Disruption of virus-host cell interactions and cell signaling pathways as an anti-viral approach against influenza virus infections

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Abstract
Influenza is still one of the major plagues worldwide with the threatening potential to cause pandemics. In recent years, increasing levels of resistance to the four FDA approved anti-influenza virus drugs have been described. This situation underlines the urgent need for novel anti-virals in preparation for future influenza epidemics or pandemics. Although the anti-virals currently in use target viral factors such as the neuraminidase or the M2 ion channel, there is an increase in pre-clinical approaches that focus on cellular factors or pathways that directly or indirectly interact with virus replication. This does not only include inhibitors of virus-supportive signaling cascades but also interaction blockers of viral proteins with host cell proteins. This review aims to highlight some of these novel approaches that represent a paradigm change in anti-viral strategies against the influenza virus. Although most of these approaches are still in an early phase of preclinical development they might be very promising particularly with respect to the prevention of viral resistance to potential drugs.

Keywords: anti-viral drugs; influenza A virus; natural products; signal transduction; virus-cell interaction.

Introduction
The biology of influenza A viruses
Influenza viruses belong to either the genus influenza A-, B- or C-virus in the order Orthomyxoviridae. Among these three types, influenza A viruses exhibit the broadest host spectrum (birds, humans and other mammals) and are unique in their capacity to develop highly pathogenic strains (Wright et al., 2007). These enveloped viruses possess a segmented single-stranded RNA genome of negative polarity (Shaw, 2007; Wright et al., 2007). The virion attaches to the host cell via one of its surface glycoproteins, the hemagglutinin (HA) that binds to sialic acid receptors at the cell surface (Figure 1). The virion is internalized via receptor-mediated endocytosis. Upon acidification of late endosomes the viral membrane fuses with the endosomal membrane and the genome is released to enter the nucleus. The genome is transcribed and replicated in the nucleus by the viral RNA-dependent RNA-polymerase (RDRP), consisting of three different subunits, PB2, PB1 and PA (Figure 1). The newly produced vRNA assembles with the viral polymerase and the nucleocapsid protein to form the biologically active ribonucleoprotein complexes (RNPs). After packaging of the RNPs into new virions the virus is released from the cell surface by the action of the second viral glycoprotein, the neuraminidase (NA) (Figure 1). The nuclear replication strategy requires a highly regulated bi-directional nuclear transport. Newly generated RNPs are transported out of the nucleus, a process that involves RNP binding to the matrix protein (M1) and the nuclear export protein (NEP/NS2) that harbors a nuclear export signal. The virus further encodes for two non-structural proteins, the non-structural protein one (NS1) (Hale et al., 2008) and the PB1-F2 protein that is derived from a +1 reading frame of the PB1 gene segment and is not expressed by all influenza viruses (Shaw, 2007). These proteins are primarily required to manipulate host cell functions for efficient replication. The NS1 protein is not only able to interfere with signaling inducers and mediators but also suppresses processing of cellular pre-mRNA at later stages of infection and thereby maintains interferon (IFN) α/β and cytokine induction in a tolerable limit (Hale et al., 2008). NS1 mediates its multiple functions via binding to a variety of cellular factors (Hale et al., 2008). Recent additions to the growing list of NS1 interaction partners are the TRIM25 ubiquitin ligase that is involved in viral activation of the RIG-I pathway (Gack et al., 2009; Inn et al., 2011), and CRK family adaptor proteins (Heikkinen et al., 2008) that regulate virus-induced JNK and ATF-2 activation (Hrincius et al., 2010). Based on these functions NS1 became primarily known as a cellular signaling-blocker, however, recently it was also shown that NS1 actively induces signal transduction events such as activation of the phosphatidylinositol-3-kinase (PI3K) by direct binding to the regulatory subunit p85 of the kinase (Hale et al., 2008; Ehrhardt and Ludwig, 2009). The function of PB1-F2 in the infected cell is less clear (Conenello and Palese, 2007). It has been reported that the protein acts as an apoptosis promoter by interfering with the mitochondrial ion channels VDAC-1 and ANT3 (Zamarin et al., 2005). This function may be highly cell type specific and so far was only conclusively shown in infected monocytic cells. In epithelial cells, PB1-F2 was reported to bind to the PB1 polymerase and to alter its localization.
and polymerase activity (Mazur et al., 2008); however, this function appears to be virus-strain dependent (McAuley et al., 2010). The protein has also been shown to exhibit type I interferon antagonistic activity, a function that was strongly enhanced by the presence of a serine at position 66 of PB1-F2 (Varga et al., 2011). It should however be mentioned that PB1-F2 deficiency did not lead to dramatically impaired progeny virus titers in cell culture. Although several in vivo models have clearly demonstrated the crucial role of PB1-F2 as a pathogenicity factor in the animal (Conenello and Palese, 2007), the restoration of the PB1-F2 reading frame in pandemic H1N1v viruses did not impact pathogenicity (Hai et al., 2010).

