Current and Future Anti-Influenza Virus Drugs

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Abstract: In 2009, we have been experiencing a new pandemic of novel influenza virus type A (H1N1) infection. The human beings still face the threat of highly pathogenic avian influenza A (H5N1) virus. Many patients with influenza virus infection have died due to severe complications even though receiving intensive care. This suggests the need for new treatment strategies of severe influenza-associated complications. In cases of severe influenza-associated complications, pathological manifestations are as a result of complex biological consequences, such as apoptosis induction, macrophage activation, oxidative damage and increased production of pro-inflammatory cytokines. Recent studies have revealed that the pathogenesis of severe influenza-associated complications involves not only the virus replication-mediated apoptotic cell death in the infected cells but also non-infected cell injury by toxicity of reactive oxygen species derived from macrophages phagocytosing apoptotic cells, and that pro-inflammatory cytokines produced by the virus-infected host cells play a critical role in the activation of macrophages. These findings provide a possibility that an agent with antiviral and antioxidant activities can be a drug of choice for the treatment of patients with severe influenza-associated complications. Selected antioxidants, such as pyrrolidine dithiocarbamate, N-acetyl-L-cysteine, glutathione, nordihydroguaiaretic acid, thujaplicin and certain types of flavonoids, possess both activities. The combination of antioxidants, such as superoxide dismutase and N-acetyl-L-cysteine, with antiviral drug ribavirin synergistically reduced the lethal effect of influenza virus infection. Accumulating a number of evidence highlights a potential of selected antioxidants for treatment of severe influenza-associated complications and a possibility that combination of antioxidants with current anti-influenza drugs can improve conventional influenza chemotherapy.

Keywords: Influenza virus, apoptosis, macrophage, reactive oxygen species, anti oxidant.

1. INTRODUCTION

In 2009, we have been experiencing a new pandemic of novel influenza A (H1N1) virus infection [1, 2], containing genes from avian, human and swine influenza viruses [3]. The clinical spectrum of pandemic influenza A (H1N1) 2009 virus infection is broad, from mild upper respiratory tract illness with or without fever and occasional gastrointestinal symptoms such as vomiting or diarrhea and exacerbation of underlying conditions, to severe complications such as pneumonia resulting in respiratory failure, acute respiratory distress syndrome, multi-organ failure, encephalopathy and death [4]. As of 17 October 2009, there have been more than 414,000 laboratory confirmed cases of pandemic A (H1N1) influenza 2009 and nearly 5,000 deaths (1.2%) reported to World Health Organization (WHO) [5]. Every year, the global burden of seasonal influenza epidemics is believed to be 3-5 million cases of severe illness and 300,000-500,000 deaths [6]. Beside seasonal influenza infection, we still face the threat of highly pathogenic avian influenza A (H5N1) virus infection.

In May 2009, WHO had reported that 2-6% of confirmed cases with pandemic A (H1N1) influenza virus infection had been admitted to hospital [4]. The main reason for hospitalization remains lower respiratory illness due to primary viral pneumonia, often described as “viral pneumonitis,” reflecting direct viral invasion in lung tissues. The relative portion of hospitalized patients and approximately 80% of fatal cases have had underlying medical conditions considered high risk for seasonal influenza, such as chronic lung disease (including asthma), heart disease, kidney disease, immunosuppression and pregnancy. Severe cases and deaths have been reported in pregnant women from all sites, especially in their third trimesters, resulting in intrauterine fetal demise, spontaneous abortion, premature rupture of membranes and emergency cesarean section. Pediatric cases of pandemic A (H1N1) influenza 2009-associated encephalopathy have been reported in the USA, Chile and other countries. Seasonal influenza virus infection has also been associated with severe complications, such as encephalopathy, transverse myelitis, myositis, myocarditis, pericarditis, Reye’s syndrome, hepatitis and placentitis [6-9], it is reasonable to assume that the rate of such severe complications would be increased by the pandemic A (H1N1) influenza 2009.

Currently, three classes of anti-influenza drugs have been used for chemoprophylaxis and treatment for influenza infection [10]; (1) amantadine and rimantadine inhibit viral membrane protein (M2) of proton channel that is necessary for uncoating; (2) oseltamivir and zanamivir inhibit viral neuraminidase (NA) that is necessary for virion release; and (3) ribavirin inhibits enzyme activity for viral replication.

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Initial testing found that 2009 novel influenza virus was susceptible to NA inhibitors but resistant to M2 inhibitors [11]. Therefore, NA inhibitors have been used widely for treatment and chemoprophylaxis of pandemic A (H1N1) influenza [2]. Sporadic cases of oseltamivir-resistant pandemic A (H1N1) influenza virus have been reported worldwide [12]. In case of development of oseltamivir-resistance, treatment options are limited because zanamivir is not licensed for treatment of children under 7 years old and is contraindicated among persons with underlying airway disease. These results suggest that the need for development of new anti-influenza drugs utilizing alternative antiviral mechanisms and consideration of using anti-influenza drug combinations.

NA inhibitors have demonstrated efficacy in reducing the incidence of influenza-associated complications [13]. However, patients with severe influenza-associated complications have received conventional treatment with anti-influenza virus drugs available now, they have died due to organ failures. The mortality may attribute to the lack of appropriate treatment strategies for severe influenza-associated complications. Therefore, understanding the pathogenesis of severe influenza-associated complications is a serious issue to provide effective treatment strategies. In cases of severe influenza-associated complications, the pathological manifestations are result from complex biological consequences, such as apoptosis induction, macrophage activation, oxidative tissue damage and higher contents of pro-inflammatory cytokines [14, 15]. Many recent studies have clarified that the pathogenesis of severe influenza-associated complications involves not only the virus replication-mediated apoptotic cell death in the infected cells but also the injury of non-infected cells by reactive oxygen species (ROS) derived from activated phagocytes (i.e., macrophages and neutrophils) infiltrated into the virus-infected organs. These findings provide that an agent with antiviral and antioxidant activities can be a drug of choice for the treatment of patients with severe influenza-associated complications [16, 17]. This article reviews recent knowledge regarding: (1) pathogenesis of severe influenza-associated complications: focusing on apoptosis induction and macrophage activation, and (2) current and future anti-influenza drugs.

