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REVIEW ARTICLE

Recent Advances in Inhaled Nanoformulations of Vaccines and Therapeutics Targeting Respiratory Viral Infections

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Abstract

With the rapid outbreak of respiratory viral infections, various biological (e.g. vaccines, peptides, recombinant proteins, antibodies and genes) and antiviral agents (e.g. ribavirin, palivizumab and valaciclovir) have been successfully developed for the treatment of respiratory virus infections such as influenza, respiratory syncytial virus and SARS-CoV-2 infections. These therapeutics are conventionally delivered via oral, intramuscular or injection route and are associated with several adverse events due to systemic toxicity. The inherent *in vivo* instability of biological therapeutics may hinder them from being administered without proper formulations. Therefore, we have witnessed a boom in nanotechnology coupled with a needle-free administration approach such as the inhalation route for the delivery of complex therapeutics to treat respiratory infections. This review discussed the recent advances in the inhalation strategies of nanoformulations that target virus respiratory infections.

Key Words inhaled vaccines and therapies · nanoformulation · respiratory viral infections

Abbreviations

ACE2	Angiotensin-converting enzyme 2
BOS	Bronchiolitis obliterans syndrome
BAL	Bronchoalveolar lavage
CD	Cluster of differentiation
CpG	Cytosine-guanine rich oligonucleotide motifs
DPI	Dry powder inhalation
FPD	Fine particle dose
FPF	Fine particle fraction
FliC	Flagellin
AuNPs	Gold nanoparticles
HA	Haemagglutinin
Th2	Helper type 2
HSCT	Hematopoietic stem cell transplant
HCNP	Hesperidin/chitosan nanoparticles
HCoV	Human coronaviruses
HSA	Human serum albumin
HCQ	Hydroxychloroquine
HPMC	Hydroxy Propyl Methyl Cellulose
IgG1	Immunoglobulin subclass 1
IgG2a	Immunoglobulin subclass 2a
M2e	Influenza matrix protein 2 ectodomain
IFN-γ	Interferon gamma
IL	Interleukin
IM	Intramuscular
IV	Intravenous
kg	Kilogram
LNP	Lipid nanoparticles
LRTI	Lower respiratory tract infection
ML	Maleic anhydride
MMAD	Mass median aerodynamic diameter
mRNA	Messenger RNA
hMPV	Metapneumovirus
µL	Microlitre
mg	Miligram
nm	Nanometer
NA	Neuraminidase
PIV	Parainfluenza virus
PIV2	Parainfluenza virus type 2
PIV3	Parainfluenza virus type 3

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PFU	Plaque-forming unit
pDNA	Plasmids DNA
PACE	Poly(amine-co-ester)
PEI	Polyethyleneimine
PLGA	Poly(lactic-co-glycolic acid)
Poly(I:C)	Polyinosinic: polycytidylic acid
RBD	Receptor-binding domain
RSV	Respiratory syncytial virus
HRV	Rhinovirus
SeV	Sendai virus
SARS-CoV	Severe Acute Respiratory Syndrome CoV
siRNA	Small interfering RNA
Th	T helper
TNF-α	Tumour necrosis factor alpha
TLR	Toll-like receptor
VIPER	Virus inspired for endosomal release
VLP	Virus-like particle

Introduction

According to the World Health Organization (WHO), respiratory infections caused by bacteria, virus, fungi and parasites constitute approximately 6% of all global disease and account for 17 million deaths globally [1]. Among 6.6 million mortality cases annually in children below five years old, respiratory viral infections such as influenza virus, respiratory syncytial virus (RSV), human coronaviruses (HCoV), rhinovirus (HRV) and metapneumovirus (hMPV) are common legal pathogens [2]. Since the pandemic outbreak of Spanish flu (Influenza virus A/H1N1) in 1918, the world has witnessed several pandemics caused by influenza virus: Asian flu (A/H2N2 in 1957), Hong Kong flu (A/H3N2 in 1968), Russian flu (A/H1N1 in 1977) and swine flu pandemics (A/H1N1 in 2009) [3]. In addition, two pandemics caused by coronavirus have been detected: the Severe Acute Respiratory Syndrome CoV (SARS-CoV) and SARS-CoV-2 (more popularly known as Covid-19) [3]. These viruses share a few common characteristics. They are RNA viruses, which are capable of gene reassortment or recombination, resulting in newer respiratory virus species that are resistant to existing therapies and have improved diverse strategies to survive in host cells. Respiratory tract infections have been classified as critical public health issues due to the implications on health and economic burden to both patient and countries. This calls for the development of therapeutics to tackle respiratory virus infections. Various antiviral drugs and biopharmaceutics (recombinant proteins, plasmids DNA (pDNA), messenger RNA (mRNA) and small interfering RNA (siRNA), antibodies) have been used to treat respiratory viral infections [4–9]. However, most of these antiviral drugs exhibit low solubility, low stability, short half-life, high occurrence of adverse event and toxicity (Table 1 and Table 2). The biopharmaceutics, on the other hand, require sophisticated manufacturing and handling methods, as they are susceptible to inactivation as a response towards small environmental changes. The use of nanotechnology to deliver antiviral drugs and vaccines are breakthrough in targeting viral respiratory infections. The different types of nanoparticles such as lipid-based (liposome, solid lipid nanoparticle and exosomes nanoparticle), polymeric-based [poly(lactic-*co*-glycolic acid) PLGA, chitosan, hydrogel], micelles, inorganic and metal-based nanoparticles provide solutions to the instability, low solubility and toxicities challenges. For instance, lipid nanoparticles have been employed as a delivery system for RNA-based vaccines. These nanoparticles serve to stabilize the RNA vaccines and ensure that they are delivered within the body. Nanoparticles could be used to target the surface proteins to prevent the viral attachment and entry to host cells. Nanoparticles as drug delivery carriers are also associated with lower systemic toxicity, presumably due to reduced dosing frequency and smaller

Table 1 Limitations of Selected Commercially Available Drugs used for Infections

Drug name	Route of administration	Human health related issue	Physiochemical related limitations	References
Ivermectin	Oral	Disturbed breathing, low blood pressure and fast heart rate	Solubility less than 5 mg/L	[13]
Niclosamide	Oral	Gastrointestinal discomfort, headache and appetite loss	Solubility less than 8 mg/L	[14]
Hydroxychloroquine	Oral	Increased cardiac toxicity including arrhythmias, kidney injury, liver failure, diarrhea, nausea, and vomiting	Very soluble in water	[15]
Favipiravir	Oral	Reduced neutrophils, diarrhea and increased blood uric acid		[16]
Remdesivir	Intravenous	Promote liver injury, induced lower oxygen level and disturbed breathing and heart rate	Solubility less than 0.03 mg/mL	[17]
Lopinavir	Oral	Induced nausea, vomiting, fatigue	Solubility less than 0.002 mg/mL	[18]
Ribavirin	Oral	Anemia	Very soluble in water	[19]

Table 2 Examples of Commercially Available Treatment for Respiratory Viral Infections

Type of respiratory viral infection	Marketed drugs (trademark)	Mode of administration	Status
SARS-CoV-2 (Covid-19)	Combination of nirmatrelvir and ritonavir (Paxlovid)	Oral	Not yet approved by FDA
	Molnupiravir (LAGEVRIO®)	Oral	Obtained FDA approval
	Remdesivir (Veklury)	Intravenous	Obtained FDA approval
	Bebtelovimab	Intravenous	Authorised for emergency use by FDA
	Tocilizumab (Actemra)	Intravenous	Obtained FDA approval
	Baricitinib (Olumiant)	Oral	Obtained FDA approval
	Combination of casirivimab and imdevimab (REGEN-COV)	Intravenous	Not yet approved by FDA
	Pfizer–BioNTech Covid-19 vaccine (Comirnaty)	Intramuscular	Obtained FDA approval
	Oxford/AstraZeneca Covid-19 vaccine (Covishield and Vaxzevria)	Intramuscular	
Influenza (A and B)	Quadrivalent Influenza Vaccine (AFLURIA, Fluarix, FluLaval, Flucelvax and Fluzone)	Intramuscular, intranasal and intradermal	Obtained FDA approval
	Amantadine (Gocovri)	Oral	Obtained FDA approval
	Oseltamivir (Tamiflu®)	Oral	Obtained FDA approval
	Zanamivir (RELENZA)	Inhalation	Obtained FDA approval
	Peramivir (RAPIVAB™)	Intravenous	Obtained FDA approval
	Laninamivir	Inhalation	Not yet approved by FDA
	Favipiravir	Oral	Approved in Japan
	Baloxavir (Xofluza®)	Oral	Obtained FDA approval
Respiratory syncytial virus (RSV)	Palivizumab (Synagis®)	Intramuscular	Obtained FDA approval
	Ribavirin (Virazole)	Inhalation	Obtained FDA approval
Rhinovirus	Pleconaril (Picovir)	Oral	Obtained FDA approval

dosage than the equivalent amount of free drugs needed to achieve a similar therapeutic effect.

