Targeting cell signalling pathways to fight the flu: towards a paradigm change in anti-influenza therapy

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Influenza is still one of the major plagues worldwide with the potential to cause pandemics. The increasing frequency of viral resistance to the four US Food and Drug Administration (FDA)-approved anti-influenza virus drugs underlines the urgent need for novel antivirals to be prepared for future influenza epidemics or pandemics. While the antivirals currently in use exclusively target viral factors, such as neuraminidase or the M2 ion channel, several pre-clinical approaches now focus on cellular factors or pathways that directly or indirectly interact with virus replication. Among these, inhibitors of intracellular signalling cascades that are essential for virus replication have been unravelled as the most promising candidates. This short article aims to highlight two of these novel approaches, namely, inhibition of the classical mitogenic Raf/MEK/ERK kinase cascade and blockade of the pathway that leads to activation of the transcription factor NF-κB. It has been shown that inhibition of both virus-induced pathways leads to impaired virus production *in vitro* and *in vivo* without side effects or the tendency to induce resistant virus variants. Besides the direct antiviral effect, such inhibitors may also exert additional beneficial effects by blocking the cytokine burst that contributes to the severity of infections by highly pathogenic influenza virus strains. Although these novel strategies are still in an early phase of pre-clinical development they might be very promising, especially with regard to prevention of viral resistance.

Keywords: influenza virus, drug development, cellular drug targets, signalling pathways, resistance

Influenza: the burden of the disease

Influenza is still a major cause of morbidity and mortality, even in developed countries. Each year influenza viruses are responsible for 20000–40000 deaths and up to 300000 hospitalizations in the USA alone and also cause an enormous economical burden.[1] Besides these yearly epidemic outbreaks, influenza viruses also have the potency to cause severe pandemics. The continuing transmissions of highly pathogenic avian influenza viruses of the H5N1 type to humans highlight the constant threat of the emergence of a novel pandemic virus strain to which no vaccine will be available. Thus, effective antiviral therapy is not an adjunct but an essential component of our options in the fight against influenza.

Inhibiting viral factors: neuraminidase (NA) inhibitors and M2 ion channel blockers

To date there are two classes of clinically approved antiviral agents against influenza: M2 inhibitors (rimantadine and amantadine), which block a viral ion channel; and NA inhibitors (zanamivir and oseltamivir), which inhibit the viral NA activity and prevent release of novel virus particles. M2 inhibitors are limited in clinical practice by their lack of activity against influenza B viruses and rapid emergence of drug-resistant variants, which retain their ability to cause disease and to transmit from person to person.[2,3] A high frequency of resistance in clinical isolates in the USA has 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.[4] In addition, increasing numbers of H5N1 virus isolates from humans and birds exhibit genotypic resistance to M2 inhibitors.[5] While the agents still may be beneficial in acute and severe cases, WHO only recommends use of M2 blockers as a first-line treatment if local surveillance data show that the H5N1 virus is known or likely to be susceptible to these drugs.

The NA inhibitors zanamivir and oseltamivir entered clinical practice in 1999 and the drugs have been proven to reduce the time of recovery following influenza virus infection provided that the drugs are administered early following onset of symptoms.[6] While in clinical trials of oseltamivir in seasonal influenza only a low percentage of resistance has been reported,[7]

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much more worrying rates have been detected in a smaller study in Japanese children where 18% of all isolates were resistant.[8] Since then, the number of reports on insensitivity to oseltamivir has rapidly increased, including findings on the emergence of influenza B viruses as well as H5N1 influenza A viruses that are resistant to the drug. Moreover, some of these resistant mutants retained full replication competence and transmissibility.[3] Lastly, with the beginning of the 2007–08 influenza season in the northern hemisphere, oseltamivir-resistant influenza H1N1 variants emerged globally at a very high percentage.[9] Notably, this phenomenon has occurred in the absence of selective drug pressure, indicating that these resistant variants are efficiently transmitted from person to person. It may be only a matter of time until zanamivir shares this fate.

These high rates of resistance to the currently available drugs raise the concern that influenza viruses sooner or later will lose sensitivity to any drug that directly targets the virus. 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.

Influenza virus, like any other virus, exploits the cellular machinery to replicate. Blocking of cellular mechanisms required for viral replication may, thus, be an alternative approach to inhibit virus growth. Inhibitors of virus-induced intracellular signalling cascades came into focus, since the respective signalling processes are central regulators of many cellular responses that may support virus replication. The big advantage is that the virus cannot replace the missing cellular function and, thus, emergence of resistance should not easily occur. In the following, two recent strategies to target cellular signalling factors for anti-influenza therapy will be highlighted.

Inhibitors of cellular factors: blocking virus-supportive signalling processes

Infection of cells with influenza viruses results in the activation of a variety of intracellular signalling pathways[10] that are in part exploited by the virus to ensure efficient replication.[11] These dependencies may be used to develop novel antiviral drugs that disrupt signal transmission.[12] Two signalling pathways that are required for efficient influenza virus propagation have attracted some attention as suitable targets for an antiviral approach, the Raf/MEK/ERK mitogenic kinase cascade[13] and the IKK/NF-κB module.[14]

The Raf/MEK/ERK signalling pathway belongs to the family of the so-called mitogen-activated protein kinase (MAPK) cascades. Signalling 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 (reviewed in Widmann *et al.*[15]).

