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NADPH OXIDASES AS NOVEL PHARMACOLOGICAL TARGETS AGAINST INFLUENZA A VIRUS INFECTION

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ABSTRACT

Influenza A viruses represent a major global healthcare challenge with imminent pandemics, emerging anti-viral resistance and long lag times for vaccine development raising a pressing need for novel pharmacological strategies that ideally target the pathology irrespective of the infecting strain. Reactive oxygen species (ROS) pervade all facets of cell biology with both detrimental and protective properties. Indeed there is compelling evidence that activation of the nicotinamide adenine dinucleotide phosphate-oxidase NADPH oxidase 2 (NOX2) isoform of the NADPH oxidase family of ROS-producing enzymes promotes lung oxidative stress, inflammation, injury and dysfunction to influenza A viruses of low to high pathogenicity, as well as impeding virus clearance. By contrast the DUOX isoforms produce ROS that provide vital protective antiviral effects for the host. In this review, we propose that inhibitors of NOX2 are better alternatives than broad-spectrum antioxidant approaches for treatment of influenza pathologies, for which clinical efficacy may have been limited owing to poor bioavailability and inadvertent removal of beneficial ROS. Finally, we briefly describe the current suite of NADPH oxidase inhibitors and the molecular features of the NADPH oxidase enzymes that could be exploited by drug discovery for development of more specific and novel inhibitors to prevent or treat disease caused by influenza.

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INTRODUCTION

Influenza A viral infections cause significant global morbidity and mortality, and are a tremendous economic burden worldwide with annual medical costs in the US being approximately $90 billion (WHO, 2009). Influenza A viruses can cause incapacitating respiratory illness in humans, and severe and often fatal respiratory and systemic complications from pandemic influenza such as the 1918 strain and the highly pathogenic H5N1 virus (Doherty *et al.*, 2006). Annually, influenza epidemics are responsible for ~5 million cases of severe illness and 200,000-500,000 deaths worldwide (WHO, 2009). In the last century up to 100 million deaths occurred worldwide to the four major pandemics (WHO, 2009).

Currently, two therapeutic options exist to combat seasonal and pandemic influenza A virus outbreaks (Osterholm, 2005); the use of anti-virals (e.g. the neuraminidase (NA) inhibitors zanamivi*r* (Relenza®) and oseltamivir (Tamiflu®*)* (Moscona, 2008; Moscona, 2005a) and strain-specific vaccination. The anti-virals exert their therapeutic actions by preventing release of influenza virus progeny from infected cells and transmission of the virus. However, certain strains of influenza A viruses are developing resistance to these compounds (Moscona, 2005b). Current vaccines bolster host immune responses to surface influenza A virus glycoproteins but are limiting, as they are only effective against current circulating viruses. However, the global population is at continual risk because of the long lag time (~6 months) for vaccine production and the ongoing threat of new pandemic strains.

The adverse effects of severe influenza infections are believed to be due to inappropriate and heightened airways and lung inflammation. (La Gruta *et al.*, 2007). This is the outcome of an immune response mounted by the host against influenza virus as a result of respiratory epithelial cell and alveolar macrophage infection. The activation of airway epithelium and alveolar macrophages by internalized influenza virus results in a burst of

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cytokine release including a number of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, interlukin-6 (IL-6), interferon (IFN)-γ as well as chemokines including IL-8, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2) and MIP-1α (Doherty *et al.*, 2006) (Perrone *et al.*, 2008). This process of cytokine and chemokine release is crucial for the recruitment of additional inflammatory cells including neutrophils and blood borne monocytes that differentiate into macrophages, which are important for viral clearance. However, this early innate immune response sets off an inflammatory cascade that causes persistent trafficking of inflammatory cells into the lung and an excessive production of pro-inflammatory cytokines and chemokines in what is referred to as the "*cytokine storm"* (La Gruta *et al.*, 2007). This has been considered the underlying contributor to lethal disease caused by H5N1 viruses and the 1918 pandemic strain (Tumpey *et al.*, 2005). Thus, the recruitment and activation of infiltrating macrophages and neutrophils must be tightly regulated during influenza infection to promote viral clearance and to minimize excessive damage of surrounding lung tissue. A developing area of research implicates oxidative stress or an overproduction of ROS, as a cause of lung tissue damage to influenza, which we propose might be a novel target for pharmacological modulation (Figure 1).

Reactive oxygen species – general properties

ROS are generally formed from the one electron reduction of molecular oxygen, giving rise to the parent ROS specie superoxide anion (Equation 1). Superoxide anions act locally at their site of production and generally fail to cross cellular membranes (although may pass through some ion channels) because they are negatively charged molecules. This localized property of superoxide compartmentalises its oxidising power, making it along with its derivative i.e. hypochlorous acid (HOCl-) ideal microbicidal molecules to kill invading

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pathogens within the confines of phagosomes of neutrophils and macrophages. This phagosomally located ROS production protects the cell from the potential damaging effects of ROS throughout the rest of the cell. An additional single electron reduction of superoxide produces hydrogen peroxide (H_2O_2) (Equation 2), which is uncharged and free to diffuse across cellular membranes.

$$2O_2 + 2e- \rightarrow 2O_2-\bullet \quad (1)$$

$$2O_2-\bullet + 2H+ \rightarrow H_2O_2 + O_2 \quad (2)$$

Both superoxide and hydrogen peroxide influence varied biological targets, with superoxide preferentially reacting with iron sulfur clusters, found on many transcription factors, whereas H_2O_2 reacts with susceptible Cys residues found on enzyme proteins such as peroxidases, kinases, phosphatases etc (Imlay, 2008; Salmeen *et al.*, 2003).

As with all signalling molecules, superoxide and H_2O_2 levels are tightly controlled to prevent excessive levels causing inappropriate activation of redox sensitive signalling pathways, and oxidative damage. For example, the reaction between superoxide and H_2O_2 gives rise to the highly reactive hydroxyl radical (OH•) via the Haber-Weiss reaction (Equation 3) (Haber *et al.*, 1932), which is greatly enhanced in the presence of free transition metal ions (e.g. Fe^{2+}, Cu^{2+}; known as the Fenton reaction when iron catalysed) (Equation 4) (Chance *et al.*, 1979). Hydroxyl radicals are the most powerful oxidizing agents yet identified, and they react indiscriminately with most biological molecules at near diffusion-limited rates.

$$O_2-\bullet + H_2O_2 \rightarrow OH\bullet + OH- + O_2 \quad (3)$$

$$H_2O_2 + Fe^{2+} \rightarrow OH\bullet + OH- + Fe^{3+} \quad (4)$$

A particularly relevant reaction occurring in inflammatory cells such as macrophages is that between superoxide and nitric oxide (NO• (Equation 5). The second order rate constant for this radical-radical reaction is ~10^{10} $M^{-1}s^{-1}$, and therefore its generation is most probably

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limited only by the diffusion of the two substrates (Beckman *et al.*, 1990). The product of this reaction is peroxynitrite ($ONOO^-$), which has a high degree of oxidising potential, perhaps as high as hydroxyl radicals and has been implicated as a major mediator of tissue injury to virus infections (Szabo *et al.*, 2007).