The main objective of this review is to highlight the recent advancements in the development of inhalable formulations using nanotechnologies to treat respiratory viral infections. The rationale and considerations of pulmonary delivery are addressed in the review. Finally, the review will outline recent nanotechnological advancements in the delivery of therapeutics (vaccine, antiviral drug, mRNA and siRNA) in the form of either oral inhalation or intranasal delivery to treat influenza, SARS-CoV-2 and RSV.

Rationale and Considerations of Pulmonary Delivery

To date, the majority of nanoparticle-based vaccine or nanoparticle-based antiviral formulations are developed for administration via oral, intramuscular or intravenous routes. Considering the location of where the virus resides and propagates, it is more appropriate and effective to construct formulations which can be delivered directly to the lung. In fact, the inhalation route is highly recommended for patients suffering from lung diseases such as asthma, chronic pulmonary obstructive disease (COPD) and cystic fibrosis in order to achieve rapid alleviation of the symptoms. Both intranasal and orally inhaled formulations could deliver the therapeutics directly to the lung and ensure high local concentration and absorption of drugs/vaccine. From a lung physiology perspective, more than 300 million alveoli are present in the lung that provide huge surface area (70–140 m^2) for drug absorption [10]. Owing to the continuous exposure of lung to foreign particles, the airways house various resident macrophages specialised in overcoming the invasion of a potential antigen by activating local immune response [10]. Therefore, in the case of a vaccine, the inhalation method can deliver therapeutics directly to the lung to induce local immune response. Furthermore, the inhalation route is ideal for therapeutics that are sensitive to first-pass metabolism and enzymatic attack as opposed to the systemic delivery routes [11]. Pulmonary administration is suitable for peptides, proteins, RNA, DNA, and large molecular complex compounds. This administration route ensures high deposition of drugs locally while minimising systemic side effects. To achieve this, the antiviral agents or vaccines could be engineered as dry powder inhalation (DPI), metered dose, and nebulised formulations in accordance to the patients' needs and the physicochemical properties of drugs.

Aerosol therapy is convenient for self-administration of therapeutics for pulmonary diseases such as seasonal flu. However, the risks associated with fugitive emissions or

exhaled droplets should be taken into consideration especially for those with highly contagious respiratory droplets. Several studies have demonstrated that at least 50% of the exhaled aerosols remain airborne in the environment for hours [12]. Therefore, it increases the risk of disease transmission to caregivers and healthcare professionals due to unintended inhalation of the exhaled aerosol during the inhalation therapy. It is advisable that formulation and device suitability should be patient-specific. For instance, patients with mild Covid-19 with inhalation capability can use pressurised metered dose inhaler and DPI [12]. For critically-ill patients receiving ventilator supports, mesh nebulisers should be considered [12]. Again, the use of nebulisers should proceed with extra precaution and preferably in closed circuit, as the exhaled aerosols could transmit into the hospital environment and put other patients and healthcare professionals at risk. It is advisable to use HEPA filters for patients on ventilators to prevent the transmission of infectious droplets. With this in mind, jet nebulisers have been fitted with filters that are capable of capturing 93% of exhaled aerosol droplets, specifically for delivering medication to patients with highly contagious pathogen (e.g. Covid-19) [12].

Inhalation Nanotherapies for Viral Respiratory Infections

SARS-CoV-2

SARS-CoV-2 virus enters and deposits on the airway epithelia through the throat or mouth. The spike (S) glycoprotein expressed on the surface of coronavirus is the major immunodominant antigen which acts to bind to the angiotensin-converting enzyme 2 (ACE2) receptor on host cells to facilitate its entry [20, 21]. Several instances of drug resistance in coronavirus have been reported due to drug selection pressure. Despite this, the mutants retain the S protein binding to ACE2 receptors in human. The first clinical trial for inhaled Covid-19 vaccine reported the safety and immunogenicity of adenovirus type-5 vector-based Covid-19 (Ad5-nCoV) in healthy subjects [22]. In a two-dose aerosol inhalation with a 28-day interval phase 1 trial, adults were randomised into five groups to receive either nebulised Ad5-nCoV vaccine only, intramuscular injection of Ad5-nCoV vaccine, or both. Both the nebulised and intramuscular delivery of Ad5-nCoV were well-tolerated and showed similar immune reactions by inducing Th1 dominant response as well as IgG and antibody levels [22]. Recently, Li *et al*. reported the safety and immunogenicity of aerosolised Ad5-nCoV vaccine as a heterologous boost to immunisation after two-dose priming with inactivated SARS-CoV-2 vaccine (Sinovac CoronaVac) [23]. Eligible adults were randomly assigned to either receive a low (0.1 mL) or high dose (0.2 mL) of aerosolised Ad5-nCoV vaccine. Homologous booster vaccination with intramuscular injection of inactivated SARS-CoV-2 vaccine (CoronaVac) was also performed for comparison [23]. Similar to previous findings, aerosolised Ad5-nCoV vaccine was safe and highly immunogenic. Meanwhile, the inhalation of aerosolised Ad5-nCoV vaccine heterologous vaccination induced substantially higher levels of serum neutralising antibody than those required for 50% efficacy against SARS-CoV-2 infections [23].

Heavy-chain-only antibodies or nanobodies are a new class of recombinant antibody that have been explored as a potential Covid-19 treatment. Nanobodies consist of two heavy chains of immunoglobulin without the presence of the effector domain (Fig. 1). It is believed that nanobodies bind to ACE2 receptor-associated epitopes on the receptor-binding domain (RBD) to inhibit the viral entry. Therefore, the viral S protein is the main target in the development of new nanobody formulations against SARS-CoV-2. In comparison to monoclonal antibodies (mAb), nanobodies are relatively smaller and more stable, allowing them to be formulated as inhalable aerosols for direct deposition to the infected lung. Ultrapotent Pittsburgh inhalable nanobodies (PiN-21 nanobodies) bind tightly to the spike protein and effectively neutralise SARS-CoV-2 in cells at concentrations as low as 0.1 ng/mL [24]. In the *in vivo* model of infected Syrian hamsters, PiN-21 nanobodies administered intranasally at 0.6 mg/kg substantially decreased virus gRNA in lung and viral titers in nasal washes and throat swabs [25]. Meanwhile, the inhalation dose of PiN-21 when delivered using nebuliser was 0.2 mg/kg after 8 h with predominant neutralising activities found in bronchoalveolar lavage (BAL) fluid, tracheal aspirate, larynx and nasal wash [25]. Similarly, in another study, NIH-CoVnb-112 nanobodies were shown to retain strong antiviral replication inhibition efficacy towards SARS-CoV-2 in infected Syrian hamsters after nebulisation [26]. Humanised Nb11-59 nanobodies derived from camels immunised with recombinant SARS-CoV-2 spike RBD demonstrated strong neutralising activity at $IC_{50} < 1$ µg/mL [27]. Huo *et al*. have reported four isolated nanobodies (C5, H3, C1, F2) that displayed differential epitope binding affinity at pM potency [28]. The recognition sites for both C5 and H3 overlapped at the ACE2 binding site. The C5 nanobody showed complete inhibition of infectivity in Vero cells at < 100 pM. A single dose of C5 (4 mg/kg) administered intranasally or intraperitoneally to infected Syrian hamsters was associated with minimal weight loss and limited pulmonary infections [28]. However, faster recovery was observed in the treatment group that received intranasal C5 nanobody, which could be due to direct deposition and thus higher C5 concentrations in the infected site in the lung [28]. Recently, Li *et al*. developed two humanised nanobodies (n3113v and n3130v) that bind to the highly conserved regions on

