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The efficacy of a 2,4-diaminoquinazoline compound as an intranasal vaccine adjuvant to protect against influenza A virus infection *in vivo*

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Adjuvants are substances added to vaccines to enhance antigen-specific immune responses or to protect antigens from rapid elimination. As pattern recognition receptors, Toll-like receptors 7 (TLR7) and 8 (TLR8) activate the innate immune system by sensing endosomal single-stranded RNA of RNA viruses. Here, we investigated if a 2,4-diaminoquinazoline-based TLR7/8 agonist, (*S*)-3-((2-amino-8-fluoroquinazolin-4-yl)amino)hexan-1-ol (named compound 31), could be used as an adjuvant to enhance the serological and mucosal immunity of an inactivated influenza A virus vaccine. The compound induced the production of proinflammatory cytokines in macrophages. In a dose-response analysis, intranasal administration of 1 μg compound 31 together with an inactivated vaccine (0.5 μg) to mice not only enhanced virus-specific IgG and IgA production but also neutralized influenza A virus with statistical significance. Notably, in a virus-challenge model, the combination of the vaccine and compound 31 alleviated viral infection-mediated loss of body weight and increased survival rates by 40% compared with vaccine only-treated mice. We suggest that compound 31 is a promising lead compound for developing mucosal vaccine adjuvants to protect against respiratory RNA viruses such as influenza viruses and potentially coronaviruses.

Keywords: influenza virus, chemical vaccine adjuvant, TLR7/8 agonist, mucosal immunity, nasal vaccine

Introduction

Influenza viruses, which belong to the *Orthomyxoviridae* family, have an eight-segmented negative-stranded RNA genome and are categorized into four types: A, B, C, and D (Hause *et al.*, 2014) (https://www.cdc.gov/flu/about/viruses/types.htm). Among types A and B, which can infect humans seasonally, influenza A virus is further divided into subtypes according to the presence of combinations of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), whereas influenza B virus is divided into two antigenically distinct lineages, Victoria and Yamagata (Rota *et al.*, 1990; Wu *et al.*, 2014). Influenza A virus infects a broad range of hosts including pigs, horses, and birds, as well as humans. The inter-species transmission of the virus continuously generates antigenic-shift variants, providing a source of pandemic strains and posing potential public health problems (Ambrose *et al.*, 2008; Bouvier and Palese, 2008; Yoon *et al.*, 2014).

To protect against influenza virus infection, quadrivalent vaccines that are composed of vaccine strains of A/H1N1, A/H3N2, B/Victoria lineage, and B/Yamagata lineage viruses have been approved globally. Most vaccines are administered as intramuscular immunization of inactivated split vaccines and are manufactured through large-scale inoculation of biannual vaccine strains in animal cells or fertilized chicken eggs. Nevertheless, the protective efficacy of existing vaccines is not sufficient, averaging just about 40%, due to antigenic mismatch and high mutation rates during circulation (Flannery *et al.*, 2019; Tenforde *et al.*, 2020). To enhance protective immunity, live-attenuated intranasal vaccines, which mimic the natural infection route, have been developed and are administered as needle-free nasal sprays. However, safety concerns regarding the potential to revert to a pathogenic form or to hyper-stimulate innate immune responses have limited the approval of these vaccines for use in healthy, non-pregnant people aged 2 to 49 years (Ambrose *et al.*, 2008; Carter and Curran, 2011).