$$O_2\text{-}\bullet + NO\bullet \rightarrow ONOO\text{-} \qquad (5)$$

ROS influence lung pathology caused by influenza A viruses

Accumulating evidence from circa late 1980s suggest ROS, including superoxide and its derivatives, promote the lung injury and inflammation to influenza A virus infection (Akaike *et al.*, 1996; Imai *et al.*, 2008). The first study implicating superoxide demonstrated that inactivation of this radical by administration of pyran polymer conjugated superoxide dismutase (SOD) improved the mortality of mice against a lethal influenza virus infection (Oda *et al.*, 1989). From a therapeutic point of view this was a vital observation as it demonstrated that protection could occur if SOD was administered 5 days post infection. In keeping with a role for superoxide as a culprit molecule it was subsequently shown that mice with selective over-expression of extracellular SOD displayed significantly less lung injury to influenza virus (Suliman *et al.*, 2001). These beneficial effects of superoxide inactivation may be due to inhibition of its downstream derivative, peroxynitrite, which causes significant alterations in the activation of small molecule antioxidants such as glutathione, cysteine and tetrahydrobiopterin, and enzymes SOD, glutathione reductase and glutaredoxin (Szabo *et al.*, 2007) by oxidation-dependent inactivation. Indeed, peroxynitrite generation is associated with epithelial cell apoptosis and lung injury to influenza A virus infection (Vlahos *et al.*, 2011). Moreover, peroxynitrite suppresses Na^+ channels on the apical membrane of airway epithelial cells (Lazrak *et al.*, 2009) causing an imbalance in fluid homeostasis leading to airways oedema. In addition, manipulation of superoxide production will decrease H_2O_2

levels. This could result in alterations in the master transcription factor nuclear factor kappa B (NF-κB) and expression of pro-inflammatory genes that influence lung inflammation and damage (Rahman *et al.*, 2006, Takada *et al.*, 2003, Rahman *et al.*, 2002). We have recently shown that the antioxidant enzyme glutathione peroxidase-1 (GPx-1), which converts H_2O_2 into water and oxygen, protects against influenza A virus-induced lung inflammation and pathology (Yatmaz *et al.*, 2013). In support of this, mice treated with the SOD/catalase mimetic EUK beginning 3 days postinfection with the 1918 influenza A virus resulted in significantly increased survival and reduced lung pathology without a reduction in viral titers (Kash *et al.*, 2014). Immunohistochemical analysis showed a reduction in the detection of the apoptosis marker cleaved caspase-3 and the oxidative stress marker 8-oxo-2'-deoxyguanosine in lungs of EUK-207-treated animals compared to vehicle controls (Kash *et al.*, 2014). Together these findings highlight the pathological role of H_2O_2 in influenza infection.

The bulk of the experimental data thus far places ROS as culprit mediators of lung injury to influenza A virus infections. However, it is noteworthy that there is an emergence of literature that indicates ROS as possessing important antiviral effects against influenza viruses. In fact the key study by Strengert *et al*., 2014 showed that H_2O_2 suppresses virus replication and release of virus by airway epithelial cells (Strengert *et al.*, 2014).

It is unquestionable that ROS promote the lung injury to influenza A virus infections and that suppressing their levels and therefore effects might be beneficial. There are several ways by which ROS can be manipulated however, given that some of them i.e. superoxide, hydroxyl radicals and peroxynitrite are highly and indiscriminately reactive and will essentially react with substrates within their vicinity with high avidity, perhaps the most effective form of defence against them is to prevent their formation. There are many ways by which this might be achieved but here we highlight two that have received the most attention.

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1. Limit the formation of their parent ROS superoxide, H_2O_2 and/or NO, although it is important to maintain the balance of good antiviral effects of ROS versus the detrimental effects of excessive ROS. This might be achieved by targeting specific sources of ROS.

2. Regulate superoxide and H_2O_2 metabolism by stoichiometric scavenging with expendable molecules such as ascorbate, vitamin E, etc. or with specific antioxidant enzyme systems such as superoxide dismutases (SODs), catalase, glutathione peroxidases, thioredoxins, etc.

Both of these scenarios have been exploited with pharmacological inhibitors to varying degrees. However, this review will focus on Point 1, namely regulation of the production of superoxide and H_2O_2 through inhibition of their major enzymatic sources – the NADPH oxidases.

ROS generating enzyme systems in mammalian cells

A number of enzyme systems transfer electrons to molecular oxygen to produce ROS, including NADH dehydrogenase and ubiquinone-cytochrome bc1 of the mitochondrial electron transport chain (Boveris *et al.*, 1975; Turrens, 2003; Orrenius *et al.*, 2007), NO synthases (NOS) (Alp *et al.*, 2004; Vasquez-Vivar *et al.*, 2000; Vasquez-Vivar *et al.*, 1998), cyclooxygenases (Simmons *et al.*, 2004), lipoxygenases (Wittwer *et al.*, 2007), cytochrome P450 reductases (Zangar *et al.*, 2004) and xanthine oxidase (McCord *et al.*, 1968; Pacher *et al.*, 2006). However, for all of these systems, ROS production does not represent the main catalytic function of the enzyme but instead occurs as a by-product of another reaction (e.g. as in the mitochondrial electron transport chain) or from a dysfunctional variant of the enzyme (e.g. uncoupled NOS, xanthine dehydrogenase → xanthine oxidase). The *only* enzymes whose sole function is to generate ROS are the NADPH oxidases.

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NADPH oxidase was first identified in phagocytic cells (Rossi *et al.*, 1963) as the enzyme responsible for the respiratory burst (Baldrigde *et al.*, 1933) essential to the microbicidal function of these cells (Iyer *et al.*, 1963; Babior *et al.*, 1973; Babior *et al.*, 2002; McPhail *et al.*, 1976; Segal, 2005; Segal *et al.*, 1978). However, it later became apparent that these enzymes are also expressed in a variety of non-phagocytic cells, including endothelial cells, smooth muscle cells and adventitial fibroblasts of blood vessels following on from the seminal observations by Griendling *et al*. that vascular smooth muscle cells also display NADPH oxidase activity (Griendling *et al.*, 1994).

NADPH oxidases are a family of enzyme complexes of multiple isoforms, which primarily are distinguished by their membrane-spanning catalytic 'NOX' or 'DUOX' subunit that it utilizes to transfer electrons from NADPH to molecular oxygen. Seven members of the NADPH oxidase family have been identified to date in mammalian species, including NOX1- through 5- as well as DUOX1- and 2- containing oxidases (Ris-Stalpers, 2006; (Lambeth *et al.*, 2007). Moreover, full activity of the individual NADPH oxidase isoforms rely to varying degrees on the association of certain regulatory proteins with the NOX catalytic subunit. For instance, NOX1- and NOX2 associate with a smaller integral membrane protein, p22phox (Ambasta *et al.*, 2004; Hanna *et al.*, 2004), which appears to stabilise the Nox protein within various cellular membranes, as well as an 'organiser' protein (p47phox or NoxO1), an 'activator' protein (p67phox or NoxA1) and a small GTPase, Rac1. NOX4, by contrast, may only require the association of p22phox for full activity, while NOX5 appears to function independently of any associated regulatory proteins.

NADPH oxidases are expressed in key cell types of the airways

All cells of the airways express NADPH oxidase isoforms (Figure 1). The key Nox isoform expressed in macrophages and neutrophils is the NOX2 oxidase, whereas airway and alveolar

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epithelial cells express NOX1 (Carnesecchi *et al.*, 2009), NOX2 (Fink *et al.*, 2008; Soucy-Faulkner *et al.*, 2010; Takemura *et al.*, 2010; Tickner *et al.* 2011) and DUOX1 and 2. In vascular endothelial cells there is a high abundance of both NOX2 and NOX4 oxidases, and in vascular smooth muscle there is a strong expression of NOX4 (Drummond *et al.*, 2011; Peshavariya *et al.*, 2009a; Peshavariya *et al.*, 2009b; Peshavariya *et al.*, 2007; Selemidis, 2008; Selemidis *et al.*, 2007). Unlike NOX2 and NOX1, which produce superoxide and then H_2O_2 following dismutation of superoxide by SOD, NOX4, DUOX1 and DUOX2 enzymes generate H_2O_2 directly (Drummond *et al.*, 2011).

There has been a strong focus on identifying the roles of NOX2 in the context of influenza A virus infection, as it is (i) highly expressed in inflammatory cells that are both resident (i.e. alveolar macrophages) and recruited to the lungs (i.e. macrophages and neutrophils) and in epithelial cells (which are directly infected by virus), (ii) generates a large burst of superoxide and (iii) is responsible for peroxynitrite production. Despite a clear message that suppression of ROS production from NOX2 is beneficial against primary influenza A virus infection, recent work on other NADPH oxidase isoforms in particular NOX1 and DUOX indicate the contrary. In fact, it appears that both NOX1 and DUOX 2 protect the host against these types of infections (Selemidis *et al.*, 2013; Strengert *et al.*, 2014).

