

# **Practical Strategies for Targeting NF-kappaB and NADPH Oxidase May Improve Survival During Lethal Influenza Epidemics**

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## **Abstract**

The most foolproof way to promote survival in epidemics of potentially lethal influenza is to target, not highly mutable viral proteins, but rather intracellular signaling pathways which promote viral propagation or lung inflammation. NF-kappaB, activated in influenza-infected lung epithelial cells and macrophages, is one likely target in this regard, as it plays a role both in viral replication and in the excessive lung inflammation often evoked by influenza infection. Indeed, salicylates, which suppress NF-kappaB activation, have been shown to reduce the lethality of H5N1 avian-type influenza in mice. Another potential target is NADPH oxidase, as this may be a major source of influenza-evoked oxidant stress in lung epithelial cells as well as in phagocytes attracted to lung parenchyma. A number of studies demonstrate that oxidant stress contributes to overexuberant lung inflammation and lethality in influenza-infected mice. The documented utility of N-acetylcysteine, a glutathione precursor, for promoting survival in influenza-infected mice, and diminishing the severity of influenza-like infections in elderly humans, presumably reflects a key role for oxidative stress in influenza. The lethality of influenza is also reduced in mice pretreated with adenovirus carrying the gene for heme oxygenase-1; this benefit may be mediated, at least in part, by the ability of bilirubin to inhibit NADPH oxidase. It may be feasible to replicate this benefit clinically by administering biliverdin or its homolog phycocyanobilin, richly supplied by spirulina. If this latter speculation can be confirmed in rodent studies, a practical and inexpensive regimen consisting of high-dose salicylates, spirulina, and N-acetylcysteine, initiated at the earliest feasible time, may prove to have life-saving efficacy when the next killer influenza pandemic strikes.

## **Targeting Intracellular Signaling Pathways in Flu Therapy**

The influenza epidemic of 1919 is estimated to have killed 20-40 million people, and there is considerable concern that avian flu (influenza A, H5N1), which has killed about half of the people who have managed to acquire it, might someday mutate to a strain that is readily transmitted from person to person, giving rise to another lethal pandemic. Efforts to protect against this eventuality have focused on vaccine production and the development of additional drugs that target the function of viral proteins. Unfortunately, influenza viruses are capable of mutating rapidly, implying that they can often quickly develop immunity to vaccines and drugs targeting their proteins.<sup>1-3</sup> Thus, some insightful researchers have suggested that it may be more fruitful to focus on human cellular signaling pathways that the virus exploits to proliferate and spread, or that contribute to the inflammatory pneumonitis that can make influenza infection lethal.<sup>4-6</sup> In particular, attention has been drawn to the fact that an overexuberant inflammatory response in lung parenchyma is largely responsible for the lethality of “killer flus”.<sup>7-10</sup> Indeed, the most lethal strains of influenza are characterized by increased capacity to induce

proinflammatory cytokines in human macrophages.<sup>8</sup> Thus, measures which dampen the influx and activation of leukocytes during influenza infection may aid survival – even if they don't promote clearance of the virus.

### **Multiple Roles for NF-kappaB in Influenza Infection**

One such potential target is NF-kappaB. This family of transcription factors is activated in influenza-infected pulmonary epithelial cells,<sup>4, 11-13</sup> and this activation appears to contribute to the life cycle of the virus by aiding the caspase-mediated nuclear export of viral nucleoproteins.<sup>14</sup> NF-kappaB also protects the virus by inhibiting the ability of type-1 interferons to induce antiviral proteins.<sup>15</sup> Moreover, NF-kappaB activation evidently plays a role in the inflammatory response to influenza infection, as it promotes the transcription of a number of proinflammatory cytokines; thus, inhibitors of IkappaB kinase suppress the production of interleukin-8 in human epithelial cells infected with influenza A.<sup>9</sup> Mazur and colleagues have recently shown that aspirin, which in high concentrations prevents NF-kappaB activation by blocking IkappaB kinase-beta activity,<sup>16, 17</sup> suppresses the propagation of an Asian influenza virus in human cell cultures; this effect was not replicated by indomethacin, suggesting that inhibition of NF-kappaB, rather than cyclo-oxygenase, was responsible for aspirin's activity.<sup>6</sup> Moreover, aspirin administered in drinking water or via aerosol reduced the lethality of this virus in mice. This study did not determine the extent to which a direct anti-viral effect, as opposed to anti-inflammatory activity, was responsible for this protection, although lung viral titers were indeed noted to be lower in mice receiving aerosolic aspirin. These findings suggest that prompt administration of oral or aerosolized aspirin, in high doses capable of influencing NF-kappaB activity, might improve survival during life-threatening influenza infections. A better option in this regard might be salicylate (clinically available in various forms such as sodium salicylate or salsalate), which is no less effective than its derivative aspirin in inhibiting NF-kappaB, but, owing to its weak and transient impact on cyclooxygenase activity, does not increase risk for gastrointestinal bleeding.<sup>18-21</sup> The high oral doses of aspirin required for NF-kappaB suppression in contrast, would pose a considerable risk in this regard.