Toll-like receptors 7 (TLR7) and 8 (TLR8), which are mainly distributed in the endosomal membrane of immune cells, including dendritic cells, macrophages, or B cells, sense purine-rich single-stranded RNA as non-self when cells are infected with RNA viruses (Dowling, 2018; Bhagchandani *et al.*, 2021). These pattern recognition receptors (PRRs) trigger innate immune responses and elicit strong antigen-specific humoral responses to protect against viral infection. Activated endosomal TLR7 and TLR8 stimulate an antiviral response by phosphorylating interferon regulatory factor 7 (IRF7) via activation of the myeloid differentiation factor 88 (MyD88)- and TNF receptor-associated factor 6 (TRAF6)-dependent

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pathway, eventually inducing the production of type I interferons (IFNs) (Kawai *et al.*, 2004). The TLR7-selective agonist imiquimod (also named R837) is an imidazoquinoline derivative that was first approved by the United States Food and Drug Administration (FDA) for the topical treatment of genital and perianal warts (De Clercq and Li, 2016). Following the discovery of TLR7-selective or TLR7/8 dual-function compounds, dozens of synthetic agonists are now in phase I and II clinical trials mainly as anti-cancer immunotherapies. Among them, some compounds, including resiquimod (also named R484), selgantolimod (also named GS-9688), RG-7864 (also named RO7020531), JNJ-4964 (also named TQ-A3334), and vesatolimod (also named GS-9620), have been evaluated for safety and antiviral efficacy against hepatitis B virus (HBV) or human immunodeficiency virus (HIV) infection (Janssen *et al.*, 2018; Luk *et al.*, 2020; Amin *et al.*, 2021; Hu *et al.*, 2021). In addition to antiviral applications, several reports show the potential of the synthetic TLR7/8 agonists, imiquimod and resiquimod, as vaccine adjuvants with stimulation of both cellular and humoral immune responses in antigen-presenting cells (Ahonen *et al.*, 1999; Pieters *et al.*, 2018). A lipidated form of the TLR7/8 agonist imidazoquinoline (also named UM-3005) enhances the protective efficacy of an A/H3N2 split vaccine in intramuscularly vaccinated mice (Miller *et al.*, 2020), indicating that TLR7/8 agonists improve vaccination against viral infection.

Recently, starting from a pyrimidine-based TLR7/8 dual agonist numbered as compound **2** in the original publication (McGowan *et al.*, 2016) and subsequent systematic structure-activity relationship studies, a series of 2,4-diaminoquinazoline derivatives have been identified (Embrechts *et al.*, 2018; Pieters *et al.*, 2018). Among them, compound **31** with the full name of (*S*)-3-([2-amino-8-fluoroquinazolin-4-yl]amino) hexan-1-ol (Fig. 1A) has the most promising dual agonistic activity (lowest effective concentration [LEC]: 0.15 μM for human TLR7 and 0.16 μM for human TLR8), as well as low cytotoxicity and few off-target effects. In mice and cynomolgus monkeys, orally administrated compound **31** led to the production of IFN-α and proinflammatory cytokines (Embrechts *et al.*, 2018). The immunostimulatory efficacy of compound **31** has largely been evaluated for the treatment of chronic HBV infection. Given that compound **31** strongly elicits inflammatory cytokine and chemokine production, we anticipate that compound **31** could function as a vaccine adjuvant.