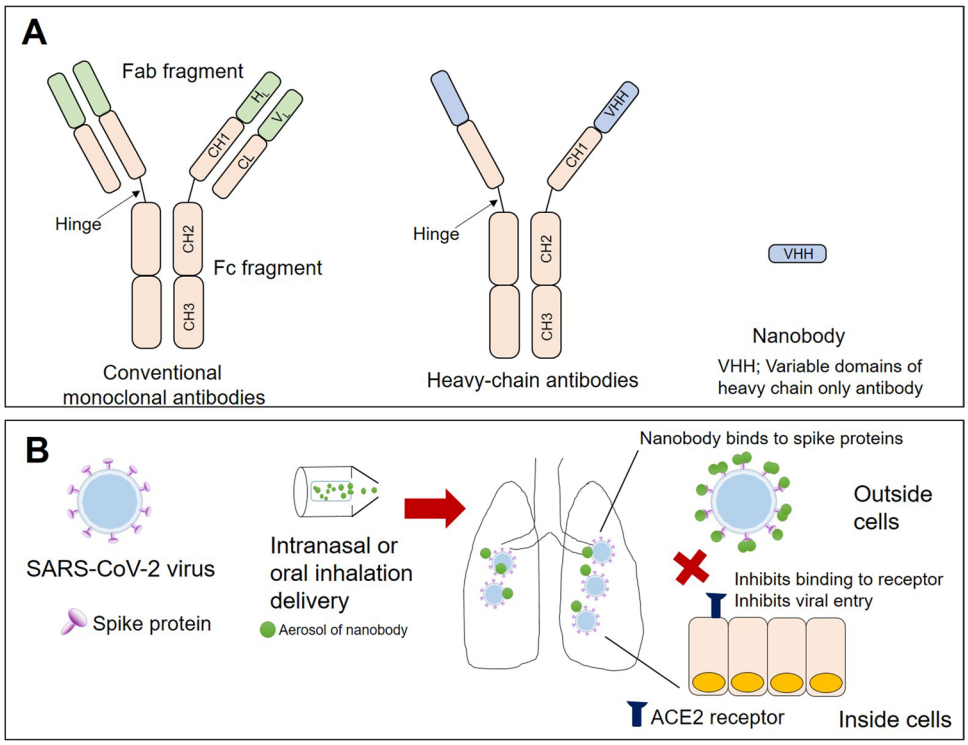

Fig. 1 (**A**) Schematic diagram of conventional immunoglobulin (IgG) antibody, camelid heavy-chain only antibody and isolated nanobody. (**B**) Nanobody acts to inhibit viral entry into the host cell through interaction with viral spike proteins. This prevents the binding of viral particles to ACE2 receptor-associated epitopes on the receptor-binding domain (RBD) and finally inhibits viral infection inside the cells

the Omicron variant receptor-binding domain [29]. Both n3113v and n3130v nanobodies were conjugated to form a small bispecific humanised nanobody (bn03) conjugate. The bn03 conjugate was fourfold more potent in neutralising SARS-Co2-V compared to the n3113v and n3130v. Bn03 retained its RBD binding efficacy and was stable after aerosolisation without aggregation or degradation. A single *in vivo* inhalation dose (5 or 25 mg/kg) also led to higher nanobody retention and concentration compared to the IV route. The inhaled route was also accompanied with significantly stronger neutralizing action against SARS-CoV-2 *in vivo* [29].

The first few mRNA vaccines encapsulated in lipid nanoparticles (LNPs) for Covid-19 infections were developed by BioNTech/Pfizer and Moderna for intramuscular administration [6, 7]. However, the translational delivery of LNP-based mRNA vaccines using inhalation is not easy. One of the challenges is the induction of inflammation in the respiratory mucosa following the intranasal delivery of mRNA/LNP vaccines, presumably caused by the LNP excipients [30]. To overcome this, Suberi *et al*. developed an inhalable and non-inflammatory polymeric based vehicle for the delivery of mRNA vaccine [31].

Suberi *et al*. found that a biodegradable poly(amine-co-ester) (PACE)-mRNA polyplex could be expressed in epithelial lung and antigen presenting cells without causing pro-inflammatory responses. The inhaled PACE-mRNA polyplex successfully induced both systemic and local production of antibodies against SARS-CoV-2 [31]. In another study, a microfluidic microsphere-based inhaled aerosol was distributed throughout the respiratory tract in mice following inhalation [32]. The aerosolised nanovesicle-in-microspheres neutralised the viral effectivity and hyperinflammatory state in infected mice via competitive binding of PACE-mRNA polyplex with ACE2/S protein receptors [31].

Small interfering RNA (siRNA) is specifically designed to interfere with expressions of vital genes and ultimately leading to inhibition in viral replication. A siRNA-modified peptide dendrimer formulation (siR-7-EM/KK-46) demonstrated significant reduction of viral burden and lung inflammation following the exposure of a Syrian hamster at three increasing doses (0.7, 1.96 or 5.6 mg/kg) using a mesh nebuliser [33]. To enhance the therapeutic activity of siRNA, a virus inspired for endosomal release (VIPER) was complexed with siRNA to increase the endosomal escape and subsequently downregulate the viral replication *in vivo* [4].

Remdesivir (GS-5734) is a prodrug of nucleoside monophosphate, which acts to inhibit the activities of viral RNA polymerase that could be exploited to target RNA viruses such as Ebola, MERS-CoV and SARS-CoV-2 infections [34–37]. Despite this, the current IV administration protocol of remdesivir is unlikely to achieve a satisfactory outcome owing to its instability and low penetration and distribution in lung tissues. Several studies using monkeys demonstrated that remdesivir was undetectable and the concentrations of its active metabolite (nucleoside triphosphate, Nuc-TP) were merely 0.8 – 1.5 μM in the lung following the IV administration dose of 10 mg/kg (equivalent to a dose

of 200–300 mg in humans) [38, 39]. This implied that the current IV dose regimen in humans was unlikely to be clinically effective in the lung against SARS-CoV-2 infections. To improve the bioavailability of remdesivir in the lung, Sahakijpijarn *et al*. developed aerosolisable nanostructured aggregates of remdesivir using the thin film freezing technique [9]. The nanoaggregates were sheared into respirable microparticles upon fluidization and exhibited excellent fine particle fraction (i.e. 93%). When administered to Syrian hamsters at a dose of 10 mg/kg, the plasma levels of remdesivir and its metabolites were sufficiently higher than the concentrations needed to exert antiviral activities over 20 h [40]. The authors also suggested a twice-daily dosing regimen of inhaled remdesivir based on its half-life of 7 h. In other studies, aerosolised liposomal remdesivir and remdesivir-loaded dendrimer showed promising results to be developed as inhalation therapy for SARS-CoV-2 [41–43]. Negatively charged liposomal remdesivir (2.5 mg drug/mL liposome) with a hydrodynamic diameter of approximately 72 nm was stable and exerted minimal toxicity in A549 cells [41]. The nebulised liposomal remdesivir exhibited mass median aerodynamic diameter (MMAD) and fine particle fraction (FPF) of 4.56 µm and 74.4%, respectively. As the projected *in vivo* dose of aerosolised remdesivir should be 10.5 mg to induce higher antiviral efficacy, the authors suggested that a nebulisation of 6 mL would be theoretically sufficient to achieve the drug concentration in the lung [41]. In another study, the Nuc-TP concentration in the lung was 100-fold higher when liposomal remdesivir was delivered via inhalation as compared to the IV route [42], which is attributable to increased cellular uptake and lung retention. The expression of pro-inflammatory cytokines such as IL-6, TNF-α and HMGB-1 in BALB/c mice was negligible and comparable for the aerosolised liposomal remdesivir and the control group, suggesting that the liposomal formulation was safe for inhalation delivery [42].

Hydroxychloroquine (HCQ), an anti-malarial drug, has been repurposed to treat SARS-CoV-2 infections via inhibition of viral entry into host cells through impairment of terminal glycosylation of the ACE2 enzyme. This drug is also believed to block endosomal acidification and subsequent autophagosome-lysosome fusion as well as attenuate the cytokine production. The current oral dosing regimen of HCQ (800 mg daily followed with a maintenance dose of 400 mg/day for 4 days) might not achieve the clinically effective HCQ concentration (6.7 µg/mL) in the lung needed to kill the virus [44]. In addition, cardiotoxicity and QTc prolongation were noted in patients receiving large oral doses of HCQ (600 mg/day) [45]. Therefore, pulmonary delivery of HCQ at a lower dose of 10–20 mg/day of inhaled HCQ sulfate (equivalent to 7.7–15.7 mg/day free-base) is believed to achieve the recommended maintenance dose of (6.4 mg/kg/day) HCQ in the lung [46]. Klimke *et al*. proposed that a dose of 2–4 mg HCQ aerosol delivered via nebulisation was well-tolerated with negligible side effects [47]. Recently, it was demonstrated that approximately 60% of HCQ isotonic nebulised solution (20–100 mg/mL) was within the respirable range (˂5 µm) [15]. In a study by Tai *et al*., a desirable pharmacokinetic (PK) profile in rats following the inhalation of liposomal HCQ (0.284 mg HCQ sulfate/animal) was demonstrated, including increased exposure and residency of drugs in the lung as well as reduced systemic heart distribution [48]. A single dose of inhaled liposomal HCQ achieved 129.9 µg/g peak plasma concentration and at least 29-fold higher lung exposure compared to the IV normalised dose [48]. Recently, inhalable crystalline powders of jet-milled HCQ sulfate were successfully produced with suitable aerosol performance (FPF: 60 – 63%, volume median diameter: 1.7 µm) [49]. The authors demonstrated that the conditioning storage of jet-milled powders at 43%, 53% RH and 58% RH produced optimal aerodynamic properties for inhalation [49]. To assess the tolerability and safety of HCQ aerosols, 12 healthy volunteers were administered with HCQ DPI in single ascending doses of 5 mg, 10 mg or 20 mg [50]. The DPI formulations were well tolerated without significant changes in QTc intervals except for minor coughing and a reported transient bitter taste [50]. A lipid nanostructured carrier encapsulating HCQ (HCQ-LNP) was successfully synthesised using almond oil, Compritol® 888 ATO, and L-phosphatidylcholine [51]. The IT administration of HCQ-LNP at the dose of 8.78 mg/kg was superior in reducing the expression of inflammation markers compared to oral dose HCQ at the higher dose (70 mg/kg) [51]. It is recently reported that polymeric nanomicelles prepared from pluronic F127 exerted higher antiviral activity compared to polymeric nanoparticles of PLA/PVA [52]. At the concentration of 3.125 µg/mL HCQ, only polymeric nanomicelles were able to reduce the viral load to undetectable RNA copies. In addition, HCQ loaded micelles were mostly distributed and retained for 24 h in the lung after pulmonary instillation of the formulations into Wistar rats [52].

