected cells. These pathways represent part of pathogenesis of influenza virus infection. Selected antioxidants with superoxide-scavenging activity, PDTC ammonium salt and NDGA inhibit the proliferation of influenza viruses, resulting in the suppression of apoptosis induction and pro-inflammatory cytokine gene expression in the virus-infected cells. The selected antioxidants can terminate the pathological pathways during influenza virus infection.

for influenza chemotherapy as a multifunctional drug with antiviral and antioxidant activities [41, 54]. Further studies are needed to elucidate the precise mechanism of their inhibitory effect on influenza virus replication, and to extend their potency using influenza animal models in order to develop a new anti-influenza drug.

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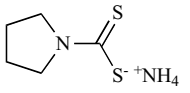
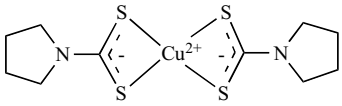
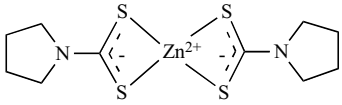


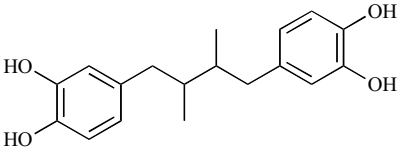
1. PDTC



2. PDTC-copper complex



3. PDTC-zinc complex



4. NDGA

Fig. (2). Anti-influenza virus agents with superoxide-scavenging activity.

ABBREVIATIONS

BiP	=	Immunoglobulin heavy-chain binding protein
C-C	=	Cysteine-cysteine
C-X-C	=	Cysteine-X-cysteine
ER	=	Endoplasmic reticulum
gp	=	Glycoprotein
HA	=	Hemagglutinin
IFN	=	Interferon
IL	=	Interleukin
IP	=	Interferon-inducible protein
MAP	=	Mitogen-activated protein
MCP	=	Monocyte chemoattractant protein
MIP	=	Macrophage inflammatory protein
M2	=	Membrane protein 2
NA	=	Neuraminidase
NADPH	=	Reduced nicotinamide adenine dinucleotide phosphate
NDGA	=	Nordihydroguaiaretic acid
p	=	Protein
PDTC	=	Pyrrolidine dithiocarbamate
RANTES	=	Regulated on activation, normal T cell expressed and secreted
RDRP	=	RNA-dependent RNA polymerase(s)
SOD	=	Superoxide dismutase
SR-A	=	Class A scavenger receptor
TNF	=	Tumor necrosis factor

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