Influenza – the burden of the disease

Influenza is still a major cause of morbidity and mortality, even in developed countries. According to a recent calculation of the Center of Diseases Control (CDC) influenza viruses were responsible for 3000–49,000 deaths annually over the last 30 years in the United States alone. Although mortality in non-pandemic years is concentrated in the elderly, influenza also causes an enormous economical burden due to work loss in adults (Nichol et al., 1995). Besides these yearly epidemic outbreaks, influenza viruses are even more threatening pathogens due to their potency to cause pandemics, as occurred in 2009 by the emergence and worldwide spread of the pandemic H1N1v viruses. Influenza viruses cannot be eradicated due to the enormous virus reservoir in water fowl, providing an unlimited gene pool to create novel pathogenic strains or reassortants that could also infect humans (Wright et al., 2007). The continuing transmissions of highly pathogenic avian influenza viruses (HPAIV) of the H5N1 type to humans highlights the constant threat of the emergence of a novel pandemic virus strain that could be more aggressive than the H1N1v viruses and to which no vaccine will be available. Current vaccine technology requires an approximate 6-month lead time and there might still be the possibility of an occasional mismatch to the prevalent strain at the time when the vaccination can start. Thus, effective anti-viral therapy is not an adjunct but an essential component of our options in the fight against influenza. Recent years has seen a significant increase in the circulation of influenza viruses that are resistant to licensed anti-influenza drugs (see chapters below), thereby blunting our anti-viral arsenal against these dangerous pathogens. This unfortunate development highlights that it is a risky strategy to solely rely on the existing compounds both in the control of seasonal influenza outbreaks and in the preparation for the next pandemic outbreak. Recent studies also showed that the severity of infections with highly virulent influenza viruses is aggravated by a deregulated cytokine response (Peiris, 2006), which is currently neither understood on the mechanistic level nor can be treated in patients. A future pandemic virus could also possess such a property. Thus, there is a pressing need to explore and develop novel avenues for influenza-specific anti-infectiva and treatment options that are less prone to the emergence of resistance than the currently available drugs.

Figure 1  Novel experimental and pre-clinical approaches to fight influenza virus infections. Schematic representation of the influenza virus life cycle and the different steps of replication that are targeted by the various novel anti-viral approaches discussed here. For further details see text.
Licenced anti-virals against the flu – M2 blockers and NA inhibitors

There are two classes of clinically approved anti-viral agents against influenza: M2 channel inhibitors (rimantadine and amantadine) and neuraminidase (NA) inhibitors (zanamivir and oseltamivir). M2 inhibitors specifically block an ion channel in the viral envelope formed by the viral M2 protein that is derived from a spliced mRNA from RNA segment seven. These inhibitors are however limited in clinical practice due to their toxicity, lack of activity against influenza B and rapid emergence of drug resistance. Both amantadine and rimantadine rapidly select for resistant mutants, which retain their ability to cause disease and to transmit from person to person (Hayden and Hay, 1992; Pinto and Lamb, 2006; Pinto and Lamb, 2007). High frequency of resistance in clinical isolates in the US have led to the conclusion that M2 inhibitors should not be used for the treatment and prophylaxis of influenza until susceptibility to these drugs has been re-established among circulating influenza A isolates (Bright et al., 2006). In addition, most of the recent H5N1 virus isolates from humans and birds exhibit genotypic resistance to M2 inhibitors (De Clercq and Neyts, 2007). Thus, they are also likely to be of very limited use in an evolving H5 pandemic.

