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-\(\kappa\)B. It has been shown that inhibition of both virus-induced pathways leads to impaired virus production \textit{in vitro} and \textit{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 economic burden. 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. 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. In addition, increasing numbers of H5N1 virus isolates from humans and birds exhibit genotypic resistance to M2 inhibitors. 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. While in clinical trials of oseltamivir in seasonal influenza only a low percentage of resistance has been reported,
Infection of cells with influenza viruses results in the activation virus-supportive signalling processes. Inhibitors of cellular factors: blocking Lastly, with the beginning of the 2007–08 influenza season in retained full replication competence and transmissibility.3 Influenza B viruses as well as H5N1 influenza A viruses that are has rapidly increased, including findings on the emergence of more worrying rates have been detected in a smaller study 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, in an in vivo mouse model 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 storm. 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 1 IFN defence programme.23 Although NF-κB is generally regarded as a central factor in the innate immune defence, two independent studies demonstrated that replication of influenza viruses was impaired rather than enhanced in cells where the pathway was blocked.24,25

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, and subsequent activation of caspases.7 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 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) 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

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 that are in part exploited by the virus to ensure efficient replication.10 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 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, in an in vivo mouse model 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 storm. 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.

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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 FasL28 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 pores28,29. 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.

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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 multipassing 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
models35 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 exclu-
sively 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 alterna-
tive 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 arbidol36 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 sig-
nalling 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 develop-
ment, 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 detri-
ment of cytokine burst. Finally, there are already many inhibitors
of MEK and NF-κB under clinical investigation for other pur-
poses and a lot of clinical data have been accumulated. It may
be more attractive for a pharmaceutical company to start a de-
velopment 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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