### **NADPH Oxidase as a Potential Target**

Another potential target in the treatment of influenza is NADPH oxidase. Influenza has been shown to induce oxidant stress in the cells it infects, an effect mediated at least in part by the viral hemagglutinin protein;<sup>11</sup> this evoked oxidant stress may contribute to activation of NF-kappaB.<sup>11, 12</sup> Lung epithelial cells express several different isoforms of NADPH oxidase,<sup>22-24</sup> and it seems likely that this enzyme complex is a major source of excess oxidant stress in influenza-infected cells – albeit xanthine oxidase may also contribute in this regard,<sup>25</sup> and a decrease in intracellular glutathione levels exacerbates the impact of oxidants in infected cells.<sup>26, 27</sup> The phagocytic form of NADPH oxidase expressed by leukocytes infiltrating infected lung tissue also evidently contributes to total oxidative stress in infected lung parenchyma.

The increased oxidant stress evoked by influenza infection may contribute to the efficient viral propagation, while also playing a key role in inflammatory lung damage. Measures which boost glutathione levels in infected cells have been shown to decrease the transcription of late viral

proteins.<sup>26</sup> Administration of N-acetylcysteine, which promotes glutathione synthesis by increasing intracellular availability of free cysteine,<sup>28</sup> diminishes mortality in influenza-infected mice.<sup>29</sup> In a double-blind placebo-controlled study, supplementation with N-acetylcysteine (1200 mg daily) was found to decrease both the number and the severity of influenza-like episodes during the winter in initially healthy elderly volunteers.<sup>30</sup> (No attempts have been made to replicate this remarkable study, published in 1997.) In transgenic mice overexpressing extracellular superoxide dismutase, influenza infection evokes far less lung pathology than in wild-type mice.<sup>7</sup> Induction of the inducible isoform of nitric oxide synthase also contributes notably to lung damage during influenza infection in mice, as such infection was less lethal and produced less lung pathology in mice genetically deficient in iNOS,<sup>31</sup> in wild type mice, administration of the iNOS inhibitor L-NMMA reduced the lethality of influenza.<sup>32</sup> This likely points to a role for peroxynitrite in influenza-associated lung pathology – implying that a reduction in superoxide production could provide protection by decreasing peroxynitrite formation. Reduced inflammation of lung parenchyma has also been noted during influenza infection in mice that lack the phagocytic form of NADPH oxidase or that are treated with a cell-permeable mimic of superoxide dismutase.<sup>10</sup>

Of particular interest is a study demonstrating that adenovirus-mediated transfer of heme oxygenase-1 (HO-1) cDNA reduces the lung damage and influx of leukocytes in influenza-infected mice, while markedly boosting survival (60% vs. 0% in controls).<sup>33</sup> HO-1 generates bilirubin, which recently has been shown to be a potent physiological inhibitor of NADPH oxidase activity.<sup>34-37</sup> The possibility that concurrent generation of carbon monoxide also contributed to the observed protection merits consideration, but it is reasonable to suspect that the potent antioxidant activity of bilirubin contributed prominently to the protection afforded by HO-1 transfection. This conclusion is of particular interest in light of suggestions that oral administration of biliverdin, or of its homolog phycocyanobilin (PCB - richly supplied by spirulina), can mimic the NADPH oxidase-inhibitory activity of endogenous bilirubin.<sup>37, 38</sup> It would thus be of great interest to examine the impact of oral biliverdin, dietary spirulina, or PCB-enriched spirulina extracts on the course of influenza infection in mice.

### **Protective Potential of Combination Regimens**

If NADPH oxidase proves to be a useful target in influenza therapy, it is reasonable to expect that jointly targeting both this enzyme complex *and* NF-kappaB would produce a more substantial impact on the survivability of potentially lethal influenza strains. If this strategy showed promising results in mice, joint administration of ample doses of spirulina and salicylate, initiated as soon as feasible during infection, might be expected to have life saving potential during dangerous flu epidemics. This would be a reasonably feasible and inexpensive strategy, as spirulina and salicylate could be stockpiled in anticipation of such an epidemic. Inclusion of N-acetylcysteine in this regime – also inexpensive and readily available - might further boost its protective efficacy, and it would of course be reasonable to use this regimen in conjunction with any drugs targeting viral proteins that would seem likely to have efficacy. In this regard, administration of N-acetylcysteine in conjunction with antiviral drugs (ribavirin or oseltamivir) produces a more substantial reduction in influenza lethality in mice than either agent administered alone.<sup>39, 40</sup>

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