Much hope has been given to the viral NA as a more attractive target for drug action, as it is essential for infectivity and has a highly conserved active site across influenza A and B viruses (Shaw, 2007; Wright et al., 2007). The approaches that have been started to target the viral NA were based on the thoughtful rationale that inhibition of a viral surface enzyme should not only suppress viral replication but also prevent generation of resistant variants because insensitive mutant strains should be attenuated due to impaired NA activity. Several highly selective competitive NA inhibitors have been developed that bind tightly to the active site of the enzyme (Oxford et al., 2002; Crusat and de Jong, 2007). They inhibit the release of new virion progeny from an infected cell, prevent cleavage of neuraminic acids bound to components of the mucus and thereby reduce the ability of the virus to spread through the respiratory epithelium. Zanamivir, a dehydrated sialic acid derivative and oseltamivir, the oral pro-drug of the active oseltamivir carboxylate both entered clinical practice in 1999. These drugs effectively inhibited viral replication in cell culture of many clinical influenza A and B virus isolates, although the concentration required for inhibition could vary significantly from strain to strain (Gubareva et al., 2000). In animal models such as mice and ferrets, the drugs were effective as therapeutic and prophylactic agents. Clinical trials in humans revealed that both zanamivir and oseltamivir carboxylate reduce the time of recovery following influenza virus infection by approximately one to two days, provided that the drugs are administered beginning one to two days following the onset of symptoms (Gubareva et al., 2000). During clinical trials of oseltamivir in seasonal influenza, only a low percentage of resistance has been reported, with cumulative data from all Roche-sponsored studies indicating an incidence of resistance of 0.4% in adults and 4.1% in children (Aoki et al., 2007). However, more worrying rates of resistance were first detected in a smaller study in Japanese children, where 18% of all isolates were resistant (Kiso et al., 2004). Since then, the number of reports on viral resistance to oseltamivir has rapidly increased, including findings on the emergence of influenza B viruses as well as influenza A viruses of the H5N1 and pandemic H1N1v type that are insensitive against the compound. Usually, resistant mutants exhibited reduced infectivity and virulence in animal models; however, there are also reports that some resistance mutations are associated with full replication competence and transmissibility (Hayden, 2009). Finally during the 2007–2008 northern hemisphere influenza season, oseltamivir-resistant influenza H1N1 variants emerged globally at a very high percentage (Lackenby et al., 2008). Notably this phenomenon has occurred in the absence of selective drug pressure indicating that these resistant variants are efficiently transmitted from person to person. The frequency of oseltamivir resistance in H1N1 viruses was unexpected and the reason why a high percentage of these viruses were resistant is currently unknown. However, this was not just a sporadic phenomenon because the percentage of resistance among H1N1 strains still increased in the 2008–2009 season (Thorlund et al., 2011).

Given these high rates of resistance to the currently available anti-influenza drugs that target viral factors, it could be concluded that influenza viruses will sooner or later lose sensitivity to any drug that targets the virus directly due to escape mutations. Thus, there is a new concept needed to overcome the problem of resistance. Influenza virus, like any other virus exploits the cellular machinery to replicate. This implies that there are multiple interactions not only between viral proteins but also of viral proteins with cellular proteins. Blockade of such an interaction might be an alternative approach to inhibit viral replication. The big advantage may be that the virus cannot replace the missing cellular function and thus emergence of resistance should not easily occur. Some of the recent advances to target cellular factors for anti-influenza therapy will be highlighted below.

Approaches to disrupt virus-host cell or protein interactions

The earliest step in the virus life cycle where interaction of the pathogen with the host cell occurs, is the attachment of the virion to sialic acids on the cell surface via the viral HA protein. Consequently, multiple strategies to prevent attachment have been explored as a novel anti-viral approach (Figure 1). One attempt was the removal of the cellular receptor determinant from the surface by a sialidase. DAS181 is a fusion construct that incorporates the sialidase from Actinomyces viscosus, a common oral bacterium linked to a human epithelium-anchoring domain. It can be mass produced in Escherichia coli (Malakhov et al., 2006). When this molecule is exposed to cells, it cleaves off the surface receptors on respiratory epithelium that are recognized by the HA, both the α2,6-sialic acid-linked receptors to which human viruses attach and the α2,3-sialic acid-linked receptors to which avian viruses attach. DAS181 is inhibitory for a range of influenza A and B viruses, with in vitro EC90 in the
low nanomolar range. The molecule did not inhibit growth of human cells. Intranasal dosing has shown prophylactic and therapeutic activity in mice and anti-viral effects with reduced inflammatory responses in ferrets (Malakhov et al., 2006). Furthermore, in a murine model of highly pathogenic avian influenza using influenza A/Vietnam/1203/2004(H5N1) DAS181 was active both prophylactically and therapeutically (Belser et al., 2007). Although there was doubt by some experts whether receptors could be safely denuded in the lung of patients for extended time periods DAS181 is now under further clinical development. Interestingly, in a recent study DAS181-based virus therapy protected against secondary pneumococcal infection of mice (Hedlund et al., 2010), a finding that could have important implications regarding the problem of bacterial co-infections.