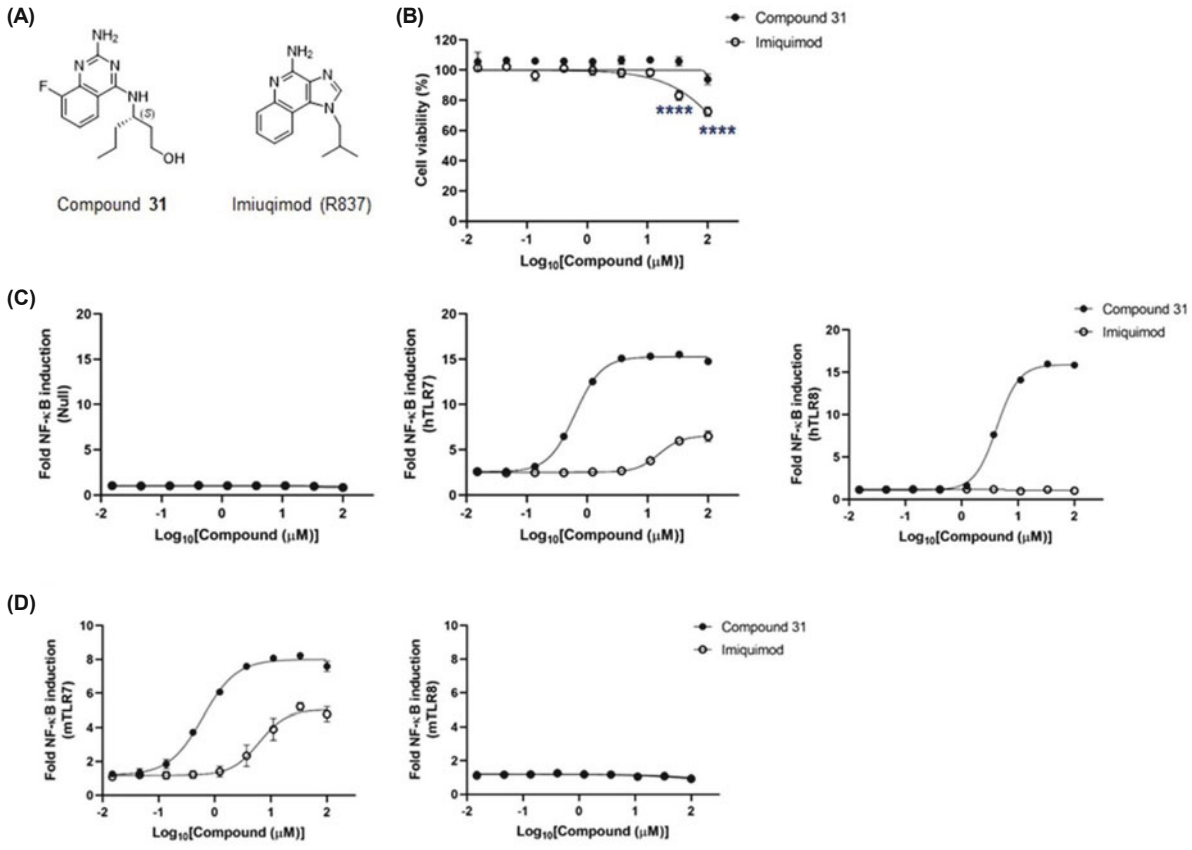

Fig. 1. TLR7 and TLR8 agonistic effects of compound 31. (A) The chemical structure of compound **31** and imiquimod. (B) The cytotoxicity of compound **31** (black circles) and imiquimod (white circles) to HEK-Blue Null1 cells. Cell viability was measured by an MTT assay one day after treatment of cells with increasing concentrations of the compounds. Significances refer to the DMSO-treated controls. ****$P < 0.0001$. (C) The agonistic effects of compound **31** on human TLR7 (hTLR7) and TLR8 (hTLR8). HEK-Blue cells expressing null (Null1; left), hTLR7 (middle), and hTLR8 (right) were treated with increasing concentrations of compound **31** (black circles) or imiquimod (white circles) as the control. The next day, NF-κB promoter activity was measured by assaying SEAP levels in the culture supernatants. Fold-changes are expressed relative to each DMSO-treated mock control. (D) Compound **31** was an agonist at mouse TLR7 (mTLR7) but not at mouse TLR8 (mTLR8). HEK-Blue cells expressing mTLR7 (left) and mTLR8 (right) were treated either with compound **31** or imiquimod as a control. The NF-κB promoter activity measured the next day is expressed as the fold-change relative to each DMSO control.

Here, we examined the effect of compound **31** on mucosal immune stimulation when combined with an inactivated, nasally administered influenza vaccine. Our results show that this compound enhances both proinflammatory cytokines in mouse macrophages *in vitro* and antigen-specific neutralizing antibodies at an optimal dose in mice, finally increasing the protection rate of mice challenged with influenza A virus when compared to the vaccine only group. The experimental results could provide insights into the effects of vaccine formulations containing a 2,4-diaminoquinazoline analogue, as TLR7/8 agonist, with inactivated vaccine or recombinant antigens on prevention of infection with respiratory viruses by intensifying mucosal immunity.