NADPH oxidase in influenza A virus-induced lung injury: rationale for targeting the NOX2 isoform

NOX2 oxidase is a key player in the pathogenesis of influenza A virus infection via its production of ROS. Indeed for nearly 30 years now its known that influenza A viruses initiate the respiratory burst characterised by the production of large amounts of superoxide and other ROS in inflammatory cells (Mills *et al.*, 1981; Oda *et al.*, 1989). In some cases,

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inflammatory cells isolated by bronchoalveolar lavage of mice infected with lethal doses of influenza virus (PR8 strain) demonstrate ~15-70 times more superoxide than cells from uninfected mice (Buffinton *et al.*, 1992). We have recently shown, *ex vivo* infection of alveolar macrophages results in a significant elevation in stimulated superoxide production via NOX2 oxidase (To *et al.*, 2014). *In vivo*, abolition of NOX2 (i.e. by use of the NOX2-deficient mouse), leads to a reduction in lung injury and improvement in lung function following influenza A virus infection (Snelgrove *et al.*, 2006). Moreover, macrophages are crucial for causing oxidative stress-dependent acute lung injury following H5N1 virus infections most likely via superoxide production and oxidized phospholipid generation *by* NOX2 oxidase (Imai *et al.*, 2008). Suppression of the regulatory subunit of NOX2 i.e. p47phox reduced lung pathology and oedema following H5N1 (Imai *et al.*, 2008). Influenza A infection increases superoxide production by BALF inflammatory cells that was solely due to NOX2 oxidase, and importantly, this gave raise to peroxynitrite production in the lungs that contributed to the lung injury (Vlahos *et al.*, 2011). In addition, $NOX2^{-/y}$ mice had substantially reduced airways inflammation and alveolar epithelial apoptosis following infection with low (HKx31) and high pathogenicity (PR8) influenza A viruses (Vlahos *et al.*, 2011). Strikingly, inhibition of NOX2 oxidase resulted in reduced viral titers in the lungs (Snelgrove *et al.*, 2006; Vlahos *et al.*, 2011) following influenza A virus infection even in the face of a suppression of airways inflammation. Vital components of the adaptive immune system that clear influenza A viruses from the lungs, were preserved following inhibition of NOX2 oxidase activity (Snelgrove *et al.*, 2006; Vlahos *et al.*, 2011). For instance, the degree of airway infiltration of influenza A virus specific $CD8^+$ T cells (i.e. $CD8^+DbNP366^+$ and $DbPA224^+$ T cells) including $CD8^+$ T cells that express IFN-γ, TNF-α or IL-2 was either unaffected (Vlahos *et al.*, 2011) or enhanced (Snelgrove *et al.*, 2006).

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The evidence thus far would suggest that inhibition of NOX2 oxidase in vivo could lead to an improvement in phenotype against influenza A virus infections of mild to high pathogenic strains of virus. However, studies in mice deficient in the NOX1 oxidase have resulted in some conflicting results. For instance, NOX1 has been demonstrated to be protective to the host from influenza A virus infection (Selemidis *et al.*, 2013). Indeed, NOX1 oxidase was shown to critically inhibit the early burst in lung pro-inflammatory cytokine expression, inflammation and oxidative stress caused by influenza A virus infection. In contrast, a separate study has shown that deletion of NOX1 resulted in an improvement in survival to a lethal dose of the PR/8 strain of influenza (Hofstetter *et al.*, 2013). It is unclear as to why there appears to be conflicting data between these two studies, however, it may be attributed to the differences in pathogenicity of the viruses used in each of the studies, i.e. a mild X-31 infection in the case of Selemidis et al., 2013 versus a highly lethal dose of PR/8 in the Hofstetter et al., 2013 study. This however, remains to be determined.

A key difference between NOX1 and NOX2 oxidases that is likely to underpin their distinct effects is their cellular localization pattern. NOX1 was found to be highly expressed in alveolar epithelial cells and vascular endothelial cells, whereas NOX2 is most highly expressed in inflammatory cells and to a lesser degree in epithelial and endothelial cells. Intriguingly, DUOX2, which is highly expressed in airway epithelial cells and the major source of H_2O_2 by these cells, like NOX1 appears to protect the host from influenza A virus infections. DUOX2 activity was associated with a marked reduction in influenza replication within epithelial cells resulting in protection in the early phases of infection (Strengert *et al.*, 2014). Overall it appears ROS have contrasting roles on influenza virus infections depending in the cell type they are generated within. For instance, epithelial ROS by DUOX (and perhaps NOX1) are protective, whereas inflammatory cell NOX2 is detrimental. These contrasting roles of ROS not only highlight the pressing need for future studies utilizing cell

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specific inhibition of NADPH oxidase isoforms on the overall host anti-viral response but also that use of broad base antioxidant approaches to scavenge ROS indiscriminately of their source, might not be the most effective approach. Thus selective enzyme inhibitors may prove to be the best approach. Here we suggest, given that bulk of the evidence, that specific inhibitors of NOX2 oxidase might be the most effective approach to reduce the burden of the oxidative stress-dependent lung injury to influenza A virus infections.

NADPH oxidases and other lung conditions

In addition to the disease caused by influenza A virus infection, NOX-generated ROS have been recognized to play key roles in the pathogenesis of a number of diverse chronic lung disorders that result in obstructive physiology, in particular asthma, cystic fibrosis, and emphysema (Griffith *et al.*, 2009). Mice deficient in p47phox or NOX2 exhibit increased cigarette smoke–induced lung inflammation and emphysema despite decreased ROS production (Yao *et al.*, 2008). The lung responses in p47phox- and NOX2-null mice were associated with increased production of proinflammatory cytokines and chemokines via a TLR4-NF-kB pathway, indicating that NOX2 may mediate anti-inflammatory functions by restraining TLR4 activation (Yao *et al.*, 2008). However, another group reported that p47phox-null mice have less inflammation, IL-6, keratinocyte-derived chemokine, and MCP-1 in lung-lavage specimens after cigarette-smoke exposure compared with WT mice (Lagente *et al.*, 2008). The differences observed by these groups may be due to variability in lung compartment sampling, cellular distributions, and chronicity of cigarette-smoke exposure.

MOLECULAR DESCRIPTION OF NADPH OXIDASES

The purpose of this section is not to provide a comprehensive review of the molecular biology and biochemistry of the various NADPH oxidase family members, as this has been

achieved by several reviews (Vignais, 2002; Sumimoto *et al.*, 2005; Groemping *et al.*, 2005; Selemidis *et al.*, 2008; Drummond *et al.*, 2011; Harrison *et al.*, 2014). Instead, we highlight key molecular sites involved in NADPH oxidase activation, particularly which are unique to specific NADPH oxidase isoforms, and may therefore be exploited by pharmacological means.