Another attachment-disrupting agent has been identified by the analysis of a panel of cell-penetrating peptides for anti-influenza activity. A 20-amino acid peptide derived from the fibroblast growth factor 4 (FGF-4) signal sequence, displayed significant broad-spectrum anti-viral activity against influenza viruses both in vitro and in a mouse model. The peptide prevented virion attachment to host cells most likely through an interaction with the HA protein (Jones et al., 2006).

An additional strategy to disrupt viral attachment to cells would be the inhibition of adhesion with the help of soluble receptor analogs. This concept has been brought forward in a study that uses multimeric sialylglycoconjugates to inhibit influenza virus replication in vitro and in a mouse model (Bovin et al., 2004).

An approach that should also be mentioned here because it would also lead to the prevention of viral entry is the use of protease inhibitors. It is long known that the HA of influenza viruses has to be cleaved by cellular proteases for full activity (Garten et al., 1981). More recent work has identified the type II transmembrane serine proteases TMPRSS2 and HAT as HA activating enzymes in the human respiratory epithelium (Boettcher et al., 2006) and the search for respective inhibitors is now ongoing. A first proof of concept for this strategy has been provided quite early on in animal models and in human volunteers, where anti-viral action of relatively unspecific protease inhibitors has been demonstrated in vivo (Zhirkov et al., 1984).

With regard to interaction blockers of intracellular events during replication, two viral target structures have received special attention so far: the viral polymerase complex and the interferon antagonistic NS1 protein (Figure 1). Although there are also attempts to target the viral polymerase activity directly by polymerase inhibitors [e.g., T-705 (Furuta et al., 2002)] or endonuclease inhibitors [e.g., L-735 822 (Tomassini et al., 1996)], some approaches focus on the interaction of polymerase proteins with each other or with cellular binding partners. Because the viral polymerase consists of three subunits that have to interact and it has been shown that disruption of the association results in impaired polymerase activity and subsequent inhibition of virus propagation (Maenz et al., 2011). It was further demonstrated that introduction of peptides that were derived from the PA-binding domain of PB1 indeed resulted in a drop of progeny virus titers (Ghanem et al., 2007; Wunderlich et al., 2009). The approach could also work if the interaction of the polymerase complex with cellular interaction partners is blocked. The cellular chaperone heat shock protein 90 (Hsp90) was shown to play a role in nuclear import and assembly of the trimeric polymerase complex by binding to PB1 and PB2. Accordingly, it was shown that Hsp90 inhibitors lead to impaired viral replication in cell culture and the authors suggested that Hsp90 inhibitors might represent a new class of anti-viral compounds against the influenza viruses (Chase et al., 2008).

The NS1 was considered another suitable target because this protein binds to a multitude of cellular factors to manipulate cell functions in favor of virus propagation (Hale et al., 2008). One of the cellular binding partners of the NS1 of influenza A viruses is the CPSF30 protein, which mediates inhibition of 3′-end processing of cellular pre-mRNAs by NS1. In MDCK cells that constitutively expressed an NS1 binding-motif peptide of CPSF30 in the nucleus, influenza A virus replication was inhibited, although no inhibition was observed with influenza B virus whose NS1B protein lacks a binding site for CPSF30 (Twu et al., 2006). These results clearly indicate that influenza A virus replication is inhibited by disrupting the interaction of endogenous CPSF30 to the NS1 protein (Twu et al., 2006).

Just recently a yeast-based assay has been developed to identify chemical inhibitors that phenotypically suppress NS1 functions, most likely by inhibiting NS1 interactions with other proteins (Basu et al., 2009). In this screen several inhibitors have been identified that exhibited a specific activity against influenza virus but not against respiratory syncitial virus (e.g., JJ 3297). Interestingly, viruses were resistant against these compounds in interferon deficient systems, indicating that the agents act via disruption of the interferon antagonistic activity of NS1 (Basu et al., 2009; Walkiwickicz et al., 2011). Another screening approach has been developed to search for compounds that disrupt interaction of the NS1 protein with RNA, based on the rationale that some functions of NS1 are executed by the ability of the protein to bind various types of RNA molecules from both viral and non-viral origin (Maroto et al., 2008). Several compounds were identified that not only inhibited NS1 binding to viral RNA but also resulted in impaired virus replication, indicated by a reduction of the cytopathic effect during the infection. Taken together, these results support the hypothesis that the binding surfaces of NS1 with cellular proteins or RNA could be a novel target for the development of anti-influenza drugs.