Materials and Methods

Cells and viruses

Human embryonic kidney (HEK) Blue cells expressing null (Null1), human TLR7 (hTLR7), human TLR8 (hTLR8), mouse TLR7 (mTLR7), and mouse TLR8 (mTLR8) were purchased from InvivoGen. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals) and antibiotics, such as blasticidin, Zeocin, and Normocin, according to the manufacturer's instructions. The murine macrophage cell line Raw 264.7 was purchased from the American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 10% FBS. Influenza virus A/Puerto Rico/8/34 (H1N1; PR8) was obtained from ATCC, while mouse-adapted PR8 virus (maPR8) was kindly provided as a gift by Prof. H. Kim (Chung-Ang University). Viruses were propagated in the allantoic cavities of 10-day-old chicken eggs (Jang *et al.*, 2018).

Reagents

Compound **31** (purity, 99.2%) was synthesized in-house and characterized by NMR according to a previous report (Embrechts *et al.*, 2018). Imiquimod (purity, 98%), purchased from Tokyo Chemical Industry, was used as a TLR7-selective agonist control. Resiquimod (purity, 98%), purchased from Sigma-Aldrich, was used as a TLR7/8-dual agonist control.

Cytotoxicity test

HEK-Blue Null1 cells were seeded onto 96-well plates (4×10^4 cells/well). The next day, cells were treated with increasing concentrations of compound **31** or imiquimod. After 24 h, cell viability was measured by treating with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) (Sigma-Aldrich) according to our previous report (Jang *et al.*, 2014).

TLR7 and TLR8 stimulation assay

HEK-Blue cells expressing null, hTLR7, hTLR8, mTLR7, or mTLR8 were seeded onto 96-well plates and cultured overnight as described above. The cells were treated with increasing concentrations of compound **31** and imiquimod (as a control) for 24 h. NF-κB promoter activity was determined by measuring secreted embryonic alkaline phosphate (SEAP) levels in the cell-culture supernatants using QUANTI-Blue (InvivoGen) according to the manufacturer's instructions.

Cytokine analysis

Raw 264.7 cells grown in 12-well plates (5×10^5 cells/well) were treated with 100 μM compound **31** or resiquimod dissolved in culture media. Supernatants were harvested in triplicate 0, 1, 2, 4, 6, 8, 12, and 24 h after treatment. Two-fold dilutions of each sample were subjected to enzyme-linked immunosorbent assays (ELISA) (Cusabio) to measure levels of the proinflammatory cytokines IL-6 and TNF-α. The absolute concentration of cytokines was determined from the corresponding linear standard curves.

Preparation of inactivated influenza A virus vaccine

Inactivated PR8 was used as a vaccine and was prepared at 4°C according to our previous reports (Kim *et al.*, 2013; Lee *et al.*, 2020). In brief, 50 ml PR8 virus (A/H1N1) amplified in embryonated chicken eggs was centrifuged 1,600 × *g* for 10 min, and the supernatants were passed through a 0.22 μm filter. The supernatant was then ultracentrifuged at 26,700 × *g* for 2 h in an SW 32 Ti rotor (Beckman Coulter). The viral pellet was resuspended in 2 ml PBS and inactivated with 0.02% formalin at 4°C overnight. To remove formalin, it was overlaid with a 20% sucrose cushion for a second ultracentrifugation at 165,000 × *g* for 1 h in an SW 60 Ti rotor. The viral pellet was finally dissolved in 1 ml PBS at 4°C overnight. The absence of infectious viral particles was verified by a plaque assay, and the protein concentration was determined using a Bradford assay kit (Bio-Rad).