Favipiravir, another potential candidate to treat SARS-CoV-2 infections, is an RNA-dependent RNA polymerase inhibitor that acts to inhibit viral transcription and replication. The oral administration of favipiravir is associated with systemic side effects in susceptible patients, which include alterations in QTc, hepatic and renal functions [53, 54]. However, a recent study demonstrated that *in vivo* inhalation delivery of favipiravir using a soft-mist inhaler was tolerated without appreciable systemic toxicities based on the negligible changes in cardiac rhythm, hepatic and renal functions in rats [55]. The rats were randomised to receive either 1, 2.5, 5 or 10 mg favipiravir/kg/mL saline for five consecutive days. The oxidative-stress associated lung injury was not observed in rats following inhalation of favipiravir [55].

Pekoz *et al*. reported that favipiravir inhalation solution in PBS buffer is stable for over a year when stored at the refrigerated condition [56]. The authors also found that nebulisation of 2 mg/mL favipiravir is optimal to achieve both antiviral and minimal toxicity in Vero-EG infected cells and Calu-3 lung epithelial cells, respectively [56]. A favipiravir solid lipid nanoparticle formulation for inhalation was developed with promising aerosolisation and antiviral properties [8]. The nanoformulation could be reconstituted for nebulisation with FPF of 60%. In addition, the IC50 value against SARS-CoV-2 was 29.9 µg/mL [8]. Heparin, an analogue of heparin sulfate, interacts with S protein to hinder its binding with host cell receptor and subsequently inhibits the viral entry into cells [57]. An inhaled heparin nanodecoy that competitively binds the S protein showed significant neutralising action against SARS-CoV-2 (including the wild-type, Delta, and Delta plus variants) *in vivo*. The neutralised pseudovirus-heparin nanodecoy complex was eliminated from the lung via the phagocytosis action of macrophages [57].

Jin *et al*. reported that hesperidin/chitosan nanoparticles (HCNP) were effective to suppress cytokine storm syndrome, which naturally occurs following sepsis or infections with SARS-CoV-2 or MERS-CoV [58]. An *in vitro* dose of 10 µg/mL HCNP significantly attenuated the inflammatory cytokine expression (IL-6, NO) in lipopolysaccharide-induced RAW264.7 cells. *In vivo* nasal delivery of 10 mg/kg HCNP towards acute inflammatory lung injury in the mouse model reduced the pro-inflammatory cytokine (TNF-α and IL-17) and suppressed vascular permeability [58]. A liposomal nanotrap formulation consisting of polylactic acid (PLA), polymeric core, a liposome shell, surface ACE2/neutralising antibodies, and phosphatidylserine ligands demonstrated complete inhibition of the pseudotyped SARS-CoV-2 entry into susceptible cells *in vitro* as well as 10-fold neutralising efficacy compared to its solution counterparts [59]. The virus-bound nanotrap could be efficiently cleared by action of phagocytosis without causing infections to macrophages [59].

A cell-mimicking ACE2 nanodecoy derived from human lung spheroid cells protected host cells from Covid-19 infection via binding and neutralisation to the SARS-CoV-2 spike protein *in vitro* [60] (Table 3). Nebulised ACE2 nanodecoys were retained in the mice lungs for over 72 h with negligible toxicity. In addition, in cynomolgus macaques challenged with live SARS-CoV-2 virus, four doses of nebulised nanodecoys resulted in increased viral clearance as well as reduced lung injury [60]. Using computational techniques, Ebrahimi *et al*. showed that an increase in the inlet velocity caused an increase in the adhesion of magnetic nanoparticle-coated microcarrier to left lower lobe (LLL) and right lower lobe (RLL)-right middle lobe (RML) regions [61]. The deposition of drugs via pulmonary delivery was affected by inlet velocity, magnet position as well as the choice of microcarriers.

Another approach to target lung injury caused by Covid-19 infection is to alleviate inflammation in the lungs. For this, chitosan, a mucoadhesive compound, was used to coat inhalable albumin nanoparticles loaded with two anti-inflammatory drugs (silymarin and curcumin) [62]. The silymarin/curcumin loaded nanoparticles exhibited antiviral activity against Covid-19 *in vitro* using plaque reduction assay [62]. Both silymarin and curcumin were capable to inhibit viral entry into host cells through the inhibition of ACE2 [62, 63]. Furthermore, the IL-6 and c-reactive proteins were significantly reduced in nanoparticle treatment groups compared to the control groups [62].

In another approach which also combined both antiviral and anti-inflammatory properties in the targeting strategy, a multifunctional alveolar macrophage-like nanoparticles with photothermal inactivation action was prepared. The nanoparticles were synthesized by coating the alveolar macrophage membranes with polymeric cores while retaining the surface receptors of the alveolar macrophages including Covid-19 and cytokine receptors [64]. Coupled with photothermal irradiation, the nanoparticles decreased viral load, inflammation and lung damage in the murine model and therefore increased the survival of infected mice [64].

Jara *et al*. recently repurposed an anthelmintic medication, niclosamide, as inhalation powder for treatment of Covid-19 using the thin-film freezing technique [65]. The formulation exhibited acceptable aerosol performance with 86% in FPF, 1.11 µm in MMAD and 2.84 in GSD [65]. The histopathology analysis in rats revealed that acute 3-day, multi-dose inhalation of niclosamide was safe. Single administration of inhaled niclosamide was able to achieve the required lung concentrations (above IC_{90} levels) for 24 h in a hamster model [65] (Table 3).

Influenza A Virus

Influenza A virus, a member of the Orthomyxoviridae family, is an enveloped virus whose genome consists of eight negative-sense single-strand RNA segments. The viral genome also consists of polymerase complex that is encircled by M1 matrix and host-derived lipid bilayer envelope. The viral glycoproteins haemagglutinin (HA), neuraminidase (NA) and M2 matrix protein are located at the surface of the envelope. It is divided into a number of subtypes according to the antigenic properties of its envelope proteins (HA and NA). As the virus has zoonotic potential and is capable of an antigenic shift, the gene reassortment of influenza A viruses of different origin results in highly contagious hybrid strains that are resistant to the existing therapy.

Vaccination is the most common prevention strategy of influenza A virus infections. The commercially available

Table 3 Pre-clinical Potential Inhaled Nanoformulations for Covid-19

Formulation	Objective	Results	Reference
Magnetic nanoparticles-coated nanocarriers	To employ magnetic nanoparticle-coated nanoparticle to promote better drug targeting	The increase of inlet velocity to approximately 0.574 m/s resulted in the maximal adherence of magnetic formulation in LLL and RLL-RML regions Higher density of magnetic caused negative effect on the magnetic triggered drug delivery	[61]
Chitosan coated silymarin or curcumin loaded albumin nanoparticle	To evaluate the potential of chitosan coated albumin nanoparticles for targeting lung injury	Higher inflammation in Covid-19 is associated with lung injury. Plaque reduction assay of SARS-CoV-2 demonstrated that the viral inhibition with the nanoparticle formulation was concentration dependent. The *in vivo* model confirmed that IL-6 expression, histopathological and complete blood count assay was reduced with the nanoparticle treatment	[62]
Dry powder of ivermectin	To study the pharmacokinetic of inhaled ivermectin	Maximum ivermectin distribution C_{max} in lung tissue was 95.6 ± 41.9 µg/g after 3 h of intratracheal administration of inhaled ivermectin (3.15 mg/kg). In addition, half-life time ($t_{1/2}$) and total drug exposure were 12.6 h and 1734 µg.hr/g after 48 h of ivermectin treatment (3.15 mg/kg). The local toxicity was recorded at 10% after 48 h of administration	[66]
Inhaled suspension of budesonide, beclomethasone and fluticasone propionate	To evaluate the effectiveness of inhaled corticosteroid for targeting SARS-CoV-2 infection	Treatment with inhaled corticosteroid reduced the expression of entry receptor angiotensin-converting enzyme-2 (ACE-2) by targeting type-I interferon. This observation was noted in both COPD patients and infected mice with COPD diseases	[67]
DPI niclosamide	To measure the pharmacokinetic profile of DPI niclosamide	The prepared niclosamide formulation demonstrated suitable aerosol performance with fine particle fraction (FPF), mass median aerodynamic diameter (MMAD) and a geometric standard deviation (GSD) of 86%, 1.11 µm and 2.84, respectively. The measured concentration of drug (C_{max} and $AUC_{0\text{-}24\,h}$) increased proportionally to the admitted dose in Syrian hamster	[65]
Nebulised omega-3 fatty acids	To measure the potential of omega-3 fatty acids to treat lung inflammation	IL-6, IL-10, TNF-α, and TGF-β expression was reduced in LPS induced acute lung inflammation in rats after receiving inhaled fatty acids treatment	[68]
Human lung spheroid cells (LSCs) nanodecoys	To study the potential of LSCs-nanodecoys in targeting Covid-19	The nanoformulation greatly reduced the viral load from bronchoalveolar lavage and nasal swabs after eight days of treatment. The formulation was safe for the animal	[60]
Multifunctional alveolar macrophage-like nanoparticles	To evaluate the potential of photothermal inactivation of multifunctional nanoparticles	The near-infrared irradiation used to treat COVID-19 in a surrogate mouse model with multifunctional alveolar macrophage-like nanoparticles significantly improved the survival rate of the mice by decreasing the viral burden, cytokines levels, inflammation, and lung damage	[64]