Inhibitors of virus-supporting signaling processes

Infection with influenza viruses results in the activation of a variety of intracellular signaling responses (Ludwig et al., 2006). With regard to the function of these signaling events the overall picture has emerged that most of the processes are initiated as a cellular response to defend the invading pathogen. However, the virus has also acquired the capability to exploit some of these activities to ensure efficient replication (Ludwig, 2007a). Taking advantage of cellular signaling factors to support viral replication is very effective but also creates...
dependencies that might be used to develop novel anti-viral drugs that disrupt signal transmission (Ludwig et al., 2003). Although several inhibitors of different signaling pathways have been shown to suppress virus replication in cell culture models, only two particular virus-supportive signaling pathways have so far also been proven as suitable targets for an anti-viral approach in vivo in mouse models: the Raf/MEK/ERK mitogenic kinase cascade (Ludwig, 2007b) and the IKK/NF-κB module (Ludwig and Planz, 2008) (Figure 1).

The Raf/MEK/ERK signaling pathway belongs to the family of the so-called mitogen activated protein kinase (MAPK) cascades (Widmann et al., 1999). Signaling via this pathway is commonly initiated by receptor tyrosine kinases or G-protein-coupled receptors, which finally leads to the stepwise phosphorylation and activation of the serine threonine kinase Raf, the dual specificity kinase MEK (MAPK kinase (ERK) kinase) and the MAPK ERK (extracellular signal regulated kinase). ERK transforms the signal by phosphorylating a variety of substrates and thereby regulates many different functions in the cell (Widmann et al., 1999). The kinase is activated upon infection with all influenza A and B viruses tested so far (Pleschka et al., 2001; Ludwig et al., 2004). Strikingly, specific blockade of the pathway strongly impaired growth of avian and human influenza A as well as human B-type viruses (Pleschka et al., 2001; Ludwig et al., 2004) indicating that activation of the Raf/MEK/ERK cascade is required for efficient virus growth. Conversely, virus titers were enhanced in cells in which the pathway was pre-activated by expression of constitutively active mutants of Raf or MEK (Ludwig et al., 2004; Olschlager et al., 2004). This has also been demonstrated in vivo in infected mice expressing a constitutively active Raf kinase in the alveolar lung epithelial cells (Olschlager et al., 2004). With respect to the underlying molecular mechanisms it was shown that cascade inhibition led to nuclear retention of the viral RNP complexes in late stages of the replication cycle. The data suggested that the pathway controls the active nuclear export of RNPs, most likely due to interference with the activity of the viral nuclear export protein NEP (Pleschka et al., 2001). The findings led to the hypothesis that active RNP export is an induced rather than a constitutive event. Although the RNPs have to reside in the nucleus for sufficient replication and transcription of the viral genome in early stages they have to be exported from the nucleus late in the replication cycle in order to prevent de-envelopment of new virions at the cell membrane. This late requirement of a supporting signal correlates well with the observed late activation of ERK in the viral life cycle. The timely activation of ERK was shown to be achieved by membrane accumulation of the viral HA protein and its tight association with lipid-raft domains (Marjuki et al., 2006). This event triggered protein kinase C alpha (PKCα)-dependent activation of the Raf/MEK/ERK cascade late in the infection cycle and thereby induces RNP export (Marjuki et al., 2006). These data are strongly supported by the finding that clustering of raft-associated HA in the external membrane leaflet modulates diffusion and signaling of H-Ras, another major upstream activator of Raf (Eisenberg et al., 2006). As HA together with NA, M2 and M1 forms electron dense patches at the site of virus membrane budding, signaling components at the inner membrane layer could be affected leading to the activation of the Raf/MEK/ERK pathway. ERK activation by membrane accumulated HA might therefore represent an auto-regulative mechanism that coordinates timing of RNP export to a stage when all viral components are ready for virus budding.

The requirement of Raf/MEK/ERK activation for efficient influenza virus replication suggests that this pathway could be a promising cellular target for anti-influenza approaches. In support of this view, inhibitors of the cascade that exhibited a strong anti-viral activity showed surprisingly little toxicity in cell culture (Planz et al., 2001; Pleschka et al., 2001; Ludwig et al., 2004), in an in vivo mouse model (Sebolt-Leopold et al., 1999) and in clinical trials for the use as anti-cancer agent (Cohen, 2002). Furthermore, these compounds showed no tendency to induce formation of resistant virus variants (Ludwig et al., 2004). Finally this novel anti-viral approach of disrupting signaling via the Raf/MEK/ERK cascade was also shown to be effective in the animal model (Droebner et al., 2011; Pinto et al., 2011). An inhibitor of the central kinase MEK, U0126, resulted in reduced virus titers in the lung of infected mice after local aerosolic administration into the trachea (Droebner et al., 2011). Several inhibitors of the Raf/MEK/ERK cascade are under clinical investigation and these studies demonstrated that the cascade can be effectively inhibited in humans. It now remains to be determined whether these inhibitors would also block influenza replication during human infection.