2. PATHOGENESIS OF SEVERE INFLUENZA-ASSOCIATED COMPLICATIONS: FOCUSING ON APOPTOSIS INDUCTION AND MACROPHAGE ACTIVATION

2.1. Lessons from Postmortem Study of Pandemic A (H1N1) Influenza

In a post mortem study, the pathology of lung and extrapulmonary organs in 21 fatal cases, including 5 pregnant cases, of pandemic A (H1N1) influenza virus infection has been described precisely for the first time [18]. In fatal cases, pathological findings revealed extensive diffused alveolar tissue damage, and variable degrees of pulmonary hemorrhage and necrotizing bronchiolitis. In infected cases, there was a marked Toll-like receptor (TLR)-3 protein expression in macrophages, in alveolar epithelial cells, in vascular endothelial cells and along the alveolar capillaries. A weak immunoreactivity against interferon (IFN)-γ protein was detected in alveolar macrophages and in endothelial cells in control lungs. In infected cases, an intensive immunoreactivity against IFN-γ protein was detected in macrophages, alveolar epithelial cells and vessels, suggesting that macrophages were activated by IFN-γ. CD8+ T cells and granzyme B-positive cells were present surrounding airways and in alveolar walls in control lungs. The density of these cells was elevated in infected cases, and the cells tended to form small groups around small vessels and bronchioles. Tumor necrosis factor (TNF)-α protein was detected in alveolar macrophages and bronchial and vascular smooth muscle in both control and infected lungs. There were no signs of direct virus-induced injury in any organs examined other than the lungs. All patients had mild/moderate kidney acute tubular necrosis. All patients presented atrophic or non-reactive white pulp in the spleen. In the lymph nodes, non-reactive follicles and sinusoidal erythrophagocytosis were found. The liver showed erythrophagocytosis and a few mononuclear inflammatory cells in the sinusoids in all patients, and variable degrees of shock-related centrilobular necrosis. It should be noted that a massive hepatic necrosis was observed in a pregnant patient with hepatic failure. The placenta of the patient presented signs of intrauterine hypoxia without signs of infection. The fetus showed meconial aspiration in the lungs. No patients presented histological signs of encephalitis, myocarditis or myositis. These results suggest that in fatal cases with pandemic A (H1N1) influenza, the pathogenesis involves enhanced innate immune responses with sustained TLR-3 activation in macrophages, alveolar epithelial cells and vascular endothelial cells, and subsequent enhanced inflammation with large numbers of CD8+/granzyme B+ cytotoxic cells and local production of pro-inflammatory cytokines, such as IFN-γ and TNF-α.

2.2. Influenza Virus Infection During Pregnancy

2.2.1. Influence of Influenza Virus on Pregnant Woman and Fetus

Pregnant women are at increased risk for influenza-associated illness and death [19]. During pandemic A (H1N1) influenza 2009, pregnant women showed an increased rate of hospital admission than that of non-pregnant women, and pregnancy-associated complications, such as maternal death, premature rupture of membranes, vaginal bleeding, spontaneous abortion and emergency cesarean section, have been reported [20]. Similarly, the risk of maternal death, premature delivery, abortion and stillbirth has increased during the past pandemics of A (H1N1) Spanish virus in 1918 [21] and A (H2N2) Asian virus in 1957 [22-25], whereas no increase has been seen in the 1968-1969 pandemic of Hong Kong influenza (H3N2) [26].

During epidemics with seasonal influenza virus type A, pregnancy-associated complications have occurred. A cluster of spontaneous abortions and stillbirths has occurred within 2-4 weeks from the onset of influenza during an epidemic with seasonal influenza A (H3N2) virus in 1985-1986 [27]. In addition, some cases of pregnancy-associated complications were found. A pregnant woman at 29 weeks’ gestation was hospitalized for pneumonia caused by influenza virus type A and developed uterine contractions within a week of hospitalization. Her cervix was 3 cm dilated, and an infant was delivered by emergency cesarean section, but mother became severely hypotensive and bradycardic and died [28]. In another case, a pregnant woman at 9 weeks’ gestation was...
hospitalized for encephalopathy caused by influenza virus type A and underwent an abortion at 12 weeks’ gestation; A (H3N2) Hong Kong virus was isolated from cerebrospinal fluid sample collected at hospital admission [29]. In an additional case, a pregnant woman at 32 weeks’ gestation was hospitalized for pneumonia caused by influenza virus and subjected to emergency cesarean section at 3 days later of admission; RNA for A (subtype H1) virus was detected in serum sample collected at hospital admission [30].

Among pregnant women limited to under age 25 who would not have been previously exposed to A (H1N1) Russia virus, the rate of acute respiratory disease was increased after reappearance of the virus in Portland metropolitan area in the 1977-1978 [31]. An epidemiological study in the Tennessee from 1974 to 1993 estimated that 0.25% of pregnant women were hospitalized for the influenza infection [32]. Another epidemiological study in the Tennessee from 1985 to 1993 demonstrated that 6.0% of pregnant women with asthma or chronic obstructive pulmonary disease associated with influenza, potentially resulting in pneumonia, premature delivery, spontaneous abortion and stillbirth, and moreover that influenza can be more severe in pregnant women with underlying medical conditions than those without, resulting in hospitalization.

2.2.2. Gestational Organ and Fetus Infection

Influenza A (H3N2) virus has been isolated from the placenta and amniotic fluid during the third trimester in fatal [34-36] and non-fatal cases [37], and from the lung of stillborn infant [24] and from fetal heart [36]. Placentitis caused by influenza virus type A and B has been observed in 32 of 186 placentas, which was characterized by hyperplasia and subsequent degradation of amnion cells, cytotrophoblast cells, decidua cells and vascular endothelial cells and by the presence of viral proteins and fucsinophilic inclusions in the affected cells, and lymphoid cell infiltrations [35]. Such pathological changes were quite often observed in placentas obtained from patients with influenza virus infection [9]. Immunohistochemical analyses further demonstrated that influenza virus proteins were detected in astrocytes and neurons in the brain of infant who was delivered by emergency cesarean section [38]. Immunoglobulin M type antibody against an epidemic strain of virus has been found in umbilical cord blood sera after the community-wide epidemic of influenza virus type A [39], and lymphocytes isolated from umbilical cord blood were proliferated by the stimulation with epidemic strain of influenza A (H3N2) virus [40]. The occurrence of viremia with influenza virus has been substantiated as described in the next section. These results substantiate that influenza viruses spread from the maternal respiratory tract to the fetus, placenta and amniotic fluid via the bloodstream. By tradition, a marked maternal hypoxia due to pneumonia or febrile response has been considered as etiological factors for influenza-associated complications during pregnancy. However, results as described here clearly demonstrate that the direct virulence of influenza virus type A infection on gestational organs and fetus implicates in the etiology of pregnancy-associated complications, such as premature rupture of membranes, premature delivery due to emergency cesarean section, spontaneous abortion and stillbirth.