influenza viruses are either live, attenuated viruses or subunit/split viral antigens. As current influenza vaccines are strain-specific, they are ineffective against hybrid strains containing characteristics of the parent viruses depending on the individual genome segment makeup. Pulmonary administration of influenza vaccine or drugs has received numerous attention to ensure high deposition of drugs/vaccine in the infected site and induce local as well as systemic immune responses [69–75] (Table 4). Oseltamivir phosphate, an ethyl ester prodrug, is recommended by WHO for both treatment and prophylaxis of influenza infection. The adverse effects associated with current marketed oseltamivir phosphate formulations including dizziness, headache and agrypnia has prompted the repurposing of this drug to be delivered as inhaled formulation [73, 76]. A DPI formulation of oseltamivir phosphate prepared via spray-drying with hydroxypropylmethylcellulose (HPMC) showed acceptable aerosol performance with maximal deposition at stage 3 of the impactor and fine particle dose (FPD) of 1.08 mg [73]. In another study, liposomal oseltamivir phosphate inhalation formulation was developed using spray-drying technology composing of lactose, L-leucine and mannitol [76]. The DPI formulation of liposomal oseltamivir phosphate had an average size and FPF of 3.5 μm and 35.4%, respectively. A sustained release profile accounting for approximately 20% drug release at 2 h indicated that the drugs were released slowly from the liposomal DPI in the lungs, thus could offer the opportunity to reduce the need of frequent dosing [76]. In addition, various nanoparticle-based strategies have been designed to tackle influenza infection, including the use of a nanoparticle as carrier of the vaccine, genes and drugs [5, 77–80]. In addition, nanoparticles with anti-influenza properties are also under development [79, 81] (Fig. 2). In a study by Sawaengsak *et al*., a chitosan nanoparticle was used as the vehicle for intranasal vaccine of HA-split influenza virus [82]. The nanovaccine demonstrated high induction of IFN-γ-secreting cells in spleens coupled with 100% protection against lethal influenza virus-load in vaccinated mice [82]. Owing to the induction of inadequate levels of mucosal IgA and cellular immune response upon intramuscular injection of inactivated swine influenza vaccine, intranasal delivery could be an alternative approach to elicit strong systemic responses and confer heterologous protection. Dhakal *et al*. demonstrated that mucosal immunity in pigs against swine influenza virus was improved for inactivated vaccine (killed swine influenza H1N2 δ-lineage antigens) packaged into the chitosan nanoparticle form and delivered via the intranasal route [83]. Pigs administered twice with nanovaccine as intranasal mist exhibited enhanced IgG serum antibody and mucosal secretory IgA antibody responses in respiratory tract [83]. When challenged with heterologous virus, the vaccinated animal groups demonstrated reduced severity in pulmonary lesions and infectious viral load compared to non-vaccinated groups [83].

Dong *et al*. recently reported the development of polyethyleneimine (PEI)-functionalised graphene oxide nanoparticle (GP) influenza vaccine formulation by simple mixing of recombinant protein vaccine (influenza HA) with GP. The HA-GP vaccine formulation was recently internalised by dendritic cells and promoted the secretion of pro-inflammatory cytokine (TNF-α and IL-6) *in vitro* [5]. Following the two-dose intranasal vaccination scheme, humoral immunity was induced as evident from the higher antigen-specific IgG and mucosal IgA antibody titers for HA-GP vaccinated mice. Strong cellular immune response was observed in HA-GP vaccinated mice with an increase in CD4 + and CD8 + T cell proliferation, thus conferring protection towards homologous and heterologous influenza viruses in mice [5]. It is known that PEI-protein recombinant vaccine formulations primarily induce Th2-dominant immune response. Notably, owing to a lack of IFN-γ cytokine induction, the protective cellular responses such as cytotoxic T-lymphocyte activation are not induced with PEI. Therefore, the incorporation of unmethylated cytosine-guanine rich oligonucleotide motifs (CpG) onto PEI-HA might trigger the TLR-9 signalling pathway, thus inducing a balanced Th1/Th2 immune response [77]. Compared to the PEI-HA nanoparticle, the intranasal vaccination of PEI-HA/CpG nanoparticles led to a more balanced Th1/Th2 response with the generation of an IgG1 and IgG2a antibody as well as enhanced neutralising action and Fc-mediated antibody-dependent cellular cytotoxicity (ADCC) [77]. In addition, the cross-protective immunity lasted over 6 months in mice vaccinated with intranasal PEI-HA/CpG nanoparticle [77]. In another approach, an intranasal vaccination strategy has been developed to target the highly conserved influenza nucleoprotein (NP) using self-assembly nanolipoprotein particle (NLP) as the carrier [84]. Intranasal delivery of the antigen-specific vaccine elicited large populations of CD4 T cell populations in the lung. In addition, co-delivery of the antigen and adjuvant (CpG) using NLP induced 4.4 fold and threefold increase in IFN-γ-producing cells and IL-2 producing cells, respectively, as compared to the antigen solution [84].

In another strategy, the M2 matrix protein is used as the viral antigen to elicit both humoral and cellular immunity towards the influenza challenge. For this, M2e of influenza A virus was conjugated to gold nanoparticle (AuNPs) and administered intranasally to mice with or without the addition of soluble CpG adjuvant [78, 85]. The administration of M2e/AuNPs stimulated the production of anti-M2e serum IgG levels whereas the presence of soluble CpG induced a Th1-biased immune response [85]. In addition, the intranasal vaccination of M2/AuNPs alongside soluble M2e (500 molar) and CpG molecules elicited a strong M2e-specific

Table 4 Examples of Inhaled Formulation for Influenza Treatment*

Drug/Formulation	Inhaler	Results	Reference
Tamibarotene	DPI oral inhalation	*In vitro* aerosol performance showed that the MMAD and FPF were 1.86 ± 0.44 µm and 95%, respectively. The intratracheal administration of the DPI formulation resulted in 159-fold higher C_{max} compared to the EC_{50} of H1N1. More rapid drug absorption was observed for direct lung deposition administration compared to the intraperitoneal route	[69]
HA of NIBRG-23 WIV vaccine + adjuvant (Bacterium-like Particles (BLP) and Advax)	DPI oral inhalation	The average particle sizes for the spray freeze-dried adjuvanted vaccine formulations were between 8 and 12 µm. Passive inhalation of aerosolised vaccine powders induced significantly higher IgY levels in chickens. Animals immunised with adjuvanted vaccine formulation were protected from infection when challenged with A/turkey/Turkey/1/2005 virus. Virus shedding was not observed	[70]
Oseltamivir phosphate (OP)	DPI oral inhalation	The FPF of micronised OP/trehalose blends ranged from 40.3% to 62.5% depending on the mass of trehalose. The blends were non-toxic to Calu-3 cells *in vitro*	[71]
Thermostable LAIV (T-LAIV) virus A/17/Texas/2012/30 (H3N2) with or without delta inulin adjuvant (Advax™)	Powder intranasal spray	T-LAIV was stable at ambient temperature without significant loss of titer at 25 °C for 52 weeks. Robust serum HI responses and mucosal antibodies level were noted in ferrets vaccinated with T-LAIV powder in the presence of adjuvant or without adjuvant	[72]
Oseltamivir phosphate (OP)	DPI oral inhalation	Sustained release formulation of OP DPI was developed using Hydroxy Propyl Methyl Cellulose (HPMC) as the rate-controlling polymer. Burst release of 49% was observed within 15 min followed with sustained release for 9 h. Majority of the DPI formulation was deposited at stage 3 of mechanical impactor with FPD of 1.08 mg	[73]
Oseltamivir phosphate (OP) liposomes	DPI oral inhalation	OP liposomal formulation was spray-dried with lactose, l-leucine and mannitol as excipient. The particle size and FPF of spray-dried OP liposomal matrix were 3.5 µm and 35.4%, respectively	[76]
A/CAL/H1N1 subunit and inulin as excipient	DPI oral inhalation	The spray dried DPI formulation induced comparable serum IgG and lung IgA titers when administered via pulmonary or intramuscular route. Immunised rats did not have detected virus in the lung when challenged with TCID50 of A/Cal/2009 virus	[74]
A/Panama/H3N2 subunit stabilised with inulin	DPI oral inhalation	Spray freeze-dried inulin stabilised vaccine demonstrated superior humoral response (IgA and IgG) in serum, lung and nasal compared to aerosolised liquid formulation	[75]
Whole inactivated influenza virus A/HIR/H3N2; A/PR/8/H1N1 and inulin as stabiliser	DPI oral inhalation	Systemic immune response was observed. Comparable efficacy in terms of reducing viral load in the lung between locally administered DPI vaccine and intramuscular injection of vaccine	[94]
Whole inactivated influenza virus A/HIR/H3N2; A/PR/8/H1N1, inulin as stabiliser and monophosphoryl lipid A as adjuvant	DPI oral inhalation	Higher mucosal antibody concentration for mice immunised with adjuvanted formulation. Immunization with adjuvanted vaccine resulted in balanced IgG2a/IgG1 levels	[95]
Inactivated swine influenza A virus H1N2 antigen encapsulated in chitosan nanoparticle	Intranasal	Higher IgA titers in nasal swabs for nanovaccine compared to antigen solution	[83]