Mouse immunization and hamagglutination inhibition (HI) test

All animal experiments performed in this study followed guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at KRICT. Six- to seven-week-old female BALB/c mice (Orient Bio) were mock immunized or immunized with inactivated PR8 virus as a vaccine alone (0.5 μg/mouse) or in combination with increasing concentrations of compound **31** (0.2, 1, 2.5, or 5 μg/mouse) given intranasally (n = 9 per group for HI and IgG ELISA; n = 8 per group for IgA ELISA). The final administration volume was equalized to 50 μl. For immunological analysis, each treatment group was boosted with each vaccine formulation at 2 weeks after the prime vaccination. One week later, whole blood was harvested and centrifuged at 1,500 × *g* for 10 min at 4°C to obtain sera. For the HI test, all sera were treated with receptor-destroying enzyme (RDE) according to the WHO protocol to remove nonspecific inhibitors (WHO, 2002). The HI assay was performed according to a previous report (Hierholzer *et al.*, 1969). In brief, chicken red blood cells (RBCs) were prepared in PBS at a concentration of 0.5% (v/v). Two-fold serial dilutions of sera were mixed with an equal volume of 4 HA units of PR8 virus (25 μl each) for 30 min at room temperature in round-bottom, 96-well microtiter plates (SPL Life Sciences). Chicken RBCs were added at a final concentration of 0.25% (v/v) for an additional 30 min.

The HI titer was determined as the reciprocal of the highest fold-dilution of sera needed to completely block HA activity. Samples were tested in duplicate in two independent experiments.

Virus-specific immunoglobulin G (IgG) and IgA titration

The level of virus-specific IgG and IgA antibodies in the immunized mice was measured according to previous reports (Tumpey *et al.*, 2005; Ye *et al.*, 2019). Whole sera were obtained as mentioned above. For analyzing mucosal immunity in the respiratory tract, mouse trachea and nasal cavity tissues were collected in 500 µl PBS and secretory IgA was prepared according to a previous paper (Wolfe *et al.*, 2007). Immunoassay microtiter plates (SPL Life Sciences) were coated with whole influenza virus (3.5×10^4 plaque forming units per well) and blocked with 5% BSA in PBS with 0.05% Tween 20. Sera were diluted to 1:10,000 and 1:10 for mouse IgG and IgA ELISA, respectively. As IgA titers in nasal cavities and tracheae were low, they were analyzed without dilution. Samples were added to the PR8-coated plates (100 µl/well) and incubated for 2 h at room temperature. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (SouthernBiotech) or goat anti-mouse IgA (Invitrogen) was added. 3,3′,5,5′-Tetramethylbenzidine (TMB; Thermo Fisher Scientific) was used as a substrate for HRP. After addition of an equal volume of stop solution (1.6 M H_2SO_4), the optical density at 450 nm was determined.

In vivo protection study

Six- to seven-week-old female BALB/c mice (Orient Bio) were mock immunized or immunized with inactivated PR8 virus as a vaccine (0.5 µg/mouse) alone or in combination with compound **31** (1 µg/mouse) intranasally (n = 8 per group). Three weeks later after the prime immunization, mice were challenged intranasally with maPR8 virus 10 times the 50% mouse lethal dose (MLD_{50}). Five days after the challenge administration, three mice from each group were sacrificed to determine lung viral RNA titration or histopathology. Body weight changes and mortality were measured in the five remaining mice in each group every day for 14 days post-infection. Mice were euthanized when they lost 30% of their starting body weight.

Viral RNA quantitation

Total RNA from mouse lung homogenates (n = 3 per group) was purified using Trizol (Invitrogen) and dissolved in RNase-free water. cDNA was synthesized from 1 µg total RNA using the SuperScript III first-strand synthesis system (Invitrogen) with random hexamers. The titer of influenza viral RNA was determined by quantitative PCR using SYBR Green PCR master mix (Toyobo) and PR8 non-structural protein 2 (NS2) gene-specific primers (forward primer, 5′-CATAATGGA TTCAAACACTGTGTC-3′; reverse primer, 5′-CCTCTTA GGGATTTCTGATCTCGG-3′) (Kim *et al.*, 2013). Primers (forward primer, 5′-TGCACCACCAACTGCTTAGC-3′; reverse primer, 5′-GGCATGGACTGTGGTCATGAG-3′) detecting both human and mouse GAPDH genes (Cicinnati *et al.*, 2008) were used as an internal control to normalize the viral RNA expression levels.