Both the placenta and the 4-month-old fetus have been studied on a fatal case of 24-year-old woman infected with highly pathogenic avian influenza A (H5N1) virus [41, 42]. The placenta showed scattered foci of syncytiotrophoblast necrosis as well as necrotizing deciduitis, and diffuse villitis. Both negative- and positive-strand viral RNAs for hemagglutinin (HA) and nucleoprotein (NP) were detected in Hofbauer and cytotrophoblast cells but not syncytiotrophoblast cells in the placenta by in situ hybridization, and HA and NP proteins were consistently detected by immunohistochemical analysis. Both negative- and positive-strand viral RNAs for HA were amplified from the placental tissue using reverse transcriptase-polymerase chain reaction (RT-PCR) techniques and detected in fetal bronchi, alveolar pneumocytes, Kupfer cells, and circulating mononuclear cells by in situ hybridization. The fetal tissues mostly showed no specific histopathological features, except for some edema and a few scattered interstitial neutrophils in the lungs. These results suggest that influenza A (H5N1) virus proliferates in Hofbauer and cytotrophoblast cells but not syncytiotrophoblast cells, and support a view that the infection of gestational organs and fetus with influenza virus type A implicates in the etiology of pregnancy-associated complications.

In swine, some reports suggest that abortion and stillbirth can be associated with epidemics of swine influenza [43, 44]. Transplacental transmission of swine influenza virus has been also observed in pregnant gilts [45]. Influenza virus has been isolated from amnion and chorion tissues in pregnant guinea-pig models [46] and amniotic fluid in pregnant ferret models [47] after intracardial inoculation with the virus. These results demonstrate the occurrence of fetal membrane infection with this virus. In addition, influenza virus infection may preferably spread to chorion tissues adhered to decidua tissue via the bloodstream, since human endometrial and decidua tissues provide a preferable environment for influenza virus replication than placental tissues [48].

2.3. Viremia and Systemic Infection

Human influenza A viruses have been isolated directly from blood [49-52] or serum [53] and from various extrapulmonary tissues, fluids and excreta, such as placenta [34, 35], amniotic fluid [34, 36, 37], brain [36], meninges [54], spinal cord [36], cerebrospinal fluid [29, 53, 55-57], heart [34, 36, 58], liver [36, 50, 58], kidney [36, 58], adrenal [54], urine [57], spleen [36, 49, 58], tonsil [58], lymph node [36, 58], muscle [55] and feces [53]. Immunohistochemical analysis has demonstrated that viral proteins were detected in Purkinje cells [59], neurons [59], ependymocytes of plexus choioideus [9] in the brain, β cells [59] in the pancreas, CD8+ T lymphocytes [59] in the spleen, hepatocytes [9] and stellate endothelial cells [9] in the liver, epithelial cells of convoluted tubules [9] of the kidney and cerebrospinal fluid [56] obtained from patients with influenza virus infection. Viral RNAs were also detected in various tissues and fluids, such as brain [29, 59], cerebrospinal fluid [59], heart [29], diaphragm [29], liver [29, 59], kidney [59], lymph node [29], peripheral blood mononuclear cells [60] and serum [30] obtained from patients with influenza virus infection. Furthermore, in mouse model virus particles and mRNAs have been
been observed in demyelinated lesions in two cases. In most instances the brain was observed to have changes have been observed. In liver tissue specifically in the spleen, lymph nodes, bone marrow, lungs, and liver. In liver tissue samples in addition to the respiratory secretions. Viral antigens were detected in pneumocytes by immunohistochemical tests. These studies suggest that influenza A (H5N1) virus replicates in the respiratory and gastrointestinal tracts.

2.4. Apoptosis Induction and Macrophage Activation

An increased number of activated macrophages was found in the brain containing neurons and glial cells undergoing apoptosis in the patients with influenza-associated encephalopathy [55]. The co-existence of macrophages and degraded cells, containing viral proteins, and pyknotic and fragmented nuclei, was also observed in other extrapulmonary tissues, such as liver, kidney and intestine, obtained from patients with influenza virus infection [9]. The same phenomena were observed in mice infected with influenza virus intranasally [9]. Moreover, intranasal influenza virus infection induced apoptosis in neurons in mouse brain, in which the virus protein-positive apoptotic bodies were phagocytosed by activated macrophages [64]. Thus the co-existence of influenza virus-infected cells undergoing apoptosis and activated macrophages is observed as a common pathological manifestation of various severe complications [8, 9, 65].

Most patients with influenza virus A (H5N1) infection have initial symptoms of high fever and an influenza-like illness with lower respiratory tract symptoms, and diarrhea, vomiting, abdominal pain and pleuritic pain. Bleeding from the nose and gums have also been reported early in the course of illness in some patients [66, 67]. The pathological findings including apoptotic cell death and macrophage activation are observed in the lung and some extrapulmonary tissues obtained from patients infected with influenza A (H5N1) virus [41, 68-73]. Apoptosis was observed in alveolar epithelial cells, and numerous numbers of apoptotic leukocytes were observed in the lung of a patient [71]. Macrophages appeared to be the predominant cells within the alveoli, and T lymphocytes accompanying with or without the existence of neutrophils in the interstitium. Scattered histiocytes with hemophagocytic activity have been observed in the lungs of some cases. Reactive histiocytes with hemophagocytic activity have been noted in the spleen, lymph node, bone marrow, lungs, and liver. In liver tissue specimens, necrosis, activated Kupffer cells, cholestasis, and fatty changes have been observed. In most instances the brain was edematous without any significant histopathological change, whereas reactive histiocytes and foci of necrosis have been observed in demyelinated lesions in two cases. In other organs no remarkable histological changes have been observed. Mice infected with highly pathogenic H1N1 and H5N1 viruses exhibit significantly high numbers of macrophages and neutrophils in the lungs compared to mice infected with low pathogenic viruses [74].