*Some of the examples of inhaled formulations are not nanobased formulation

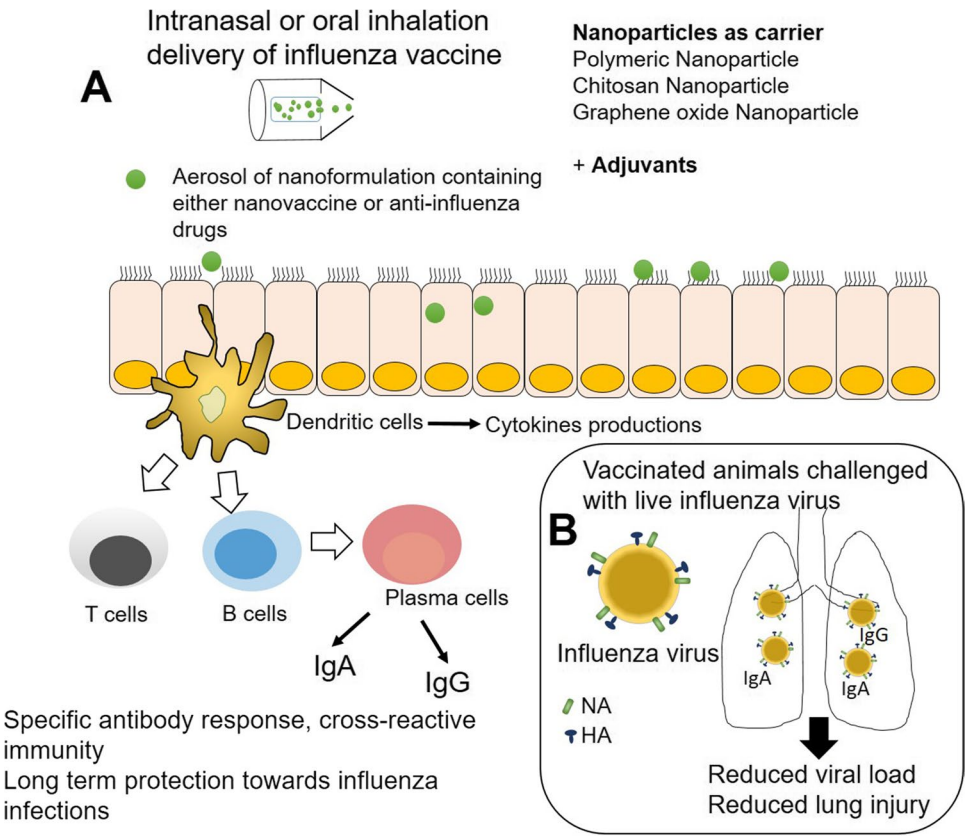

Fig. 2 (**A**) Schematic illustration of the enhancement of immune response upon intranasal or oral inhalation vaccination of influenza vaccines encapsulated within nanocarriers (i.e. chitosan based nanoparticle or graphene oxide nanoparticles). The delivery of aerosols would promote the uptake by dendritic cells which in turn induce cytokine expressions and induction of T cell-mediated responses. Plasma cells will be activated to secrete influenza specific IgA and IgG for long-term immunity towards influenza infection. (**B**) Vaccinated animals would confer protection in terms of reduced viral load and associated lung damages when challenged with live influenza infections

antibody response and conferred long-term protection from a lethal dose of A/PR/8/34 (H1N1) influenza infection [78].

In an effort to develop a universal influenza vaccine, a multicomponent nanoformulation consisting of mRNA and protein subunit vaccine was developed using chitosan nanoparticle as the delivery vehicle [86]. The anionic mRNA molecules were first complexed with a cationic chitosan nanoparticle followed with surface adsorption of H9N2 HA2 and M2e influenza proteins, resulting in nanoparticles ranging between 100–800 nm in size [86]. Following intranasal vaccination with HA2/M2e/mRNA-chitosan nanoparticles in chickens, higher systemic IgG IgA levels in lung mucosa and cellular immunity were produced compared to those receiving either chitosan nanoparticles or HA2/M2e-chitosan nanoparticles. After 28 days of post-vaccination, vaccinated animals were protected from H7N9 or H9N2 infection, demonstrating the broad protection of the nanoformulation as a universal influenza vaccine [86]. An mRNA nanoparticle that codes for a bispecific single-domain antibody that binds to the influenza A matrix protein 2 and activating mouse F receptor IV (FcγRIV) was capable of inducing a strain-specific antibody after immunization and contact with live H1N1 and H3N2 influenza virus [87]. In another study, an optimised PEG-lipid nanoparticle formulation for the nebulisation delivery of mRNA encoding the broadly neutralising antibody targeting HA was developed [88]. The aerosolised vaccination protected mice from the lethal challenge of A/Puerto Rico/8/1934 (H1N1). Meanwhile, control groups without vaccination died after substantial weight loss [88].

Wang *et al*. developed a nanovaccine formulation by conjugating both recombinant trimetric influenza A/Aichi/2/68(H3N2) HA and Toll- like receptor 5 (TLR5) agonist flagellin (FliC) onto AuNPs [89, 90]. The 18 nm AuNPs were covalently linked to long PEG spacer to facilitate the conjugation of HA and FliC onto the particles [90]. HA/FliC-AuNPs formulations induced higher antibody response when administered as a single dose intranasal vaccination to mice. The incorporation of FliC triggered the activation of TLR5 signalling pathways, which in turn elicited cell-mediated immunity [90]. In another study, the co-delivery of FliC/Au NPs and HA/Au NPs as separate individual particles established a balanced IgG2a/IgG1 immune response. The stimulation of IFN-γ-producing CD4 + T cells responses confirmed the activation of cellular immunity via this approach [89]. Similarly, Knuschke *et al*. also found that the co-delivery of immunoactive TLR9 ligand (CpG) with HA antigen using a calcium phosphate-based nanoparticle stimulated strong T cell-mediated immune response in mice [91].

A multi-shell nanoparticle consisting of a calcium phosphate core and coated with siRNA was produced via encapsulation with poly(lactic-co-glycolic acid)/ polyethylenimine

[92]. The siRNA used in the study acted to target the cytokine expression in cell-mediated lung inflammation. The intranasal delivery of siRNA nanoparticle in an influenza infection model showed a target-specific decrease in pulmonary inflammation caused by Th1 immunity as well as reduced viral load and pathology [92]. In another study, the delivery of siRNA against influenza nucleoprotein using chitosan nanoparticles protected.

BALB/c mice from a lethal influenza challenge via inhibition of viral replication [93].