NOX2-containing NADPH oxidases

NOX2-containing NADPH oxidases were the first identified enzymatic sources of superoxide, and are expressed predominantly in phagocytic cells including neutrophils and macrophages. The NOX2 catalytic domain, like all NOX homologs, harbours all the necessary redox-sensitive components for electron transfer from NADPH to molecular oxygen. The three-dimensional structure of this membrane protein has not been resolved, although the cytoplasmic C-terminal reductase domain of NOX2 has been predicted based on its sequence homology to the ferredoxin-NADP reductase family of enzymes (Taylor *et al.*, 1993). *In silico* hydrophobicity plotting, random sequence peptide phage analysis and epitope mapping has provided some topographical information of NOX2 and its molecular interactions with regulatory subunits (Burritt *et al.*, 2001; Burritt *et al.*, 2003; DeLeo *et al.*, 1995c; Imajoh-Ohmi *et al.*, 1992; Paclet *et al.*, 2004; Rotrosen *et al.*, 1990). These studies predict NOX2 to be a 570 amino acid protein with six transmembrane spanning domains and glycosylated at three asparagine residues (Asn132, Asn149, Asn240; (Wallach *et al.*, 1997; Kleinberg *et al.*, 1989; Harper *et al.*, 1985; Taylor *et al.*, 2006; Parkos *et al.*, 1987). NOX2 consists of an N-terminal segment, six predictable transmembrane a helices and a cytoplasmic C-terminal tail containing NADPH and FAD recognition sites (Babior *et al.*, 1977; Taylor *et al.*, 1993; Cheng *et al.*, 2001) which carry electrons to one of two non-identical heme moieties within the transmembrane segments of the NOX protein (Doussiere *et al.*, 1996) (Isogai *et al.*, 1995;

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Diatchuk *et al.*, 1997), which are held in place by four histidine residues (Finegold *et al.*, 1996). The first heme group is positioned towards the cytoplasmic end of the molecule while the second heme is situated towards the outer face. This outer heme might be responsible for the ultimate facilitation of electron transfer to molecular oxygen to generate superoxide either extracellularly or within the phagosome. The precise role of the C-terminal heme remains to be fully determined, but it is likely that this moiety is involved in stabilising the interaction between NOX2 and p22phox - an interaction that is critical for stability and maturation of the NOX2 protein within the membrane (Huang *et al.*, 1995; DeLeo *et al.*, 2000; Dinauer *et al.*, 1991).

p22phox is the membrane-bound binding partner of NOX1, NOX2, NOX3 and NOX4 but not NOX5 (Cheng *et al.*, 2001; Banfi *et al.*, 2004). It possesses 195 amino acids, has a molecular weight of ~21 kDa and hydrophobicity analysis of its amino acid structure indicate that p22phox most likely possesses a cytoplasmic N-terminus (residues 1-90), two transmembrane a-helices (residues 91-106 and 112-127) connected by a short extracellular loop (residues 107 – 111) (Zhu *et al.*, 2006; Taylor *et al.*, 2004) and a C-terminus that extends back into the cytoplasm (residues 128–195). The N-terminus contains two regions (residues 6-11 and 65-90), which, are essential for maturation of NOX2 into a fully glycosylated protein and its expression at the membrane. The most noticeable feature of the C-terminal end is a proline-rich region (PRR) containing a consensus motif surrounding Pro156, which plays a critical role in facilitating the association of p47phox with the membrane-bound cytochrome (Leusen *et al.*, 1994; Leto *et al.*, 1994; Sumimoto *et al.*, 1994; Groemping *et al.*, 2003). The tandem SH3 domains of p47phox form a ‘sandwich’ around the PRR of p22phox (Nobuhisa *et al.*, 2006), but the interaction is further facilitated by a contact of one of the SH3 domains with an α-helix, immediately adjacent to the PRR region (Nobuhisa *et al.*, 2006). This additional contact contributes to full activation of the NOX2

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oxidase. This is facilitated by direct interactions of the p47phox SH3 domains with specific proline and arginine residues within the PRR (especially P152, P156 and R158), as well as with certain residues in the adjacent a-helix domain of p22phox (Nobuhisa *et al.*, 2006).

The p47phox subunit (~47 kd in size; 390 amino acids) contains two SH3 domains, which are conserved regions and well known for protein-protein interactions, an auto-inhibitory region (AIR) in the C-terminal end, a Phox homology (PX) domain (Ponting, 1996; Bao *et al.*, 2001) and a PRR similar to p22phox in the N-terminal end (Figure 2). Under resting conditions, p47phox is incapable of binding to p22phox as the AIR of p47phox prevents, via an intramolecular interaction, the SH3 domains from interacting with the PRR of p22phox (Ago *et al.*, 2003) as determined by X-ray crystallographic analysis. Furthermore, and perhaps less well defined, is another intramolecular inhibitory interaction between the carboxy terminal SH3 domain of p47phox and the PX domain (Ago *et al.*, 2001). This is thought to prevent the binding of the PX domain with membrane bound phosphoinositides which presumably acts as a further hindrance to its association with the membrane during resting conditions (Kanai *et al.*, 2001; Ago *et al.*, 2001). In the presence of an appropriate stimulus such as PMA, serine residues on p47phox become phosphorylated (El Benna J, 1996); (Fontayne A, 2002; Inanami *et al.*, 1998) unleashing the auto-inhibition (Ago *et al.*, 1999; Groemping *et al.*, 2003; Yuzawa *et al.*, 2004), and the SH3 and PX domains, which associate with targets on p22phox (Leto *et al.*, 1994; Finan *et al.*, 1994; McPhail, 1994; Sumimoto *et al.*, 1994) and the plasma membrane, respectively. P47phox also binds at multiple sites with NOX2 (DeLeo *et al.*, 1995c) and this interaction is also essential in the assembly of the oxidase. On this point, interference of the molecular interaction between NOX2 and p47phox with short peptide mimetics of this region inhibits NADPH oxidase (Rey *et al.*, 2001) (see below) and this approach has given rise to one of the most selective inhibitors of NOX2 oxidases i.e. gp91dstat. Gp91dstat is effective at suppressing NOX2

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oxidase activity as it prevents, p47phox and the ternary complex it forms with two additional subunits within the cytosol, p67phox and p40phox, to interact with NOX2 and form an entire active enzyme complex.

P67phox is a 59.8 kDa protein composed of 526 amino acids, which contains at its N-terminal end four tetratricopeptide repeat (TPR) motifs and two SH3 domains, one located centrally and the second at the C-terminal end. The second SH3 domain of p67phox binds to the PRR region of p47phox (de Mendez *et al.*, 1997; de Mendez *et al.*, 1996; Kami *et al.*, 2002; Groemping *et al.*, 2005). A specific activation domain in p67phox docks to a specific region on NOX2, leading to a conformational change in NOX2 and induction of electron flow through the complex (Han *et al.*, 1998; Nisimoto *et al.*, 1999). Thus, p67phox is commonly referred to as an *activator* subunit.

P40phox is a 339 amino acid protein with a molecular weight of 39 kDa and possesses an N-terminal PX domain, one SH3 domain and an acidic OPCA motif or PBI domain — a class of domains shared by p67phox. p40phox binds to p67phox via the PBI domains (Ago *et al.*, 2001; Nakamura *et al.*, 1998; Noda *et al.*, 2003) and with plasma membrane phosphoinositides via its PX domain (Ellson *et al.*, 2001; Bissonnette *et al.*, 2008; Honbou *et al.*, 2007) - analogous to p47phox. X-ray crystallography and NMR predicts that p67phox serves as a link between p47phox and p40phox by interacting with both of these regulatory subunits. Interestingly, the SH3 domain of p40phox may interact directly with the PRR regions of p22phox and as such may act as an alternative organizer to p47phox in NOX2 activation (Tamura *et al.*, 2007), although the relevance of this direct p40phox-p22phox interaction on oxidase activation is still unknown.

Rac1 or Rac2, belongs to the Rho family of small GTPases, whose activity is governed by the guanine nucleotide that they are associated with and are essential for oxidase function (Abo *et al.*, 1991; Knaus *et al.*, 1991; Dinauer, 2003; Werner, 2004; Bishop *et al.*,

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2000; Etienne-Manneville *et al.*, 2002). Rac is inactive when bound by GDP, and active when associated with GTP (Bourne *et al.*, 1990; Bourne *et al.*, 1991), the interconversion of which is regulated by guanine-nucleotide-exchange-factors (GEFs). Rac is recruited to the membrane independently of the cytosolic ternary complex (Dorseuil *et al.*, 1995; Heyworth *et al.*, 1994; Gorzalczany *et al.*, 2002) where GDP is replaced by GTP, allowing Rac to bind to the TPR motifs in the N-terminal region of p67phox (Diekmann *et al.*, 1994; Koga *et al.*, 1999; Nisimoto *et al.*, 1997; Lapouge *et al.*, 2000). The precise mechanism by which Rac influences NADPH oxidase activity is an ongoing matter for debate. One suggestion states that the binding of Rac with p67phox induces a conformational change in p67phox that allows it to associate with NOX2, leading to superoxide production (Sarfstein *et al.*, 2004). Rac also tethers covalently to plasma membrane lipids in a process that is dependent on a post-translational modification of the protein involving addition of an isoprenoid group (i.e. geranyl-geranylation) at the cysteine residue within the C-terminal end motif consisting of C-Ali-Ali-Xaa (Didsbury *et al.*, 1990; Kinsella *et al.*, 1991; Ando *et al.*, 1992; Kreck *et al.*, 1996). One of the most commonly prescribed classes of drugs used to alleviate symptoms of cardiovascular disease, the statins, suppress the synthesis of isoprenoid derivatives such as geranyl-geranyl-pyrophosphate, thus inhibiting Rac from binding to the plasma membrane. Statins are powerful inhibitors of NADPH oxidase-derived superoxide production and this action may partially explain their clinical efficacy (Liao, 2004).