2.5. Phagocytosis of Influenza Virus-Infected Cells by Macrophages

Apoptosis, known as a programmed cell death, is involved not only in the physiological processes of development and tissue homeostasis but also in the pathological processes of a number of human diseases including influenza virus infection [75, 76]. Apoptotic cell death occurs sporadically during the development and tissue homeostasis [77]. Resident macrophages present in normal, non-inflamed tissues in limited numbers and undertake to scavenge scattering corpses of apoptotic cells as well as non-professional phagocytes such as fibroblasts [77, 78]. In contrast, apoptotic cell death induced by viral pathogens occurs focally and extensively in order to destruct the harmful cells producing infectious virus particles and block the spread of virus infection [79, 80]. It has been observed that a plenty of professional phagocytes (i.e., macrophages and neutrophils) are recruited into the site of infection with influenza virus in order to scavenge a large number of corpses of apoptotic cells resulting from the virus infection [81].

Recent studies suggest that the phagocytosis of influenza virus-infected cells undergoing apoptosis by macrophages plays a critical role in the presentation of viral antigen to T lymphocytes [82], the arrest of virus growth [83] and the prevention of virus dissemination in the infected organs [64]. The virus-infected cells were phagocytosed by macrophages anchored with phosphatidylserine expressing on the surface of infected cells during the process of apoptosis [84, 85]. The phosphatidylserine-mediated phagocytotic reaction was stimulated through the desialylation of macrophage surface by viral NA of the virus-infected cells [86]. Two types of phagocytes (i.e., macrophages and granulocytes) equally contributed to the phagocytotic elimination of apoptotic cells in the lung of mice infected with the virus, and the administration of annexin V, phosphatidylserine-binding protein, reduced the level of phagocytosis by alveolar macrophages, resulting in the augmentation of lethality in mice and inflammation in the lung [81]. Alveolar macrophages prepared from the virus-infected mice showed greater phagocytotic activity than those from uninfected mice [87]. Moreover, Fc receptor-mediated phagocytosis by macrophages contributed to the elimination of the virus particles in vivo [88]. Depletion of alveolar macrophage increased the virus titers in the lung of mice infected with the virus as compared with those of control the virus-infected mice [89]. Thus phagocytosis of influenza virus-infected cells undergoing apoptosis by macrophages is recognized as a protective host response to eliminate the virus-infected harmful cells and the viral pathogens from the body.

2.6. Superoxide Production by Activated Macrophages

Subsequent to phagocytosis by macrophages, an abrupt increase in superoxide production by macrophages, known as the oxidative burst, occurs, which is catalyzed by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex [90]. The production of superoxide by...
phagocytosis is necessary for remodeling tissues damaged by infectious agents [91]. However, it has been evidenced that superoxide is one of molecules responsible for death in individuals infected with influenza virus [92, 93], and that a controlled superoxide production by phagocytes is critical for the pathogenesis of influenza virus infection as evidenced by a study using mouse model lacking functional phagocyte NADPH oxidase [94]. It is most likely that necrotic focus in the virus-infected organs is formed by the cytotoxic effect of superoxide. When a wide area in organ is infected with the virus, massive necrosis occurs, resulting in organ failure. Therefore, phagocytosis of influenza virus-infected cells undergoing apoptosis by macrophages appears to implicate in the development of severe influenza-associated complications resulting from organ failure.

2.7. Macrophage Activation Factors Derived from Influenza Virus-Infected Cells Undergoing Apoptosis

It has been postulated that immature monocytes circulating in the bloodstream are able to infiltrate into the site of infection and differentiate to mature macrophages according to the theory of mononuclear phagocyte system [95]. Interaction of peripheral blood mononuclear cells with vessel wall involves initial tethering, rolling and firm adhesion to the endothelium, followed by their extravasation to the subendothelial space [96]. Following transendothelial migration, monocytes reside in close proximity to subendothelial matrix macromolecules. It has been demonstrated that monocytes are differentiated to macrophages by contacting with matrix proteins, such as type I and IV collagen and fibronectin [97]. This suggests that macrophages are recruited into normal tissues under physiological conditions. In contrast, various infectious agents induce the expression of pro-inflammatory cytokine genes. It has been demonstrated that pro-inflammatory cytokines, such as interleukin (IL)-1α and TNF-α, stimulate the adhesion and transendothelial migration of monocytes [98]. Interestingly, we found that influenza virus infection induced apoptosis in cultured chorion cells, and the secretion of a factor with monocyte differentiation-inducing (MDI) activity (MDI factor) from the virus-infected cells. Monocytes were differentiated to mature macrophages by the MDI factor, which is composed of IL-6, TNF-α and IFN-β at least, without the contact with matrix protein. Accordingly, in pathological conditions, it is likely that many matured macrophages are recruited into the inflamed tissues infected with influenza virus infection by the MDI factor secreted from the host cells. Consequently, in vitro influenza placentitis model using chorion cells is valuable to investigate the interaction between monocytes/macrophages and the virus-infected cells undergoing apoptosis.