Lung histopathology

Lung samples (n = 3 per group) were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections (3–4 µm thick) were prepared and stained with hematoxylin and eosin dyes (H&E). Images were acquired using a bright-field microscope (BX51TF; Olympus) and analyzed using Nuance software v.3.0.2 (Perkin Elmer).

Statistical analysis

All experiments were performed in triplicate, and data are expressed as mean ± standard deviation. Ordinary one-way ANOVA followed by Dunnett's multiple comparisons and two-way ANOVA followed by Sidak's multiple comparisons were performed using GraphPad Prism v.8.4.2 (GraphPad Software).

Results

Agonistic activity of compound 31 on TLR7 and TLR8 stimulation

Before investigating the vaccine adjuvant efficacy of compound **31**, we tested whether the in-house synthesized product could stimulate both TLR7 and TLR8 *in vitro*, as previously reported (Embrechts *et al.*, 2018). The cytotoxicity of compound **31** was compared with that of imiquimod in HEK-Blue Null1 cells (Fig. 1A and B; Table 1). The MTT assay showed that compound **31** hardly affected cell viability (93.8%) even at the maximum concentration (100 µM), whereas imiquimod reduced cell viability to 83.1% and 72.5% at 33 µM and 100 µM, respectively. This result indicated that imiquimod produces partial cytotoxicity in the HEK-Blue-based reporter system above 33 µM. To assess the agonistic effects of compound **31** and imiquimod on hTLR7 and hTLR8, HEK-Blue hTLR7 and hTLR8 cells were treated individually with each compound (Fig. 1C). As expected, the SEAP levels as a

Table 1. **The TLR7 and TLR8 agonistic activity of compound 31**

Compound	Cell viability (%)[a]	EC_{50} (µM)[b]				Maximal response (fold)[c]			
		hTLR7	hTLR8	mTLR7	mTLR8	hTLR7	hTLR8	mTLR7	mTLR8
31	93.8 ± 3.5	0.6 ± 1.0	4.2 ± 1.0	0.6 ± 1.1	n.d.[d]	15.3 ± 0.1	15.9 ± 0.1	8.0 ± 0.1	1.2 ± 0.0
Imiquimod	72.5 ± 3.0	15.4 ± 1.1	n.d.	6.2 ± 1.2	n.d.	6.6 ± 0.1	1.2 ± 0.7	5.1 ± 0.2	1.2 ± 0.0

[a]The cell viability of HEK-Blue Null1 cells in response to 100 µM of each compound was measured by an MTT assay;
[b]Half-maximal effective concentration from triplicate samples;
[c]Maximal fold-induction relative to the mock control;
[d]Not determined due to a lack of concentration-response or no observed activity.
The values present mean ± standard deviation from three independent experiments.