Respiratory Syncytial Virus (RSV)

Respiratory syncytial virus (RSV) is known to cause bronchiolitis and otitis media in young children as well as pneumonia in adults and elderly. It is noted that natural immunity to RSV is incomplete with re-infection occurring throughout life [96]. One of concerns for RSV vaccine is the risk of enhanced respiratory disease which might happen upon natural infection of vaccinated infants [96]. This enhanced respiratory disease or known as 'immunopotentiation', was observed in the late 1960s following the vaccination of young children with alum-precipitated formalin-inactivated RSV vaccine which resulted in exacerbated disease upon RSV infection. Studies have demonstrated that enhanced respiratory disease is associated with induction of poorly neutralising antibodies coupled with a helper type 2 (Th2) biased T-cell response [97, 98]. Therefore, an ideal RSV vaccine should elicit neutralising antibodies without a Th2-biased T-cell response. For this reason, owing to the presence of several antigenic sites and subsequent potential to induce neutralising antibodies in humans, the surface prefusion (F)-glycoprotein is a popular target for the development of RSV vaccines [99]. Other vaccine antigen targets include post-F protein, G-protein, envelope associated proteins (G and SH) and internal proteins (nucleocapsid, M). To date, the RSV vaccine candidates intended to induce neutralising antibodies are developed via six approaches, which include live-attenuated virus, particle-based, chimeric, monoclonal antibodies, subunit, recombinant vector and nucleic acid vaccine [100, 101]. Systemic delivery systems [e.g. oral, intramuscular (IM), intravenous (IV) and subcutaneous routes] of RSV vaccine candidates in clinical development are often employed in the treatment or prevention of RSV. Alternatively, local delivery of RSV vaccines and antibodies either via intranasal or inhalation has gained attention because this method allows the direct diffusion of biologics to the target site region (lung epithelial for the case of RSV infection). Aerosolised ribavirin was first approved in 1985 for the treatment of RSV in paediatrics [102–105] and is also prescribed for RSV lower respiratory tract infection (LRTI) in immunocompromised adults and elderly [103, 106, 107]. The aerosols should range between 1.0 and 1.3 μm to ensure sufficient deposition in the lung [108]. A retrospective cohort study on hematopoietic stem cell transplant (HSCT) patients demonstrated no significant differences in mortality between the oral ribavirin and the aerosol ribavirin group. Both administration routes derived similar outcomes in HSCT RSV patients [103]. In another review, the mortality of HSCT patients with LRTI receiving aerosolised ribavirin was reported to be 24% [109]. A detailed systematic review on RSV management with the use of aerosolised ribavirin is published elsewhere [102]. ALX-0171, a trivalent nanobody targeting the F-glycoprotein to inhibit viral entry was formulated as a nebulised solution to enable high deposition at the infected lung site [110]. Nebulisation of ALX-0171 to newborn lambs infected with human RSV showed robust antiviral activity, reduced viral load in the airway, and simultaneously improved lung pathology [111]. A double blind, randomised, placebo-controlled, phase 2b trial (EudraCT, 2016–001,651-49) was conducted to measure the time needed for the RSV viral load to drop below the quantification limit [112]. In this study, children (between 28 days and 24 months of age) suffering from RSV infections were randomly assigned to receive ALX-0171 3 mg/kg, 6 mg/kg, 9 mg/kg, or placebo. Nebulised ALX-0171 was effective to reduce the infectious viral load below quantification limit compared to the placebo (median time 49.4 h versus 95.9 h). However, the clinical outcomes were not statistically different between the nebulised ALX-0171 and the placebo group [112]. In another study, a novel inactivated RSV vaccine was developed through direct inactivation of live RSV in nanoemulsion-based adjuvants. The nanoemulsion is an oil-in-water emulsion prepared using cetylpyridinium chloride, a cationic detergent which has been proven to be safe for human use [113]. Compared to alum, nanoemulsion-based adjuvanted inactivated RSV vaccine did not result in Th2-biased immunopotentiation response. Intranasal vaccination of mice using nanoemulsion adjuvants enhanced Th1-associated immune responses without the induction of Th2 cytokines [113]. The authors also demonstrated that the formulation effectively inactivated RSV and enhanced viral clearance while mediating RSV specific IgG and IgA responses [113].

Additionally, in a cotton rat model challenged with RSV A2 strain, the intranasal administration of W805EC or P188 nanoemulsion adjuvants promoted robust Th1 cellular immunity with high levels of IFN-γ expressions. Minimal histological effects in the lung were observed after viral challenge [114]. A maleic anhydride (ML)-modified human serum albumin (HSA) is shown to inhibit the RSV entry by blocking the attachment of virus with viral G protein [115]. The intranasal delivery of ML-HAS prior to RSV challenge demonstrated significant decrease in viral load in the lungs of mice, which is promising for further development as a pre-exposure prophylaxis of RSV infection in children [115].

Recently, Ivanov *et al*. demonstrated that intranasal and intrapulmonary administrations of M-protein deleted RSV vaccine in infant baboons was effective to alleviate clinical symptoms and reduce viral replication after RSV challenge [116]. The neutralising antibodies response after intrapulmonary vaccination (4–6 months) was stronger compared to intranasal vaccination (1 month). Their results showed that intrapulmonary vaccination appeared more effective to prevent tachypnea and to reduce work of breathing and viral replication in infant baboons [116]. In another study, long term memory immune response (15 months duration of RSV-specific neutralising antibody) in mice was induced following the single dose intranasal administration of a virus-like particle (VLP) containing recombinant matrix protein (M) and F-glycoprotein of RSV [117]. Mice immunised with RSV VLP were protected against RSV-associated illness without signs of excess inflammation and lung pathology [117]. Similar findings were reported in another VLP formulation comprising of influenza virus matrix (M1) protein and RSV fusion (F) or glycoprotein (G) whereby single intranasal administration induced IgG2a response, viral clearance and RSV neutralising activities in BALB/c mice [118]. Similarly, Garg *et al*. reported that a fusion (F) protein co-formulated with adjuvants of poly(I:C) and polyphosphazene (DF/TriAdj) was able to confer immunity for up to 5 months following a single intranasal dose [119]. High levels of IgG1, IgG2a and neutralising antibodies were detectable in the vaccinated mice, thus indicating that the fusion protein-adjuvant formulation induced the production of IgA-secreting memory B cells [119]. Furthermore, no infectious viral particles were detected in week 25 post-vaccinated mice when exposed to RSV (5×10^5 PFU/50µL) for a period of 4 days. The same group also noted that the subunit F-protein adjuvant formulation (ΔF/TriAdj) was more effective at inducing higher mucosal IgA levels compared to the live RSV [120]. The Sendai virus (SeV), a mouse parainfluenza virus, has been formulated as a vector for RSV vaccine with the insertion of either RSV F or G gene between the F and HN genes of SeV [121]. SeV vaccine was effective at conferring prophylaxis protection against most strains of RSV [122]. The vaccinated cotton rats were protected against RSV challenge 3 months post-vaccination by inducing de novo virus-specific neutralizing antibodies [123]. When tested in African green monkeys via intranasal and intratracheal routes, minimal adverse events with transient vaccine infections were observed. The vaccinated subjects were completely protected from RSV infection at the RTI [101, 124] (Table 5). Another vector-based vaccine, which uses the parainfluenza virus (PIV) to display RSV antigens (MEDI-534), is based on the modified bovine PIV3 that expresses human PIV2 fusion, the human PIV3 hemagglutinin-neuraminidase and the RSV F proteins [125]. When delivered intranasally to seropositive children, MEDI-534 was minimally immunogenic and no viral shedding was noted [126] (Table 5). In seronegative children, the vaccine virus was well tolerated in 50% of the subjects and could be detected 7 – 10 days after vaccination in a dose-dependent manner [127] (Table 5). The genetic stability of RSV-F expression of the MEDI-534 could be due to the maintenance of attenuation phenotype growth and limited proliferation in human lung cells [128].

To date, only one nanoparticle-based RSV vaccine (SynGEM) administered via intranasal delivery is ready for clinical trials. SynGEM consists of RSV fusion protein bound to bacterium-like particles [129]. The intranasal vaccination with SynGEM in cotton rat models elicited both systemic IgG and mucosal IgA antibodies coupled with a balanced IgG2a/IgG1 ratio, indicating both Th1 and Th2 activation response [129]. In a randomised, placebo-controlled phase 1 trial, healthy adults were assigned to receive either placebo or intranasal low dose and high dose of SynGEM [130]. Significant increase in RSV-specific serum antibody (palivizumab-like antibodies) was observed for at least 6 months in healthy seropositive adults [130] (Table 5).

The first candidate of siRNA developed for RSV treatment via pulmonary administration is ALN-RSV01, a naked siRNA targeting the RSV nucleocapsid protein [131, 132]. The phase 1 clinical trial (NCT00496821) showed that ALN-RSV01 could be tolerated up to 150 mg as a single dose for 5 daily doses. When delivered intranasally, the adverse event and its severity was similar between ALN-RSV01 and placebo [132]. In a phase 2b trial, intranasal delivery of ALN-RSV01 was investigated for its efficacy to prevent bronchiolitis obliterans syndrome (BOS) in RSV-infected patients following lung transplant [131]. For this trial, 87 RSV-positive patients were randomised to receive ALN-RSV01 or placebo and monitored for a period of 180 days for the occurrence of new or progressive BOS. The results showed that intranasal delivery of ALN-RSV01 marked a decrease in incidence of both new and progressive BOS [131].