A comprehensive study of in vitro influenza placentitis model using chorion cells has revealed various following pathological findings: Influenza virus type A replicates in both chorion cells and amnion cells [99]. Apoptotic cell death is induced in only chorion cells by the virus infection [99]. The virus infection results in intracellular lactate dehydrogenase (LDH) leakage, caspase-3 protein cleavage, and oligonucleosomal DNA fragmentation, all of which were inhibited by the presence of a general caspase inhibitor, N-t-But-Asp(OMe)-fluoromethylketone, except for the virus proliferation [100]. These results suggest that the LDH leakage results from the cellular degradation mediated through apoptosis induced by influenza virus infection [100]. LDH level in amniotic fluid is known to be one of markers for predicting fetal membrane damage [101, 102]. Therefore, these studies provide a possible diagnostic application of LDH level to predict the extent of tissue damage of fetal membranes via apoptosis induced by influenza virus infection. MDI activity is simultaneously increased in an extracellular medium of the virus-infected cells undergoing apoptosis [103]. Pro-inflammatory cytokines, such as IL-6, TNF-α and IFN-β, are identified as a member of MDI factor [104-106, our unpublished data]. P38 mitogen-activated protein (MAP) kinase is implicated in the process of MDI factor production induced by the virus infection [107]. Furthermore, mature macrophages induced by the MDI factor phagocytose corpses of chorion cells resulting from apoptosis induced by the virus infection [108-110]. It should be noted that these phenomena are not observed in amnion cells where apoptosis is not induced by the virus infection [99, 100, 103-106]. Therefore, it is possible that influenza virus-infected cells secrete MDI factor in order to facilitate phagocytosis of cell corpses by macrophages. It is notable that T lymphocytes secrete lymphokine with powerful MDI activity by stimulation with mitogen, but apoptosis is not induced in the cells. Therefore, it is interesting to study the biological significance of MDI factor derived from cells undergoing influenza virus-mediated apoptosis. Our previous reports have suggested a possibility that the secretion of MDI factor from fetal membrane chorion cells is implicated in the pathogenesis of premature delivery during influenza virus infection [105, 111, 112].

As listed in Table 1, in certain types of cells, such as monocytes/macrophages [113-115] or bronchial epithelial

<table>
<thead>
<tr>
<th>Cell Types</th>
<th>Cytokines</th>
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<tr>
<td>Chorion cells</td>
<td>Pro-inflammatory cytokines: IL-6, TNF-α, IFN-β</td>
</tr>
<tr>
<td></td>
<td>C-C chemokines: MCP-1, MIP-1β, RANTES</td>
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<tr>
<td></td>
<td>C-X-C chemokines: IL-8, GRO-α, GRO-β, ENA-78, IP-10</td>
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<tr>
<td>Monocytes or macrophages</td>
<td>Pro-inflammatory cytokines: IL-1, IL-6, TNF-α, IFN-αβ</td>
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<td>C-C chemokines: MCP-1, MIP-1α, MIP-1β, RANTES</td>
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<td>C-X-C chemokines: IP-10</td>
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<tr>
<td>Bronchial epithelial cells</td>
<td>Pro-inflammatory cytokine: IL-6</td>
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<td>C-C chemokine: RANTES</td>
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<td>C-X-C chemokine: IL-8</td>
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Table 1. Induction of Cytokine Gene Expression by Influenza Virus Infection in Cells Undergoing Apoptosis
cells [116, 117] as well as chorion cells [99, 104, 105], our unpublished data], it has been demonstrated that influenza virus infection induces apoptosis and the gene expression of pro-inflammatory cytokines (e.g., IL-1, IL-6, TNF-α), antiviral cytokines (e.g., IFN-α/β), monocyte directive cysteine-cysteine (C-C) chemokines (e.g., monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α/β), regulated on activation, normal T cell expressed and secreted (RANTES) and neutrophil directive cysteine-X-cysteine (C-X-C) chemokines (e.g., IL-8, growth-regulated gene (GRO)-α, GRO-β, epithelial cell-derived neutrophil-activating protein (ENA)-78, interferon inducible protein (IP)-10). These results suggest that influenza virus-infected host cells secrete a set of pro-inflammatory, antiviral and monocyte chemoattractive cytokines in order to attract immature monocytes circulating in the bloodstream into the site of infection and differentiate them to mature macrophages.

3. CURRENT AND FUTURE ANTI-INFLUENZA VIRUS DRUGS

3.1. Prophylaxis and Treatment of Influenza

Influenza vaccines are successful in preventing viral transmission. The efficacy of vaccines in preventing laboratory-confirmed illness is around 70%-90% both in children and in adults but is substantially lower in elderly people [118]. Therefore, the use of anti-influenza virus drugs is receiving much greater attention as a first-line defense against a new pandemic of influenza virus infection [119, 120]. Currently, two classes of anti-influenza virus drugs are available for chemoprophylaxis and treatment of influenza [121, 122].

The first-generation, amantadine (1-adamantanamine hydrochloride; known under several brand names) and rimantadine hydrochloride (α-methyl-1-adamantane-methyamine hydrochloride; brand name Flumadine) (Fig. 1), compounds 1 & 2, respectively target the viral M2 proton channel. The adamantanes, amantadine and rimantadine, exert their antiviral activity by blocking the M2 ion channel, preventing virion uncoating and the release of viral genome segments into the cytoplasm. They have been used almost exclusively to prevent infection or to reduce the duration of uncomplicated seasonal influenza, but their efficacy for the treatment of severe disease cases has not been defined. The adamantanes are inexpensive and highly stable in storage, but treatment is frequently complicated by side effects observed in gastrointestinal and central nervous system. Their use has also been significantly restricted by the rapid emergence of drug-resistant viruses that retain full virulence and transmissibility [123].

The second-generation, the NA inhibitors oseltamivir ((3R, 4R, 5S)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid ethyl ester; brand name Tamiflu) and zanamivir (5-acetamido-4-guanidino-6-(1, 2, 3-trihydroxypropyl)-5, 6-dihydropyridin2-carboxylic acid; brand name Relenza) (Fig. 1), compounds 3 & 4, respectively impair the release of virus particles from infected cells. The NA inhibitors are effective against all NA subtypes of influenza, whereas the M2 inhibitors are effective only against influenza A virus because influenza B virus does not have M2 protein [122].

Ribavirin (1-β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) (Fig. 1), compound 5 is a guanosine analogue that inhibits influenza virus ribonucleoprotein synthesis through reducing the size of the cellular guanosine 5’-triphosphate pool and by directly affecting viral replicative enzymes [124]. Ribavirin has been used in the treatment of human influenza A virus infections, usually administered orally or by aerosolization, and occasionally by the intravenous route for severe infections or in immunocompromised hosts [125]. However, ribavirin is not considered to be a drug of choice for influenza virus type A infection, since satisfactory clinical efficacy has not been achieved [125].