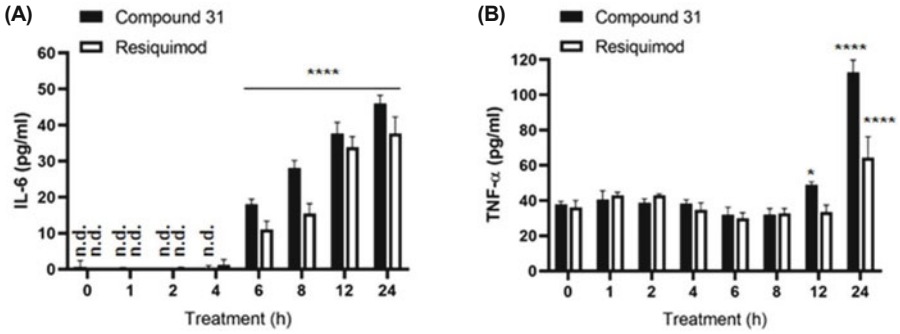

Fig. 2. Induction of proinflammatory cytokines by compound 31 in mouse macrophages. Raw 264.7 cells were treated with 100 μM compound **31** (black bars) or resiquimod (white bars). Culture supernatants were harvested for use in an ELISA assay specific for mouse IL-6 (A) and mouse TNF-α (B) at the indicated time points. Statistical analyses were performed by two-way ANOVA followed by Sidak's test. Significances refer to each '0 h' control. $^{*}P < 0.05$; $^{****}P < 0.0001$. n.d., not detected.

marker of NF-κB promoter activity verified that compound **31** is a dual TLR7/8 agonist while imiquimod is a TLR7-selective agonist. Neither compound altered SEAP levels in HEK-Blue Null1 cells. Quantitatively, compound **31** had EC_{50} values of 0.6 μM for hTLR7 and 4.2 μM for hTLR8, and maximal responses that were 15.3- and 15.9-fold higher, respectively, than the mock-treated controls (Table 1). Imiquimod selectively stimulated hTLR7 with an EC_{50} value of 15.4 μM and a maximal response 6.6-fold higher than control. As a preliminary test for potential *in vivo* vaccine adjuvant efficacy in mice, the ability of compound **31** to induce NF-κB expression was measured in HEK-Blue cells expressing mTLR7 or mTLR8 (Fig. 1D and Table 1). Compound **31** activated mTLR7 (EC_{50} value, 0.6 μM; maximal response, 8.0-fold higher than control) but not mTLR8; in terms of biased mTLR7 activation, this result is similar to results produced by imiquimod (EC_{50} value for mTLR7, 6.2 μM; maximal response, 5.1-fold higher than control). However, the lack of mTLR8 agonist activity of compound **31** might be due to intrinsic null sensing of mTLR8 rather than the selectivity of compound **31** for mTLR7 (Heil *et al.*, 2004). Together, these results suggest that highly purified compound **31** (purity, 99.2%) is biologically active and suitable for further immunological experiments.

Induction of proinflammatory cytokines by compound 31 in mouse macrophages

Several studies of vaccine adjuvants show that proinflammatory cytokines induced by TLRs play a key role in regulating cellular and humoral immune responses (McGowan *et al.*, 2016; Gutjahr *et al.*, 2020). We wondered if the TLR7/8 agonist activity of compound **31** observed in the NF-κB-as-