Another important influenza virus induced signaling process is the activation of the transcription factor NF-κB. This factor regulates expression of a variety of anti-viral cytokines including IFNβ, that is the initiator of a strong type I IFN defense program (Pahl, 1999). Due to this role in anti-viral gene expression, NF-κB and the upstream activator kinase IκB kinase (IKK) 2 were regarded as bona-fide components of the innate immune response to virus infections (Chu et al., 1999).

However, in two independent studies the surprising observation was made that replication of influenza viruses is much more efficient in cells with pre-activated NF-κB (Nimmerjahn et al., 2004; Wurzer et al., 2004). Conversely, progeny virus titers were reduced when grown in host cells in which NF-κB signaling was impaired by specific inhibitors such as BAY11-7085 or BAY11-7082, or by the use of dominant-negative mutants of IKK2 or the inhibitor of κB, IκBα (Nimmerjahn et al., 2004; Wurzer et al., 2004). From these studies it could be concluded that influenza viruses have acquired the capability to turn the anti-viral activity of NF-κB into a virus-supportive action.

The viral dependence on NF-κB function raises the question of the underlying molecular mechanism(s). To this end, it was demonstrated that the virus supportive function of NF-κB is at least in part due to the NF-κB dependent expression of factors such as TNF-related apoptosis inducing ligand (TRAIL) or FasL (Wurzer et al., 2004), which are known activators of a cell death program executed by a family of apoptosis regulating proteases termed caspases (Thornberry and Lazebnik, 1998). Accordingly, it was shown that influenza virus propagation was also strongly impaired in the presence
of either caspase inhibitors or by using siRNA to suppress expression of a major effector caspase, caspase 3 (Wurzer et al., 2003). Caspases specifically cleave cellular proteins including those of the nuclear pores resulting in an enhanced diffusion limit for protein transport in and out of the nucleus (Faleiro and Laeznik, 2000; Kramer et al., 2008). This function appears to be relevant for viral replication because in the presence of both caspase inhibitors and NF-κB inhibitors, a nuclear retention of viral RNP complexes could be observed (Wurzer et al., 2003; Mazur et al., 2007). This event most likely prevented formation of progeny virus particles. Thus, the typical anti-apoptotic function of NF-κB in response to other stimuli is converted into a pro-apoptotic function in the context of infection. Although this represents a first chain of events that could determine the virus-supportive function of NF-κB, recent data have revealed additional mechanisms that might contribute to the virus-supportive action of the transcription factor. It was shown that in cells infected with influenza viruses the suppressor of cytokine signaling-3 (SOCS-3) gene is strongly up-regulated in a NF-κB dependent manner due to the accumulation of viral 5′ triphosphate RNA (Pauli et al., 2008). SOCS proteins are efficient blockers of the Janus kinase (JAK)/Signal transducers and activators of transcription (STAT) pathway (Kubo et al., 2003). After cytokine binding to their respective receptors, associated JAKs activate STAT transcription factors by tyrosine phosphorylation, a process that can be inhibited by SOCS factors. Consistent with this function, the expression of SOCS-3 in influenza virus-infected cells limited the anti-viral gene expression responses to virus-induced type I IFN (Pauli et al., 2008) and thus resulted in enhanced virus replication. In another more recent study it was shown by means of inhibitors that NF-κB also differentially regulates viral RNA synthesis (Kumar et al., 2008). Finally, NF-κB might not only influence the pathogenesis of influenza virus by direct effects on several stages of the virus life cycle. Excessive inflammation due to overabundant production of proinflammatory cytokines and chemokines (also known as cytokine burst) is considered an important factor in disease pathogenesis. The majority of cytokines/chemokines is regulated by NF-κB (Pahl, 1999). Consequently, activation of NF-κB in airway epithelia, endothelial cells or infiltrating monocytes might strongly influence the outcome of the cytokine burst after influenza A virus infection. This is supported by findings from genome wide gene expression array assays that demonstrated a major role of NF-κB for cytokine responses induced by the HPAI virus H5N1 (Schmolke et al., 2009; Viemann et al., 2011).