![Fig. (1). Current available anti-influenza drugs.](image-url)
Moreover, an oseltamivir-resistant A (H5N1) virus has been isolated from two of eight patients who had been treated with oseltamivir, had a virus with a specific mutation (glutamic acid-to-valine substitution at position of 224 (R224V) in order to form a pocket. Zanamivir, by contrast, binds to the active site without any rearrangement of the molecule. Several mutations that limit the necessary molecular rearrangement may diminish the binding of oseltamivir. Molecular analysis shows that glutamic acid at position of 276 (E276) must rotate to bind with arginine at position of 224 (R224) in order to form a pocket for the side chain of oseltamivir. The mutations (arginine-to-tyrosine substitution at position of 292 (R292K), asparagine-to-serine substitution at position of 294 (N294S), and histidine-to-tyrosine substitution at position of 274 (H274Y)) inhibit this rotation and prevent forming the pocket, resulting in resistance to oseltamivir. The mutations nonetheless allow the binding of natural sialic acid substrate, so mutated virus can survive and propagate. In contrast, the binding of zanamivir does not require any reorientation of amino acids, so these mutated viruses remain sensitive to that drug. A mutation (glutamic acid-to-valine substitution at position of 119 (E119V)) also interferes only with oseltamivir binding, possibly because a water molecule can fit between oseltamivir and valine at the active site but cannot insinuate itself between zanamivir and valine at residue 119.

Amidst 2,287 viruses isolated globally during the first 3 years of NA inhibitors use (1999 to 2002), eight viruses had a >10-fold decrease in susceptibility to oseltamivir, 0.22% in 1999/2000, 0.36% in 2000/2001, and 0.41% in 2001/2002 [134]. Two strains (A/New York/24/2001 and A/Hokkaido/15/02) were resistant to both oseltamivir and zanamivir [134]. A 2004 study in Japan demonstrated that 9 of 50 children (18%) with influenza A (H3N2) virus infection, who had been treated with oseltamivir, had a virus with a drug-resistance mutation in the NA gene [135]. Oseltamivir-resistant viruses were transmissible in ferrets [136]. Oseltamivir-resistant A (H5N1) viruses with an amino acid substitution in the NA were isolated from two of eight patients during oseltamivir treatment, and both patients died [137]. Moreover, an oseltamivir-resistant A (H5N1) virus has been also isolated from a Vietnamese girl [138]. Initial testing found that 2009 novel influenza virus was susceptible to NA inhibitors but resistant to M2 inhibitors [11]. Therefore, NA inhibitors have been used widely for treatment and chemoprophylaxis of pandemic A (H1N1) influenza. Sporadic cases of oseltamivir-resistant pandemic A (H1N1) influenza virus have been reported worldwide [12].

A frequent emergence of influenza viruses resistant to the M2 and NA inhibitors during the treatment with current drugs used suggests the need for development of the third-generation anti-influenza virus drugs with alternative antiviral mechanisms.

3.3. Potential of Selected Antioxidants as Future Anti-Influenza Virus Drugs

3.3.1. Superoxide Dismutases

Intravenous injection of pyran polymer conjugated with copper/zinc (Cu/Zn)-superoxide dismutase (SOD) protected mice against a potentially lethal influenza virus infection [92]. Intravenous injection of manganese (Mn)-SOD to mice with influenza virus infection at a lethal dose mildly increased mean days of survival, lessened arterial oxygen saturation decline, and lowered lung consolidation [139]. A combination of Mn-SOD and ribavirin, each of which was administered with small-particle aerosol, resulted in a generally mild improvement of the disease induced by the influenza A virus compared with use of either material alone [139]. A combined application of Cu/Zn-SOD and rimantadine hydrochloride in doses, which by themselves did not protect significantly mice against the infection, resulted in a synergistically decrease in lung virus titers, lung weights and consolidation and mortality rates [140]. Treatment with allopurinol, an inhibitor of xanthine oxidase capable of generating superoxide anion, improved the survival rate of influenza virus-infected mice [141]. Thus the treatment with SOD decreased the lethal or toxic effect of influenza virus infection in mice models but did not inhibit the virus proliferation [92, 139-141]. Transgenic mice carrying overexpressed extracellular SOD exhibited less severe lung injury after influenza virus infection [93]. Mice lacking functional phagocyte NADPH oxidase exhibited the augmentation of macrophages and the reduction of apoptosis in macrophages and virus titer in bronchoalveolar space after influenza virus infection as compared to those of wild-type animals [94]. Therefore, these studies suggest that superoxide anion produced by phagocytes, especially macrophages, play a critical role in the pathogenesis of influenza virus infection.

3.3.2. Thiol Antioxidants

(a) Pyrrolidine Dithiocarbamate

Pyrrolidine dithiocarbamate (PDTC) (Fig. 2), compound 6) has been shown to scavenge hydroxyl and superoxide anion radicals, the effect of which is comparable to other free radical scavengers, such as ascorbate and glutathione [142]. Both PDTC and trolox (6-hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid, a water-soluble vitamin E analogue) suppressed the induction of ROS production in chorion cells by influenza virus infection [143, 144]. PDTC inhibited both apoptosis induction and virus proliferation in chorion cells infected with influenza virus, whereas no such
inhibitory effect was observed by trolox [143, 144]. PDTC also inhibited the cytotoxic effect of influenza virus infection on the other types of cells, such as human pulmonary epithelial A549 cells and murine macrophage J774.1 cells [145-147]. The studies using J774.1 cells also demonstrated that various other antioxidants, such as trolox, deferoximine nesiuate, dithiothreitol, N-methyl-D-arginine, catalase and SOD, did not inhibit the cytotoxic effect of influenza virus infection [146, 147]. These results suggest that the inhibition of influenza virus-induced apoptosis by PDTC is attributable to its antiviral activity rather than its antioxidant property. That is, ROS may not be responsible for apoptosis induction by influenza virus infection [143, 144]. However, as described in the previous section, it has been suggested that organ injury was occurred by ROS derived from phagocytes during influenza virus infection. Therefore, these evidence reveals that the pathogenesis of influenza virus infection involves not only the virus replication-mediated apoptotic cell death in the infected cells irrespective of ROS, but also the injury of non-infected cells by ROS derived from macrophages and neutrophils infiltrated into the virus-infected organs. The findings provide a possibility that an agent with antiviral and antioxidant activities can be a drug of choice for the treatment of patients with severe influenza-associated complications. PDTC is one of the drug candidates. Since PDTC is well tolerated in vivo at doses by 100 mg/kg (intraperitoneal injection) and exhibits the therapeutic effect on animal inflammation and tissue injury models [148-151], further studies on the therapeutic effect of PDTC on animal influenza models are warranted.