Conclusions and Outlook

Pulmonary administration of antiviral agents and vaccines is an alternative method to deliver high concentrations of therapeutics locally to the lung in order to induce local and systemic immune responses. The utilisation of nanotechnology has expanded the horizon of drug delivery for those therapeutics or biologics requiring extreme storage refrigeration for stability. Nanotechnology has also overcome challenges associated with the inherent characteristics of some drugs, including limited solubility, short half-life and poor stability, to ensure efficient local concentrations at the target site. The use of nanotechnology is evident with the success

Table 5 Clinical Trials for the Treatment of RSV Infections

Group	Drug Name	Route	Target of drug	Phase	Primary endpoint or Results	Clinical trial number or Reference
RNAi	ALN-RSV01	Inhalation	Targeting nucleocapsid gene to inhibit viral replication	2	No post-inhalation perturbation in lung function. After day 90, progressive bronchiolitis obliterans syndrome (BOS) was significantly reduced in ALN-RSV01 groups compared to placebo groups	NCT00658086, [133]
	ALN-RSV01	Intranasal	Targeting nucleocapsid gene to inhibit viral replication	2	Primary endpoint: to evaluate the safety and tolerability of intranasal ALN-RSV01 in RSV-infected patients. Study is completed but results are unavailable	NCT00496821
	ALN-RSV01	Inhalation	Targeting nucleocapsid gene to inhibit viral replication	2b	Primary endpoint: to evaluate the occurrence of new or progressive BOS grade 0p through 3 in RSV-infected lung transplant patients Study is completed but results are unavailable	NCT01065935
Monoclonal antibody (mAb)	SynGEM	Intranasal	Prefusion F	1	Induced systemic plasmablast response and increase in RSV-specific serum antibody in healthy seropositive adults	NCT02958540 [130]
Live attenuated vaccines	RSV ΔNS2/Δ1313/I1314L	Intranasal	Viral proteins	1	In RSV-seronegative children, dose was tolerated. Replication of vaccine is detected in 90% of vaccine. Immunogenic in seronegative children (geometric mean serum RSV plaque-reduction neutralizing antibody titer, 1:64)	NCT03422237, NCT01893554 [134]
	RSV LID ΔM2-2 Vaccine	Intranasal	Viral proteins	1	Vaccine induced substantial titers of RSV-neutralizing serum antibodies in seronegative infants and children. The antibodies were boosted in subsequent RSV season without RSV infection	NCT02040831 NCT02237209 [135]
	RSV LID ΔM2-2 1030 s	Intranasal	Viral proteins	1	More than fourfold rise in serum-neutralizing antibody. Median peak titer of 3.1 log10 PFU/mL and 5.1 log10 copies/mL using immune-plaque assay and reverse-transcription quantitative polymerase chain reaction, respectively	NCT02794870 NCT02952339 [136]
	RSV cps2 Vaccine	Intranasal	Viral proteins	1	Well-tolerated and moderately immunogenic in seronegative children. Median peak titer of 0.5 log10 PFU/mL and 2.9 log10 copies/mL by culture and polymerase chain reaction, respectively	NCT01968083, NCT01852266 [137]
	RSV MEDI ΔM2-2 vaccine	Intranasal	Viral proteins	1	Post-neutralizing serum antibody with geometric mean titer of 1:97. Higher antibody level detected in seronegative children with no illness reported	NCT01459198 [138]
	D46/NS2/N/ΔM2-2-HindIII	Intranasal		1	D46/NS2/N/ΔM2-2-HindIII demonstrated good infectivity and immunogenicity in RSV-seronegative children	NCT03099291 NCT03102034 [139]

Table 5 (continued)

Group	Drug Name	Route	Target of drug	Phase	Primary endpoint or Results	Clinical trial number or Reference
Live attenuated vector based vaccine	MEDI-534 Vaccine	Intranasal	Wild-type F	1	No viral shedding in seropositive children. Safe and minimally immunogenic Well tolerated in seronegative children	NCT00345670, NCT00686075 [126, 127]
Live attenuated vector based vaccine	SeVRSV	Intranasal	Wild-type F	1	Vaccine was well-tolerated in healthy adults. Minimal antibody response was observed towards the vaccine	NCT03473002 [101]
Live attenuated vector based vaccine	RSV 6120/ΔNS1; RSV 6120/F1/G2/ΔNS1	Intranasal		1	Primary endpoints: to evaluate the infectivity, safety, and immunogenicity of a vaccine in seropositive children and seronegative infants	NCT03596801
Live attenuated vector based vaccine	PanAd3-RSV	Intranasal	viral protein	1	A rise in serum RSV neutralising antibody titer in healthy adults. Anti-F IgG-, IgA-secreting B-cells and Th1 (IFNγ) responsive T-cells were present	NCT01805921 [140–142]
Live attenuated vaccine	MV-012–968				Primary endpoints: solicited adverse events after vaccine administration	NCT04909021 NCT04227210

of Pfizer-BioNTech Covid vaccine nanoformulation following the recent Covid-19 outbreak. Having said that, there are several challenges that should be considered prior to the roll-out of inhaled vaccines to the public. The lung is always present with the resident macrophages to eliminate potential pathogens. The inhaled nanovaccines could be effectively internalised by antigen presenting cells (i.e. alveolar macrophages, dendritic cells), and neutralised to elicit appropriate cellular and humoral responses specific to the targeted virus. The potential of cytokine storm could also be avoided to minimise the associated lung injuries. The choice of adjuvant used in conjunction with the vaccine formulation is therefore crucial to reduce immunopotentiation effect. In the case of RSV, nanoemulsion-based adjuvanted inactivated RSV vaccine has been proposed as an alternative to alum to inhibit the Th2-biased immunopotentiation response that could worsen the disease outbreak upon infection.

Another challenge associated with the inhalation formulation is stability of the fabricated nanoformulation during storage as well as during use. In the case of inhaled vaccine, the stability should include the preservation of active ingredients (e.g. inactivated virus particle, mRNA, subunit particle) activity. Particle agglomeration ($^{>}$ 5 μm) is likely to cause deposition of vaccine in the trachea and therefore reduce the available vaccine in the infected site. Finally, the physiochemical properties of vaccines such as solubility, stability, and effective dosage play an important role to determine whether they should be delivered in dry powder or liquid formulations.

Compared to the systemic administration methods such as intravenous injection, inhalation route should deposit higher concentration of vaccine locally into the lung which could induce higher risk of toxicity, uncontrolled inflammation as well as activated innate immune response. It is generally accepted that activated innate immune response is linked to the reduction of hepatic cytochrome P450 activity. This interaction is extended to drug transporters and other organs which finally exert impact on drug absorption, distribution, metabolism and excretion (ADME), the primary pharmacokinetic clearance pathways for complex formulations (e.g. nanoparticles, biologics) [143]. For instance, altered ADME of drugs have been observed in Covid-19 infection and vaccination. A clinical case study reported that an increased level of clozapine was observed in an individual with history of oral clozapine treatment over 10 years after receiving mRNA vaccine (Pfizer-BioNTech) for SARS-CoV-2 [143]. The individual showed symptoms of Covid-9 infection although tested negative repeatedly as well as multiple comorbidities associated with increased risk of neurologic toxicity of clozapine [143]. Therefore patients with active viral infection or individuals who have just received vaccination, may have altered innate immune response which in turn impact on the uptake and clearance of drugs. Subsequently,

these events will put the individuals at risk for subtherapeutic response or drug-associated toxicities. People at highest risk of complications would be those with underlying medical conditions such as diabetes, hypertension and hyperlipidaemia that require constant use of medicines.

It is also important to note the potential side-effect from drug deposition in the lung. For instance, Paxlovid, a solid dosage formulation of nirmatrelvir and ritonavir is introduced as oral formulation for Covid-19 treatment. Inhaled version of Paxlovid could minimise the potential of drug metabolism by liver enzymes as well as reducing side-effects such as stomach pain, diarrhoea, and high blood pressure. Although pulmonary administration has gained popularity in self-administration therapy for respiratory infections, it should be prescribed with caution for highly contagious viruses capable of eliciting pandemics such as influenza and coronavirus.

It is advisable that the inhalation is conducted in negative pressure room where all involved are equipped with personal protective equipment (cloth, mask etc.). For instance, in the case of Covid-19, different inhalation strategies have been investigated for patients with mild, moderate and severe Covid-19. Mild cases are advised to use MDI and DPI instead of nebulisers. Meanwhile in severe cases, patients under ventilator support should receive their aerosol therapy using jet mesh nebuliser that did not prevent ventilation circuit in order to prevent virus spreading. In addition, infection control during aerosol therapy is also an issue. The exhaled aerosol might contain contagious viral particles might increase the risk of infection among healthcare professionals in cases with poor adherence to infection control procedures.

In summary, we would highlight that the inhaled nanoformulations may provide more effective alternatives approaches for treatment and vaccination against viral respiratory infections. Nevertheless, to ensure the safety and efficacy of the inhaled nanoformulations, more in-depth studies are warranted to understand the interactions between the drug particles and human cells as well as the cell responses to these inhaled nanoformulations.

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Declarations

Conflict of Interest The authors declare no conflict of interest to this review.

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