NOX1-containing NADPH oxidases

The first homologue of the gp91phox subunit of the prototypical phagocytic oxidase was identified nearly 25 years after the discovery of NADPH oxidases in phagocytic cells and termed Mox1 (for mitogen oxidase 1) (Suh *et al.*, 1999). Later renamed NOX1, this protein consists of 564 amino acids, has a molecular mass of 65 kDa (Cheng *et al.*, 2001), sharing

56% homology with NOX2. It possesses some structural similarities to NOX2, but it possesses different tissue distribution (Suh *et al.*, 1999; Banfi *et al.*, 2000). NOX1 is expressed in colon and airway epithelium (Banfi *et al.*, 2003; Szanto *et al.*, 2005), stomach (Suh *et al.*, 1999), placenta (Cui *et al.*, 2006), uterus (Suh *et al.*, 1999), as well as vascular smooth muscle cells (Cheng *et al.*, 2001), and analogous to phagocytes, may play a role in cell defence at those sites (Kawahara *et al.*, 2004; Cheng *et al.*, 2004). The similarities between NOX1 and NOX2 include the C-terminal cytoplasmic tail containing the NADPH binding sites, FAD moieties and 2 heme regions as well as 6 putative transmembrane spanning regions. Like NOX2, NOX1 is complexed and stabilised with p22phox at the protein level (Ambasta *et al.*, 2004; Kawahara *et al.*, 2005)) and is tightly and efficiently regulated by several modulatory protein subunits. Indeed, NOX1-p22phox heterodimers are primarily inactive but produce superoxide in the presence of homologues of p47phox and p67phox called NOXO1 (Nox-organizer 1) and NOXA1 (Nox-activator 1), respectively (Ago *et al.*, 2003; Banfi *et al.*, 2003; Banfi *et al.*, 2003; Cheng *et al.*, 2004; Geiszt M, 2003).

Like p47phox, NOXO1 protein possesses an N-terminal PX domain, two tandem SH3 domains and a C-terminal PRR that serves as a binding site for SH3 domains (Ago *et al.*, 2001; Ago *et al.*, 2003), however they differ in that NOXO1 is only ~41 kd, and lacks an AIR (Ago *et al.*, 1999) - and this distinctive feature allows the SH3 domains of NOXO1 to interact with p22phox, even in the absence of stimulation (Ago *et al.*, 2003). Also like p47phox, the PX domain of NOXO1 associates with membrane phospholipids, and the identification of four alternative splice variants of NOXO1 (Cheng *et al.*, 2005; Ueyama *et al.*, 2007) , which vary in the nature of their PX domain, influences the subcellular localization of NOX1 and ROS production (Opitz *et al.*, 2007).

Similar to p67phox, NOXA1 (molecular size ~51 kd) contains four TPR that associate with Rac and an activation domain for NOX1 binding, which are required for full NOX1

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activity (Cheng *et al.*, 2006; Miyano *et al.*, 2006; Ueyama *et al.*, 2007). Also, like p67phox, NOXA1 contains a PBI domain, however it does not possess the conserved lysine residue and therefore it fails to interact with p40phox (Ago *et al.*, 2003). Unlike p67phox, NOXA1 contains only a single C-terminal SH3 domain that associates with NOXO1 (Ago *et al.*, 2003). These structural differences between NOXO1 and p47phox, and NOXA1 and p67phox, are likely to account for the major functional differences between NOX1- and NOX2-containing oxidases. NOX1 oxidase is likely to be constitutively active, whereas NOX2 requires activation by phosphorylation of p47phox.

NOX4-containing NADPH oxidases

NOX4 is a 578 amino acid protein that shares 39% homology with NOX2 (Geiszt *et al.*, 2000; Shiose *et al.*, 2001), but in addition to its expression in kidney cortex, NOX4 is expressed in endothelial cells (Hanna *et al.*, 2004), smooth muscle cells (Lassegue *et al.*, 2001; Wingler *et al.*, 2001; Bengtsson *et al.*, 2003; Paravicini *et al.*, 2002), heart (Byrne *et al.*, 2003; Cheng *et al.*, 2001), pancreas (Cheng *et al.*, 2001) and osteoclasts (Yang *et al.*, 2001) but in low amounts in phagocytic cells. As for NOX1 and NOX2, NOX4 possesses the same series of electron transporting moieties including the NADPH binding site, FAD and heme groups. Also NOX4 may undergo alternative splicing giving rise to at least 4 splice variants that appear not to possess some of these important moieties. Similar to NOX1 and NOX2, the full size NOX4 forms a heterodimer with p22phox, that promotes its activity and stability (Ambasta *et al.*, 2004; Martyn *et al.*, 2006) but, unlike NOX1 and NOX2, the PRR region of p22phox is not necessary for NOX4 oxidase activity. Additional molecular interactions that p22phox make with organizer proteins like p47phox and NoxO1 are not crucial for NOX4 oxidase-dependent ROS production (Kawahara *et al.*, 2005). Rac does not appear to regulate

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NOX4 activity as neither constitutively active Rac1 nor a dominant negative Rac1 influences NOX4 activity in epithelial cells (Martyn *et al.*, 2006), although in renal mesangial cells, the activation of NOX4 by angiotensin II (Ang II) has been suggested to be dependent on Rac (Gorin *et al.*, 2003). Given that activity of NOX4-containing oxidases is not dependent on at least the currently identified activator and organizer subunits which appear to be critical for activity of NOX1- and NOX2-containing oxidases, NOX4 may serve as an important constitutively active ROS generating system, whose overall ROS output is perhaps governed by its expression level (Wingler *et al.*, 2001) and post-translational modification.

NOX5

NOX5 is a 737 amino acid protein expressed in human lymphoid tissues, testis and spleen (Banfi *et al.*, 2001) and vascular endothelial cells (BelAiba *et al.*, 2007). It possesses the same redox active domains and NADPH binding sites as NOX1 to NOX4 but also a unique, cytosolic N-terminal Ca^{2+} binding domain with calmodulin-like EF-hand motifs that render this oxidase highly sensitive to Ca^{2+} (Banfi *et al.*, 2001) and to phosphorylation that increases Ca^{2+} sensitivity (Jagnandan *et al.*, 2006; Fulton, 2009) . NOX5 exhibits only ~22-27% homology to NOX1 through to NOX4 (Banfi *et al.*, 2001), but unlike those NOX proteins, NOX5 does not require p22phox for activation (Kawahara *et al.*, 2005) nor indeed any of the other known regulatory subunits. There are five splice variants identified to date, named NOX5α, NOX5β, NOX5γ, NOX5δ and NOX5s, that differ in the sequence of their Ca^{2+} binding regions as well as their tissue distribution (BelAiba *et al.*, 2007). NOX5s lacks the Ca^{2+} binding regions and is constitutively active (BelAiba *et al.*, 2007). Recent evidence shows that NOX5 is regulated by protein kinase C isoforms (Pandey *et al.*, 2011).