The mode of inhibitory effect of PDTC on influenza virus proliferation has been investigated. PDTC inhibited the synthesis of negative-strand virion RNA (vRNA) and positive-strand complementary and/or messenger RNA (c/mRNA) for influenza virus HA gene [144]. Therefore, it is likely that the inhibition of influenza virus gene replication and transcription contributes to the inhibition of virus proliferation. Dithiocarbamate can chelate various divalent metal ions, leading to the formation of a lipophilic dithiocarbamate-metal complex, and rapid transport via a lipophilic complex by PDTC has been proposed to explain the intracellular recruitment of copper and zinc ions from the extracellular medium [152]. It has been demonstrated that copper and zinc ions inhibit influenza virus RNA-dependent RNA polymerase activity, and that the inhibitory effect of bathocuproine-copper and bathocuproine-zinc complexes is greater than that of bathocuproine itself [153]. Moreover, PDTC-copper or PDTC-zinc complex inhibited the replication of coxsackievirus [154] and rhinovirus [155]. Conceivably, it is possible that PDTC inhibits influenza virus gene replication and transcription through the inhibition of viral RNA-dependent RNA polymerase activity by increasing the amount of intracellular copper and zinc ions or intracellular PDTC-copper and PDTC-zinc complexes. Further study is needed to elucidate the precise mechanism of inhibitory effect of PDTC on influenza virus gene replication and transcription.

(b) N-Acetyl-L-Cysteine

N-Acetyl-L-cysteine (NAC) (Fig. 2, compound 7), the acetylated variant of the amino acid L-cysteine, is an excellent source of thiol groups, and is converted into metabolites in the body capable of stimulating glutathione synthesis, promoting detoxification, and acting directly as free radical scavengers [156]. NAC inhibited the induction of apoptosis [157-159] and pro-inflammatory cytokines and chemokines, such as IL-6, IL-8, RANTES and IP-10, by influenza virus infection [159]. NAC inhibited the proliferation of influenza virus at an early, but not later, stage of infection [158, 159]. Administration of the NAC significantly decreased the mortality in mice infected with influenza virus [160], and combination of NAC and ribavirin synergistically reduced the lethal effect [161]. These results suggest that combination of antioxidants with current drugs used can improve the treatment for influenza virus infection.

Administration of NAC appears to reduce symptomatic conditions associated with influenza virus infection. A total of 262 subjects of both sexes were given either placebo or NAC (600 mg) orally twice daily for six months. Although incidents of seroconversion towards A (H1N1) Singapore 6/86 influenza virus was similar in the two groups, NAC treatment decreased both the incidents and severity of influenza-like episodes, and the length of time confined to bed. The authors concluded that NAC did not prevent influenza A
(H1N1) virus infection but significantly reduced the incidence of clinically apparent disease [162].

(c) Glutathione

Reduced glutathione (Fig. 2, compound 8) has an anti-influenza activity in vitro and in vivo [163]. The addition of reduced glutathione into culture medium exogenously blocked the induction of apoptosis through the inhibition of viral macromolecule synthesis in Madin-Darby canine kidney (MDCK) cells after influenza virus infection. The antiviral effect of reduced glutathione on influenza virus proliferation was also observed in normal human small airway epithelial cells. In BALB/c mice, inclusion of reduced glutathione in the drinking water decreased viral titer in both lung and trachea homogenates at 4 days after intranasal inoculation with a mouse-adopted influenza strain A/X-31. Moreover, both the levels of Bcl-2 expression and the content of intracellular reduced glutathione contribute to the ability of host cells for down-regulating influenza virus replication, although their effects are exerted at different stages of the viral life-cycle [164].

3.3.3. Hydroxyl Antioxidants

(a) Nordihydroguaiaretic Acid

Nordihydroguaiaretic acid (NDGA; 1, 4-bis (3, 4-dihydroxyphenyl)-2,3-dimethylbutane) (Fig. 2), compound 9 occurs in the resinous exudates of the creosote bush Larrea divaricata. NDGA scavenges oxygen radicals, such as peroxyxinitrite, singlet oxygen, hydroxyl and superoxide anion radicals [165]. The treatment with NDGA inhibited apoptotic DNA fragmentation and virus proliferation in choriocarcinoma cells infected with influenza virus [166]. The maximum inhibition against DNA fragmentation was observed with 500 mM NDGA. The antiviral activity of NDGA against influenza virus was more potent than that of PDTC. This study, therefore, has suggested for the first time that NDGA, a known antioxidant reagent, inhibits the induction of apoptosis in choriocarcinoma cells infected with influenza virus through the more potent antiviral activity than that of PDTC. In this regard, it should be noted that Erimos Pharmaceuticals and North Carolina State University have filed a joint patent for the use of a developmental Erimos product, EM-1421 (tetra-O-methyl NDGA).

Recently, it has been reported that several methylated derivatives of NDGA possessed an inhibitory activity on the expression of reporter genes driven by some viral promoters. Therefore, NDGA derivatives did not affect the expression of reporter genes driven by the adenovirus major late promoter and the cytomegalovirus promoter [168]. It is predicted that the antiviral activity of NDGA derivatives is selectively depending on the virus types. Influenza virus has viral RNA-dependent RNA polymerases, which contribute to the replication and transcription processes of the viral genes, probably irrespective of cellular transcription factor Sp-1. An additional mechanism of NDGA for the inhibition of influenza virus proliferation has been proposed. NDGA is shown to inhibit the intracellular transport of vesicular stomatitis virus glycoproteins [168]. Conceivably, NDGA may inhibit influenza virus proliferation via inhibition of intracellular transport of viral glycoproteins.