Strikingly, specific blockade of the Raf/MEK/ERK pathway strongly impaired growth of all influenza A and B-type viruses tested so far.[16–18] With respect to the underlying molecular mechanisms it was demonstrated that cascade inhibition led to

nuclear retention of the viral ribonucleoprotein (RNP) complexes in late stages of the replication cycle.[16] The timely activation is achieved by membrane accumulation of the viral haemagglutinin protein and subsequent protein kinase C α-dependent activation of the Raf/MEK/ERK cascade.[19]

As a proof-of-concept in an animal model, an inhibitor of the central kinase MEK, U0126, resulted in reduced virus titres in the lungs of infected mice after local aerosolic administration into the trachea. Targeting the Raf/MEK/ERK pathway at its bottleneck may have several advantages. Besides strong and broad antiviral activity, MEK inhibitors showed surprisingly little toxicity in cell culture,[16,17,20] in an *in vivo* mouse model[21] and in clinical trials for use as an anticancer agent.[22] Furthermore, one study showed that there is no tendency to induce viral resistance as assessed in a multipassaging cell culture assay.[17] Finally, the Raf/MEK/ERK cascade is also a regulator of cytokines, such as tumour necrosis factor (TNF)-α or interleukin-8, and thus inhibition may also prevent excessive inflammation due to overabundant production of proinflammatory cytokines and chemokines, also known as the cytokine burst. Several inhibitors of the Raf/MEK/ERK cascade from different pharmaceutical companies are under clinical investigation and these studies demonstrated that the cascade can indeed be effectively inhibited in humans without adverse side effects.

Another important influenza virus-induced signalling mediator is the transcription factor NF-κB. This factor regulates expression of a variety of antiviral cytokines, including interferon (IFN)-β, which is the initiator of a strong type I IFN defence programme.[23] Although NF-κB is generally regarded as a central factor in the innate immune defence,[24] two independent studies demonstrated that replication of influenza viruses was impaired rather than enhanced in cells where the pathway was blocked.[25,26]

Several molecular mechanisms have been identified to confer this virus-supportive function of NF-κB. First, it was shown that NF-κB acts via induction of proapoptotic factors, such as TNF-related apoptosis-inducing ligand (TRAIL) or FasL,[25] and subsequent activation of caspases.[27] Caspase activation resulted in an enhanced nuclear export of viral RNPs, presumably by specific cleavage of nuclear pore proteins, resulting in an enhanced diffusion limit through the pores.[28,29] A second mechanism involves NF-κB-dependent counteraction of type I IFN induced gene (ISG) expression that may occur either by up-regulation of the suppressor of cytokine signalling-3 (SOCS-3)[30] and/or by direct suppression of ISG promoter regions.[31] Lastly, it was also shown that NF-κB differentially regulates viral RNA synthesis.[32] Each of these mechanisms may contribute to a different extent to the enhancing effect of NF-κB on virus propagation, identifying the factor as a suitable target for antiviral intervention.

A 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, is an efficient and quite selective inhibitor of the NF-κB-activating kinase IKK2.[33] 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 host cells.[34] Application of the compound as an aerosol directly into the trachea of lethally infected mice reduced virus titres in the lung and significantly promoted survival.[34] The drug

was well tolerated in mice and did not exhibit harmful side effects. Strikingly, the study also shows that ASA, in contrast to amantadine or oseltamivir, did not lead to the generation of resistant virus variants in multipassaging experiments in cell culture.[34]

In the light of these data it is surprising that the antiviral action of ASA has been neither observed previously in animal models[35] nor in epidemiological studies in humans. This may be due to the fact that ASA is not usually inhaled but administered orally or by injection, which does not lead to sufficiently high concentrations in the lung. Thus, topical treatment with an aerosol would be the mandatory application route.

These promising results prompted further research with other NF-κB-inhibiting agents, such as the NF-κB inhibitor SC75741, which exhibited an even better antiviral efficacy in cells and mice without the emergence of viral resistance.

Besides the direct antiviral action, NF-κB inhibition may also indirectly influence pathogenesis of influenza virus, since the majority of cytokines/chemokines that are hyperinduced during infection with highly pathogenic viruses (cytokine burst) are regulated by NF-κB.[23]

Conclusions and perspectives

Our current options regarding clinically approved antiviral drugs against influenza are very limited. M2 inhibitors clearly cannot 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, both of circulating strains as well as of highly pathogenic avian strains of the H5N1 type. Therefore, it cannot be ruled out that a future pandemic virus may already be resistant to NA inhibitor treatment. Moreover, it might be concluded that every new drug that exclusively targets viral structures will sooner or later share the fate of M2 and NA inhibitors. This also raises concerns in the pharmaceutical industry whether to invest in viral-target approaches that may be ineffective after a few years. Thus, there is consensus among many experts that we urgently need alternative approaches for influenza therapy.

Accordingly, a wide variety of different antiviral strategies have been explored in recent years. A few examples should be mentioned here, including the use of viral attachment or fusion inhibitors, such as arbidol[36] and other compounds, IFN treatment or stimulators of the IFN system, or polyphenolic plant-derived agents,[37] which prevent resistance by unspecific blockade of virus binding.[38]

Among all the novel approaches, the targeting of cellular signalling pathways that are essential for virus propagation may be particularly promising to prevent resistance. Although these strategies are still in a very early phase of pre-clinical development, it seems that it is indeed possible to target these pathways without harmful side effects or the emergence of resistance. In addition, inhibitors of MEK and NF-κB are broadly active and may have additional beneficial effects, e.g. the suppression of overabundant cytokine expression that may prevent the detrimental cytokine burst. Finally, there are already many inhibitors of MEK and NF-κB under clinical investigation for other purposes and a lot of clinical data have been accumulated. It may be more attractive for a pharmaceutical company to start a development programme with a given drug towards an additional

antiviral use rather than to start from the very beginning. It is interesting to see that a significant portion of new drug approaches target cellular factors, indicating that start-ups and pharmaceutical companies are increasingly attracted by this novel concept.

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