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DUOX enzymes

Like NOX5, DUOX1 and DUOX2 do not require p22phox for activity and they also possess EF-hands, which render them Ca^{2+}-dependent enzymes. Distinguishing characteristics in DUOX1 and 2 versus other members of the Nox family are that they contain seven membrane-spanning domains and a peroxidase-like domain in their extracellular N-terminal region.

Pharmacology of current NADPH oxidase inhibitors

The evidence presented above provides a strong rationale for the use of pharmacological inhibitors of NOX2-containing NADPH oxidase to alleviate oxidative stress and its associated pathologies in influenza infection. Several compounds are commonly purported as being inhibitors of NADPH oxidase and these have been the covered in recent comprehensive review articles (Brandes *et al.*, 2010; Drummond *et al.*, 2011; Selemidis *et al.*, 2008). We have previously proposed that perhaps the most effective and safest way of suppressing NOX2 oxidase activity would be to inhibit the association of p47phox with NOX2 (Vlahos *et al.*, 2012). As mentioned above NOX2 oxidase relies on the organiser subunit p47phox for full activity, although the enzyme can still be partially activated in its absence (Drummond *et al.*, 2011). Given the importance of preserving some NADPH oxidase activity in immune cells, p47phox represents a clinically safer target than other enzyme subunits (i.e. p22phox and p67phox) that are absolutely required for activity.

Of the currently available inhibitors, gp91dstat and apocynin are the most likely to be specific for the phagocytic NOX2 acting to suppress the actions via interactions with p47phox. The synthetic peptide NOX2ds-tat (also known as Gp91ds-tat) was designed to penetrate cells and prevent the assembly of the NOX2 oxidase complex (see below). This peptide inhibits NOX2 oxidase activity in vitro and reduces parameters of cardiovascular

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disease in a mouse model of hypertension (Rey *et al.*, 2001.) However, being a peptide it is unlikely to be orally active or display a suitable pharmacokinetic profile to have widespread utility as a clinical drug.

Apocynin inhibits the association of membrane bound NOX2 with p47phox in a mechanism that appears to be enhanced in the presence of the MPO enzyme and H_2O_2, which renders the compound a selective inhibitor of the phagocytic NOX2 oxidase (Heumuller *et al.*, 2008) (given the high amounts of H_2O_2 generation and expression of MPO by these cells), and suitable for suppressing the oxidative stress caused by inflammatory cells in response to influenza. Indeed, apocynin administration to mice in vivo resulted in a significant reduction in inflammatory cell superoxide production and airway inflammation and enhanced the clearance of virus from the lungs following low pathogenic (i.e. HKx31 virus) or moderate pathogenic (i.e PR8 virus) virus infection in mice (Vlahos *et al.*, 2012). This protective effect of apocynin was achieved with only a 50% suppression of superoxide production by inflammatory cells, therefore apocynin might be useful in suppressing oxidative stress to influenza whilst preserving some phagocytic function.

EXPLOITING NOVEL INTERFACES IN THE NOX2 OXIDASE ENZYME COMPLEX TO UNRAVEL NOX2 SELECTIVE INHIBITORS

We suggest that selective targeting of the p47phox organizer protein of NOX2 oxidase which contributes to the pathophysiology of influenza disease could be achieved via a drug discovery approach, that begins by unraveling key p47phox interactions with NOX2 and other protein subunits, that are exclusive to NOX2 oxidase complexes (Figure 2). The best characterised protein-protein and protein-lipid interactions involving p47phox include its interactions with the p67phox or NOXA1 subunits, p22phox, and the NOX2 catalytic subunit

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as well as with phospholipids in the biological membrane to which the enzyme complex is fastened.

The p47phox–p67phox interface

As mentioned in the previous section p47phox interacts directly with an SH3 domain on p67phox via a PxxP binding motif contained within a proline-rich region (PRR) near its C terminus. Moreover, further contacts with the SH3 domain of p67phox are made by a domain on p47phox that lies distal to the PRR on its C terminus (amino acids 368–390), and these contacts are important for increasing the affinity of the protein–protein interaction (KA~20 nM) and for conferring binding partner specificity (Kami *et al.*, 2002). Therefore disruption of the interaction of the PRR of p47phox and the SH3 domain of p67phox represents a possible tactic for preventing the constitutive association of the two subunits in resting cells and for the translocation of the ternary complex to the membrane complex, thereby inhibiting NADPH oxidase activation. A succession of synthetic peptides based on the PRR of p47phox were shown to prevent the binding of p47phox to p67phox, thus providing proof of principle that this interaction may represent a future target for selective NOX2 oxidase inhibitors (Finan *et al.*, 1994).

Intramolecular interactions of the p47phox autoinhibitory region with its SH3 and Phox homology domains

In resting cells, p47phox is folded in on itself such that the bis-SH3 and Phox homology (PX) domains of the protein are concealed owing to the intramolecular interactions between its AIR and bis-SH3 domain (Groemping *et al.*, 2003; Yuzawa *et al.*, 2004) and also between its bis-SH3 domain and PX domains (Hiroaki *et al.*, 2001; Marcoux *et al.*, 2010). Serine residues within the AIR are phosphorylated by protein kinase C (PKC) or phosphoinositide 3-kinase

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(PI3K) (Ago *et al.*, 2003; Ago *et al.*, 1999; Hoyal *et al.*, 2003; Huang *et al.*, 1999) which weakens the interaction of the AIR with the bis-SH3 domain, allowing p47phox to assume an open conformation whereby its bis-SH3 and PX domains are free to interact with the p22phox PRR and membrane phospholipids, respectively (El-Benna *et al.*, 2009). Therefore prevention of phosphorylation of the AIR could lock p47phox in its closed state and thereby prevent its interaction with the NOX2-p22phox components of the NADPH oxidase enzyme. Indeed inhibitors of PKC might reduce phosphorylation of the AIR of p47phox, although inhibition of PKC is likely to have additional consequences. Perhaps a more selective approach involves modification of the sulfhydryl side-groups of the regulatory serine residues present within the AIR. This may be the mechanism by which apocynin achieves its widely reported inhibitory effects on NADPH oxidase.

The p47phox–NOX2 subunit intermolecular interface

P47phox makes multiple contacts with the NOX2 subunit including contact on the first predicted intracellular loop (amino acids 86–93) of NOX2, and on two further domains on the cytosolic C-terminal tail (amino acids 450–457 and 554–564) (DeLeo *et al.*, 1995b). Elegant studies looking for regions on p47phox that interact with the NOX2 subunit identified only one site situated within the AIR (amino acids 323–342) (De Leo *et al.*, 1996; DeLeo *et al.*, 1995a), which may indicate a single point of contact for the NOX2 subunit on p47phox. Short peptides that correspond to any one of the three p47phox-interacting domains on the NOX2 subunit acted as competitive inhibitors of NADPH oxidase activation in both cell-free systems and in electropermeabilized neutrophils (DeLeo *et al.*, 1995b; Rotrosen *et al.*, 1990). Follow-up to this work showed that the addition of a human immunodeficiency virus trans-activator of transcription (tat) peptide sequence to the NOX2 subunit-docking sequence

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between amino acids 86-93 (i.e. NOX2ds) resulted in a compound that was effective at inhibiting NOX2 oxidase activity.

The p47phox PX–membrane phospholipid interaction

The PX domain of p47phox is a conserved sequence of approximately 120 amino acids that is present in at least 100 other proteins, including p40phox and NOXO1 subunits (Cheng *et al.*, 2004; Cheng et al., 2005; Ponting, 1996). As previously mentioned, the p47phox PX domain contains a PRR that maintains p47phox in its inactive state in resting cells. The p47phox PX domain contains two highly basic pockets that bind to membrane phosphoinositides following unfolding of the protein (Kanai *et al.*, 2001; Karathanassis *et al.*, 2002), which are structurally distinct from the one that is present on the p40phox PX domain. The first pocket is comparatively large and unlikely to represent an appropriate target for small-molecule drugs and selectively binds to phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P2). The second pocket is much smaller and has strong affinities towards anionic phospholipids such as phosphatidic acid or phosphatidylserine (Karathanassis *et al.*, 2002). Future studies using virtual ligand screening of this pocket may yield novel selective NOX2 oxidase inhibitors.