The unexpected dependence of influenza viruses on NF-κB activity raised the question whether the signaling pathway could be a suitable target for anti-viral intervention. The first proof-of-concept study was performed with a NF-κB inhibiting agent that is in frequent clinical use. Acetylsalicylic acid (ASA), also known as aspirin, has been previously shown to be an efficient and quite selective inhibitor of IKK2 in low millimolar concentration ranges (Yin et al., 1998). Correspondingly, ASA efficiently blocked replication of influenza viruses including H5N1 strains, in cell culture by several orders of magnitude in a concentration range that was not toxic for the host cell (Mazur et al., 2007). Comparison to other NF-κB inhibitors or blockers of other functions that are targeted by ASA revealed that the compound indeed demonstrated anti-viral action via its NF-κB inhibiting activity. Strikingly, ASA did not lead to the generation of resistant virus variants in multi-passaging experiments (Mazur et al., 2007). Finally ASA also efficiently acted in an anti-viral manner in vivo in a mouse infection model. Application of the compound as an aerosol directly into the trachea of lethally infected mice reduced virus titers in the lung and, significantly promoted survival (Mazur et al., 2007). More recent data further showed that ASA as an aerosol is well tolerated in mice and does not exhibit harmful side effects (O. Planz, Tübingen, Germany, personal communication).

In summary, these data are promising and could be taken as a first proof of concept that NF-κB inhibitors can serve as anti influenza agents in vivo without toxic side effects or the tendency to induce viral resistance.

**Natural plant products that disrupt virus-cell interactions**

The threat of a possible pandemic in the near future has raised attention to the potential anti-viral action of natural products from plants. Although reports on an anti-influenza virus activity of plant extracts are manifold, only a few of these products have been studied in dept with regard to the molecular mechanisms and the anti-viral action in animal models. Some of the natural products under investigation appear to exhibit an action profile that fits into the scope discussed here, namely disruption of virus host cell interactions or signaling pathways. This is particularly true for plant extracts that are rich in polyphenols. Polyphenols are a large family of natural compounds widely distributed in plants and characterized by the presence of more than one phenol group per molecule. Thus, polyphenols constitute one of the most numerous groups of substances with more than 8000 phenolic structures currently known. The structure can vary from simple molecules such as phenolic acids to highly polymerized compounds (Harborne, 1980). The current knowledge of absorption, biodistribution and metabolism of polyphenols is poor; however, in general it can be stated that low molecular weight polyphenols are absorbed and exhibit bioactive properties, however, although most polymorphic polyphenols are not metabolized (Manach et al., 2004). Reports on an anti-viral and anti-bacterial potency of polyphenolic compounds are numerous (Scalbert, 1991; Cos et al., 2003a,b; Taguri et al., 2006) and several polyphenolic agents or polyphenol rich plant extracts showed effective anti-viral action against influenza viruses in vitro and in vivo (Serkedjieva, 1997; Droebner et al., 2007; Oxford et al., 2007a,b; Serkedjieva et al., 2008). The compounds have attracted some attention by the findings that polyphenols from green tea (Song et al., 2005; Oxford et al., 2007a,b; Huang et al., 2008) and red wine (Palamara et al., 2005) exhibit anti-influenza virus activity. The anti-viral action of resveratrol, a polyphenol that can be isolated from red grapes and red wine, appeared to be linked to its capacity to inhibit protein kinase C activity and its dependent pathways (Palamara et al., 2005).
Resveratrol treatment resulted in impaired nuclear-cytoplasmic translocation of viral RNPs and reduced the expression of late viral proteins. In another study a resveratrol tetramer was effective to block virus induced RANTES expression through interference with Akt and STAT phosphorylation (Huang et al., 2008). Thus, resveratrol appears to exhibit bioactive effects and acts by inhibiting cellular rather than viral functions. The potential as an anti-viral agent was highlighted by the finding that resveratrol also significantly improved survival and decreased pulmonary viral titers in influenza virus-infected mice without signs of toxicity (Palamara et al., 2005).