(b) Thujaplicin

Thujaplicins, including α-thujaplicin (2-hydroxy-3-isopropyl-2, 4, 6-cycloheptatrien-1-one), β-thujaplicin (2-hydroxy-4-isopropyl-2, 4, 6-cycloheptatrien-1-one) and γ-thujaplicin (2-hydroxy-5-isopropyl-2, 4, 6-cycloheptatrien-1-one) (Fig. 2), compound 10), are tropolone-related compounds found in the heartwood of several cupressaceous plants, such as western red cedar (Thuja plicata), eastern white cedar (Thuja occidentalis) and hinoki cypress (Chamaecyparis obtusa) [169]. All complexes of α-thujaplicin-copper, β-thujaplicin-copper and γ-thujaplicin-copper blocked the induction of apoptosis in MDCK cells by influenza virus infection through their antiviral effects [170]. While thujaplicin-ferrous, thujaplicin-ferric, thujaplicin-magnesium and thujaplicin-manganese complexes showed no inhibition of influenza virus-induced apoptosis [170]. Thujaplicin scavenges hydroxyl radical, tert-butyl peroxyl radical, hydrogen peroxide, superoxide anion radical and singlet oxygen [171].

(c) Resveratrol

Plant polyphenol resveratorol (3, 5, 4′-trihydroxy-trans-stilbene) (Fig. 2, compound 11) inhibited the progressive infection of superoxide anion and hydrogen peroxide radicals on arachidonic acid production and cyclooxygenase-2 induction in macrophages [172]. Resveratrol inhibited the replication of influenza virus in MDCK cells, as a result of the blockade of the nuclear-cytoplasmic translocation of viral ribonucleoproteins and the reduced expression of late viral protein, such as HA and matrix protein [173]. Resveratrol also improved survival and decreased pulmonary viral titers in influenza virus-infected mice [173].

(d) Ambroxol

Ambroxol (2-amino-3, 5-dibromo-N-[trans-4-hydroxyclohexyl]benzylamine) (Fig. 2, compound 12), known as a mucolytic agent, has been used for the treatment of chronic bronchitis and neonatal respiratory distress syndrome [174]. Ambroxol suppressed the proliferation of influenza virus in the mouse airway and improved the survival rate of mice [175]. Antioxidant activity of ambroxol is related to the direct scavenging effect for ROS, such as superoxide anion and hydroxyl radicals [176, 177].

3.3.4. Flavonoids

Flavonoids are a ubiquitous group of polyphenolic substances which are present in most plants, concentrating in seeds, fruit skin or peel, bark, and flowers. The structural components common to these molecules include two benzene rings on either side of a 3-carbon ring. Multiple combinations of hydroxyl groups, sugars, oxygen, and methyl groups attached to these structures create the various classes of flavonoids: flavanols, flavanones, flavones, flavan-3-ols (catechins), anthocyanins, and isoflavones. Flavonoids have been shown in a number of studies to be potent antioxidants, capable of scavenging hydroxyl radicals, superoxide anions, and lipid peroxo radicals [178].
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(a) 5, 7, 4’-Trihydroxy-8-Methoxyflavone

5, 7, 4’-trihydroxy-8-methoxyflavone (F36) (Fig. 3, compound 13) isolated from the roots of Scutellaria baicalensis was shown to have a specific inhibitory activity against influenza virus NA because it did not affect the mouse liver NA activity [179, 180]. F36 inhibited the proliferation of influenza virus in MDCK cells, in the allantoic sac of embryonal chicken egg and in vivo using BALB/c mice [180-182]. Immunoelectron microscopic analysis revealed that F36 inhibited the budding of progeny influenza virus particles from MDCK cell surface and microvilli [183].

(b) Catechins

Catechins, (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin (EGC) (Fig. 3, compounds 14, 15 and 16, respectively), from green tea have been evaluated for their ability to inhibit influenza virus replication in cell culture [184]. Among the test compounds, the EGCG and ECG were found to be potent inhibitors of influenza virus replication in MDCK cell culture, and this effect was observed in all influenza virus subtypes tested, including A (H1N1), A (H3N2) and B virus. The 50% effective inhibition concentration of EGCG, ECG, and EGC for influenza A virus were 22-28, 22-40 and 309-318 μM, respectively. EGCG and ECG exhibited inhibitory activity of hemagglutination, suppressed viral RNA synthesis in MDCK cells, and inhibited the NA activity, however, the effects of EGC were much lesser. The results show that the 3-galloyl group of catechin skeleton plays an important role on the observed antiviral activity, whereas the 5’-OH at the trihydroxy benzyl moiety at 2-position plays a minor role. Catechins have been shown to possess the ability to scavenge for superoxide anion and hydroxyl radicals [185]. Gargling with tea catechin extracts prevented influenza virus infection in elderly nursing home residents [186]. The introduction of long alkyl chains enhances anti-influenza virus activity 24-fold relative to native EGCG [187].

(c) Quercetin 3-Rhamnoside

Quercetin 3-rhamnoside (Q3R) (Fig. 3, compound 17) from Houttuynia cordata possessed strong anti-influenza A/WS/33 virus as well as oseltamivir [188]. The mode of action of Q3R involved the inhibition of virus replication in the initial stage of virus infection by indirect interaction with virus particles.

4. CONCLUSION

As illustrated in Fig. (4), host cell secretes monocyte directive C-C chemokines (e.g., MCP-1, RANTES and MIP-1α/β) and MDI factor (i.e. IL-6, TNF-α and IFN-β) in response to influenza virus infection prior to undergoing apoptotic cell degradation. The C-C chemokines act on immature monocyte circulating in the bloodstream, resulting in recruitment of monocyte into the site of infection. The MDI factor acts on the recruited monocyte, resulting in differentiation into well-matured macrophage capable of phagocytosing and producing superoxide. The activated macrophage move to the virus-infected host cell and phagocytes apoprotic cell debris resulting from the virus infection. An abrupt increase in superoxide production occurs during phagocytosis. The superoxide induces injury in non-infected cell. These MDI factor-relating pathways represent a part of mechanisms of tissue injury during severe influenza-associated complications.

Scavenge of superoxide is an important aspect to develop new strategies for prevention of organ failure during severe influenza-associated complications. Since the most important aspect in viral disease treatment is to inhibit virus replication, an agent with antiviral and antioxidant activities should be a drug of choice for the treatment of patients with severe
influenza-associated complications. Selected compounds, such as PDTC, NAC, glutathione, NDGA, thujaplicin, resveratrol, ambroxol, F36, EGCG, ECG and Q3R, possess both antiviral and antioxidant activities. Consequently, they are potential drugs of choice for severe influenza-associated complications. In theory, combination of these antioxidants with current anti-influenza drugs can improve conventional chemotherapy for severe influenza-associated complications.

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