Hybrid NOX1-p47phox-containing NADPH oxidase

The above discussion that highlights important protein and lipid domain interactions for p47phox that regulate NOX2 oxidase activity in some respects must be taken with some degree of caution. It must be noted that p47phox also serves a similar organizer functional role for the NOX1 oxidase complex that is expressed in vascular smooth muscle cells (VSMCs), by facilitating the interaction of the cytosolic NOXA1 subunit with the catalytic domain of NOX1 oxidase (Ambasta *et al.*, 2004). These mechanisms could be similar to those involved in the association of p47phox with p67phox. Upon close investigation of the C

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terminus of the NOXA1 subunit it appears to be highly homologous to that of the PxxP binding motif-containing SH3-binding domain on p67phox. Moreover there is striking homology in the domain in the C-terminal end, which is responsible for increasing the binding affinity and specificity of p67phox for p47phox (Banfi, 2003) (Takeya *et al.*, 2003). Therefore, pharmacological strategies that inhibit the interaction of the p47phox PRR and the p67phox SH3 domain are expected to block the interaction between the p47phox and NOXA1 subunits, and would thereby inhibit the production of ROS by NOX1 oxidase in VSMCs.

As for the actions of NOX2dstat, a recent study shows it to be highly specific for the NOX2 oxidase and importantly it does not interfere with the ROS producing capacity of the hybrid NOX1 oxidase in VSMCs that relies on p47phox (Csanyi *et al.*, 2011).

ALTERNATIVE APPROACHES TO SUPPRESSING NOX2 OXIDASE ACTIVITY

Splice variant NOX2β highly expressed in alveolar macrophages

Thus far our discussion has been focussed on suppressing the actions of p47phox-containing NADPH oxidase to alleviate ROS generation predominantly by inflammatory cells such as neutrophils and macrophages and thereby the burden of influenza. However, it must be noted that NOX2-containing NADPH oxidase is expressed ubiquitously and not exclusively in lung tissue. Certainly from a treatment perspective, inhibitor drugs may be delivered by inhalation minimising systemic effects, however, manipulation of NOX2 oxidase solely in the lung is the ideal scenario. We have recently identified a novel splice variant of NOX2 (the second to have been identified to date and thus named NOX2β) that is expressed highly in mouse and human alveolar macrophages (Harrison *et al.*, 2012). Using immuno-screening this splice variant of NOX2 was undetectable in other organs and tissues including heart, brain, kidney and aorta but was highly expressed in lung (Harrison *et al.*, 2012). Importantly siRNA

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transfection to downregulate its expression resulted in a significant decrease in the NOX2-containing NADPH oxidase activity of macrophages, even though the expression of the full length NOX2 was unaffected (Harrison *et al.*, 2012). These results suggest that NOX2β may represent a novel target for drugs to suppress macrophage-dependent NOX2 activity, which has been implicated in the pathobiology of influenza virus infections (Imai *et al.*, 2008).

Inhibition of NOX2 oxidase priming in neutrophils and macrophages

Work from our laboratory has recently shown that influenza A virus infection of macrophages or activation of Toll like receptor 7 (i.e. TLR7) with imiquimod, resulted in a significant elevation in the oxidative burst in macrophages (To *et al.*, 2014). The virus *per se* had very little to no effect on superoxide production; however, it substantially elevated the ability of phorbol dibutyrate (PDB) to activate NOX2 activity and the oxidative burst. This observation is analogous to the "priming effect" characterised in human neutrophils whereby an initial exposure to TNF-α results in a greater degree of superoxide production to a second stimulus such as N-formyl-methionyl-leucyl-phenylalanine (fMLF)) (El-Benna *et al.*, 2008). This primed state for NOX2 oxidase has been described as a "ready to go" state, as it results in a higher and faster response to a second stimulus (Clark *et al.*, 1990). How priming of NOX2 oxidase occurs has been elucidated and it is believed to involve p47phox. As already mentioned, the multiple serine residues on p47phox become phosphorylated to induce conformational changes in the protein that allow it and its associated subunits i.e. p67phox and p40phox, to assemble with NOX2 to form a fully functional oxidase unit capable of ROS generation. However for priming to occur, generally the priming agents cause partial phosphorylation of p47phox, in particular its serine 345 on the peptide sequence (El-Benna *et al.*, 2008; Sheppard *et al.*, 2005). This partial phosphorylation status of p47phox then results in a greater increase in phosphorylation of the remaining serine residues on p47phox upon

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subsequent stimulation with the second stimulus and a greater superoxide response. We hypothesized that the priming effect of the virus demonstrated in macrophage may involve phosphorylation of serine346 (mouse sequence differs from human which is serine 345) on the p47phox subunit, analogous to what occurs in human neutrophils (Dang *et al.*, 2006; El-Benna *et al.*, 2008; Sheppard *et al.*, 2005). To this end we custom designed in the same manner as Dang *et al*., 2006, a peptide inhibitor corresponding to amino acids 337-348 of p47phox tagged at the N-terminus to a HIV-tat based peptide (YGRKKRRQRRR) to permit membrane translocation, which we anticipate will compete against serine 346 of p47phox for the substrate that causes phosphorylation in response to influenza A virus. Our study showed that this Ser346 peptide inhibitor significantly suppressed the influenza A virus-dependent enhancement in the oxidative burst in macrophages (To *et al.*, 2014). It remains to be determined whether this phenomenon of NOX2 priming occurs *in vivo* following influenza A virus infection. Certainly, as a starting point, peptide inhibitors that span over serine 345 (Ser346 in mouse) should be tested *in vivo* against influenza A infections.

CONCLUSION

The primary focus of our mainstay therapies for treatment of disease caused by influenza A virus infections is to minimise virus infection and replication. However, with influenza viruses becoming resistant to antivirals and imminent threats of a new pandemic strain, there is a serious need to develop novel pharmacological approaches that ideally target lung injury irrespective of strain. There has been a strong movement towards understanding key features of the host immune response triggered by virus such as the lung oxidative stress induced by NOX2-containing NADPH oxidase enzymes. Thus, inhibitors of NOX2 oxidase are likely to provide some protection against these types of infection and coupled with antivirals, this type of polypharmacy could alleviate both the host immunopathology and viral burden. However,

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this field *is* still in its infancy stages in particular there is a lack of knowledge as to which cells of the immune system are responsible for this ROS generation, although macrophages have been implicated. In addition, the potential roles of other ROS generating enzymes (including NOX4, DUOX1 and DUOX2, etc.) need to be elucidated and in particular their spatial-temporal aspects are unknown. Finally, there is a dire need to establish the X-ray crystal structures of catalytic (i.e. NOX and DUOX) subunits, their splice variants, regulatory (i.e. p47phox and p67phox) subunits and their key binding sites to facilitate the development of more specific inhibitors of these enzyme complexes.

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FOOTNOTES

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Figure legends

Figure 1. Schematic diagram depicting major cell types in the lungs, sites of influenza A virus infection and expression profile of the various Nox isoforms. Influenza A virus infects airway epithelial cells, which are major sites for virus replication and cytokine release. In addition, virus infects and activates sentinel alveolar macrophages resulting in cytokine and chemokine release. Replication in alveolar macrophages is abortive for most influenza virus strains (with the exception of some H5N1 strains). The cytokine and chemokine burst is responsible for exacerbating the lung inflammation by stimulating the recruitment of additional inflammatory cells such as neutrophils (Neut) and monocytes (Mono) from the bloodstream. Alveolar macrophages, and blood-derived neutrophils and monocytes that differentiate into macrophages within the lung are major sources of NOX2 oxidase-dependent reactive oxygen species. Airway epithelial cells express predominantly NOX1 and DUOX, with lower levels of NOX2 and NOX4. Lung endothelial cells express NOX1, 2, 4 and 5. Note, this schematic focuses on innate immune cells and does not deliberately ignore the importance of plasmacytoid dendritic cells as well as cells of the adaptive immune system including T lymphocytes and B-lymphocytes. These have been omitted for clarity only. Abbreviations: ssRNA (single stranded RNA).