Although some polyphenolic agents such as resveratrol or plant extracts from Geranium sanguineum L. (Murzakhmetova et al., 2008) appear to act intracellularly, the anti-viral action of other polyphenolic plant products have been proposed to be due to direct physical interactions with the virion (Haslam, 1996). For example, polyphenolic catechins from green tea such as epigallocatechin gallate, epicatechin gallate and theaflavin digallate bind the HA of influenza viruses (Song et al., 2005) and agglutinate the particles and therefore reduce their infectivity and binding to the cell surface (Nakayama et al., 1993). Prodelphinindin B-2 3-O-gallate, also isolated from green tea leaves, similarly inhibits viral attachment and penetration (Cheng et al., 2002). The same action profile has been described for CYSTUS052, a plant extract that is very rich in highly polymeric polyphenols. The extract is a preparation of a selected variety of the biochemical polymorphic species Cistus incanus (Pink Rockrose). Cistus species are used in traditional medicine in the eastern Mediterranean for centuries (Petereit et al., 1991; Danne et al., 1993). In two recent studies CYSTUS052 exerted a potent anti-viral activity in cell culture (Droebner et al., 2007; Ehrhardt et al., 2007). On a molecular basis the extract was shown to non-specifically interfere with the virion and to prevent the viral HA from attaching to cellular receptors. Probably due to this broad and non-specific action, the emergence of resistant virus variants was not observed (Ehrhardt et al., 2007). CYSTUS052 did not exert any negative effect on proliferation, metabolism, transcriptional/translational activity or responsiveness of the cell to cytokines, probably due to the fact that the highly polymeric active component is not resorbed to cells. This would restrict an anti-viral application to the local route, e.g., inhalation as an aerosol into the lung of patients. Accordingly, potent anti-viral activity of CYSTUS052 applied by the inhalation route has been demonstrated in a mouse infection model (Droebner et al., 2007). CYSTUS052 has just entered first clinical examinations and beneficial effects for patients infected with respiratory pathogens, including influenza viruses have been demonstrated (Kalus et al., 2009, 2010).

**Conclusion and perspective**

Our current options regarding clinically approved anti-viral drugs against influenza are very limited. M2 inhibitors can clearly not be recommended due to their side effects and the frequency of resistance against the drug. There is also a worrying increase in the frequency of resistance to oseltamivir; in both circulating strains and highly pathogenic avian strains of the H5N1 type. Thus, it cannot be ruled out that a future pandemic virus might already be resistant to NA inhibitor treatment. Despite these clear limitations, the World Health Organization still supports stockpiling of anti-viral drugs but it also admits that this approach provides no guarantee of success. Thus, it is common consent among many experts that we urgently need alternative approaches for an influenza therapy. However, the increasing frequency of resistance to the current drugs that exclusively target viral factors raises concern that every new drug that targets viral structures will sooner or later share the fate of M2 and NA inhibitors. Thus, a paradigm change in drug development is urgently needed. Targeting cellular rather than viral factors could be the most promising approach to prevent the problem of resistance because the pathogen simply cannot replace the missing cellular function. The results of the various approaches discussed here, although still in a very early phase of preclinical development, so far fully support this concept. According to these studies it is indeed possible to target cellular factors without harmful side effects or the tendency of the drugs to select for resistant virus variants. In addition, some of the agents such as MEK inhibitors or NF-κB inhibitors might have additional beneficial effects, e.g., the suppression of overshooting cytokine expression that could prevent the detrimental cytokine burst, which contributes to the pathogenicity of highly pathogenic influenza viruses.

The research field of cellular drug targets in influenza therapy could receive a tremendous boost due to the results of several recent genome wide functional screening studies using siRNA or other approaches (Hao et al., 2008; Brass et al., 2009; Shapira et al., 2009; Karlas et al., 2010; Konig et al., 2010). In one of these studies, the authors additionally focussed on interactors of viral proteins (Shapira et al., 2009). Although it should be stressed that these screening results are far from being fully analysed in functional terms, these studies provide a valuable library to select and functionally evaluate novel cellular drug targets or interaction surfaces.

Unfortunately at present, most of the cell targeting strategies are in an early stage and only very few new anti-viral therapies have entered clinical testing. This could be attributable, at least in part, to the fact that pharmaceutical companies for a long time did not consider the potential profits associated with development of anti-influenza drugs to be worth the high cost and risk (Brooks et al., 2004). This situation has somewhat changed since the emergence of H5N1 viruses in humans and the stockpiling activities of anti-virals due to the concern of a near pandemic. It is interesting to see that a significant portion of new drugs under development target cellular factors, indicating that start-ups and pharmaceutical companies are attracted by this novel concept. Frustratingly it will still need many years before some of these novel drugs will enter the clinic and thus only time will tell how well they will perform.

The limitations of the current options of chemical anti-viral treatments have resulted in a new interest in natural/herbal anti-viral agents. Most notably some of these preparations were not only effective in the ferret model (Oxford et al.,
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Given the apparent limitations of current influenza drugs, use of plant extract such as CYSTUS052 could be a promising and readily available prophylactic and therapeutic option or at least a supplementary strategy for influenza prophylaxis and therapy.

References


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