Figure 2. Schematic diagram depicting the Nox2-containing NADPH oxidase. Highlighted are the key regulatory subunits and protein-protein, and protein-lipid interfaces that could be exploited by drug discovery. AIR= autoinhibitory region, PRR= polyproline rich region, PtdIns3p= phosphatidylinositol 3-phosphate

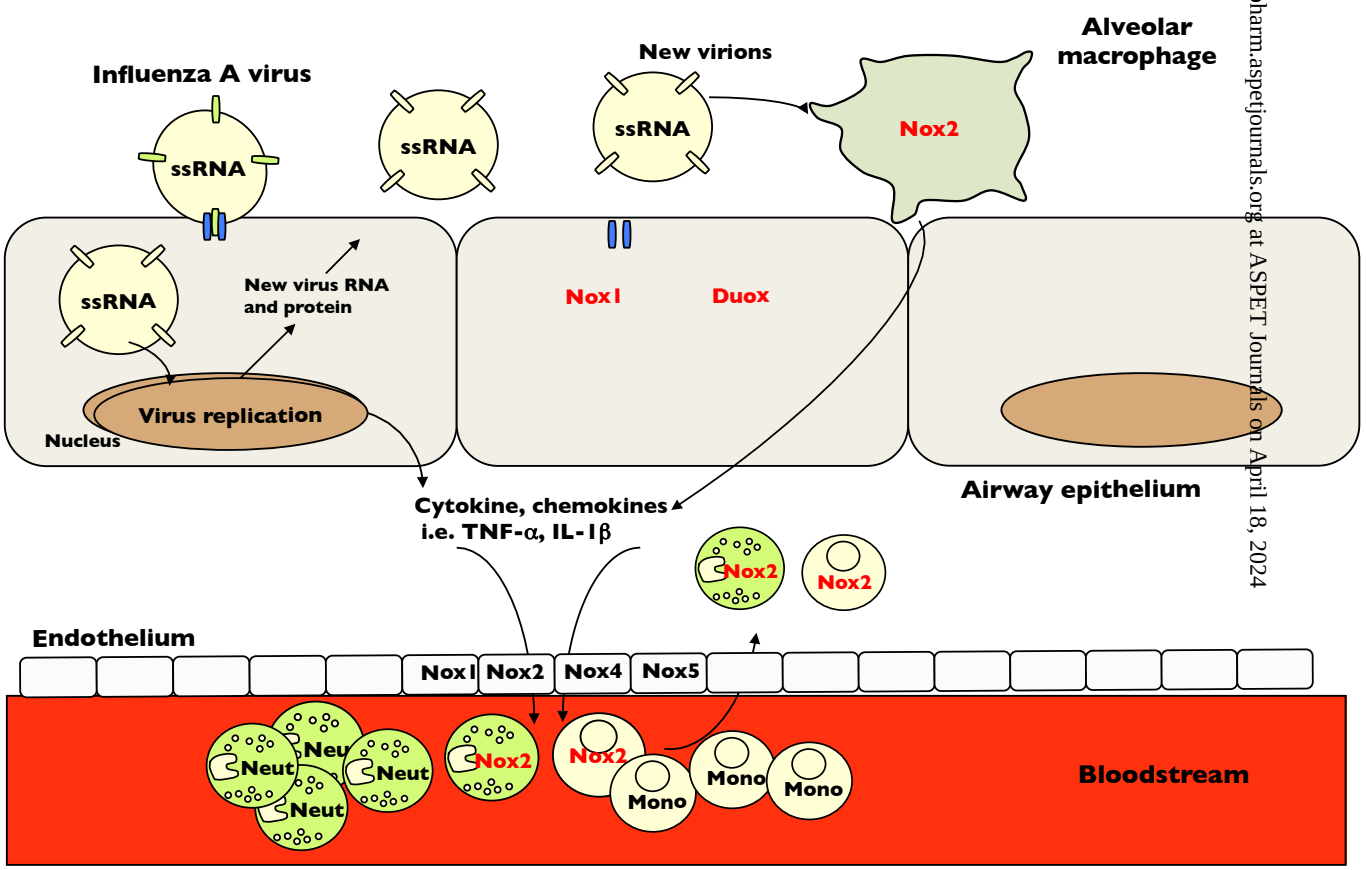
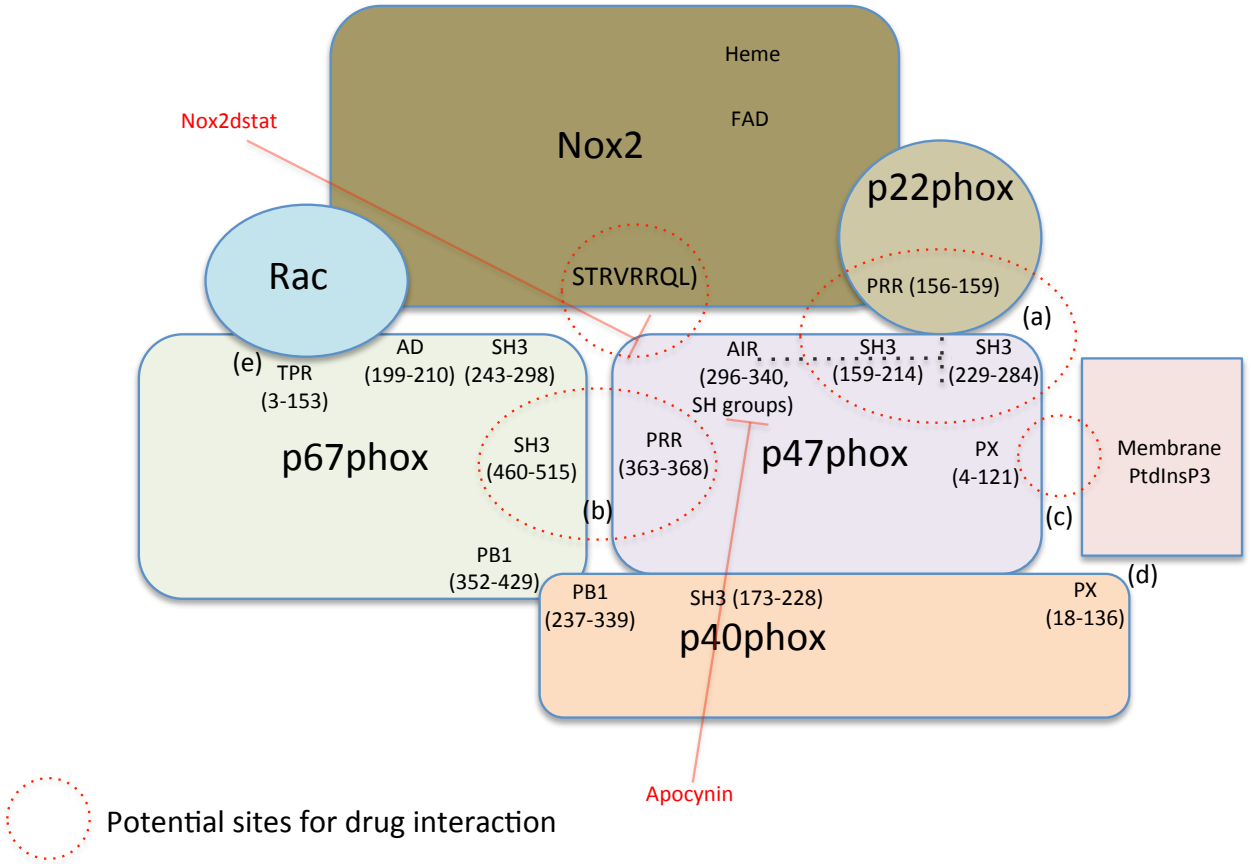


Figure 1



a) to e)- NMR and X-ray crystal structures of some key molecular interactions have been established

Figure 2