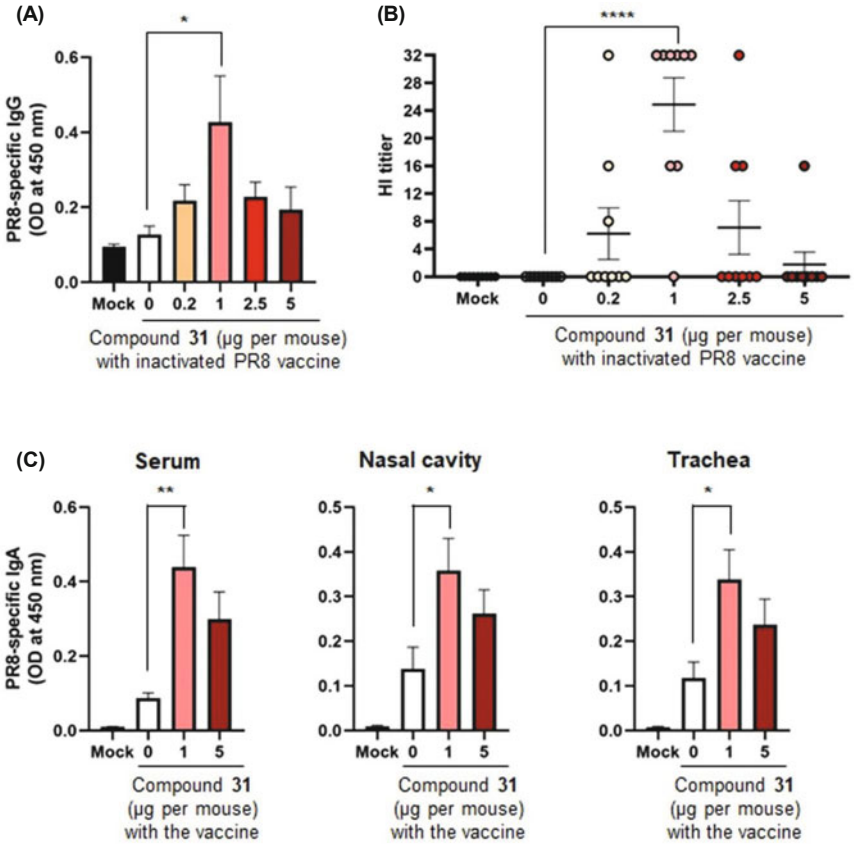

Fig. 3. Production of serological and mucosal antibodies against influenza virus. Mice were mock immunized or immunized either with inactivated vaccine alone or with compound **31**-adjuvanted vaccine, in which the amount of compound **31** ranged from 0.2 to 5 μg/mouse. Prime and second immunizations were given, and 1 week after the booster shot, the whole sera of nine mice from each group was harvested for the measurement of IgG levels specific for PR8 virus (A) and used in an HA inhibition assay using 4 HA units of virus and chicken RBCs (B). In a separate experimental set, mice were intranasally immunized with vaccine alone or with compound **31** (with eight mice per group) twice at a two-week interval. One week after the booster vaccination, whole sera, nasal cavities and tracheae were collected for PR8-specific IgA ELISA (C). Values are expressed as mean ± SEM. Significances refer to the vaccine-only control. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$.

sociated reporter system are sufficient to enhance the expression of endogenous proinflammatory cytokines in immune cells. For these *in vitro* experiments, we used mouse macrophages, Raw 264.7 cells, as they match the immunogenic background of the *in vivo* mouse vaccination model that we aimed to use later in this study. The imidazoquinoline derivative resiquimod, which is a TLR7/8 dual agonist, was used as a control. Based on our earlier data showing that mTLR8 was silent (Fig. 1D), it was expected that only mTLR7 can sense dual agonists in mouse immune cells. Raw 264.7 cells were treated with an equal concentration (100 μM) of compound **31** and resiquimod, and IL-6 and TNF-α levels were measured by ELISA at 0, 1, 2, 4, 6, 8, 12, and 24 h after treatment (Fig. 2). Compound **31** and resiquimod significantly increased the absolute concentration of IL-6 from 6 h after treatment in a time-dependent manner (Fig. 2A). The concentration of IL-6 ranged from 18.1 to 46 pg/ml in the presence of compound **31**, and from 11.1 to 37.7 pg/ml in the presence of resiquimod. By contrast, significant amounts of TNF-α were detected at later points, *i.e.*, 12 and 24 h after treatment with compound **31**, and 24 h after treatment with resiquimod (Fig. 2B). Comparative analysis at 24 h showed that the amount of TNF-α was higher in the compound **31**-treated culture supernatant (112.8 pg/ml) than in the resiquimod-treated samples (64.3 pg/ml). Together, the results indicated that by activating the mTLR7 signaling pathway, compound **31** induces the expression of endogenous proinflammatory cytokines, such as IL-6 and TNF-α, in immune cells.

Enhancement of serological and mucosal antibody responses by compound 31 in mice

We examined whether activation of the innate immune response by intranasal administration of compound **31** could lead to humoral immunity to a vaccine in mice, and what dose of compound **31** is optimal to produce antigen-specific neutralizing antibodies. Mice were immunized with inactivated, whole PR8 virus vaccine alone (0.5 μg/mouse) or in combination with compound **31** at a dose either of 0.2. 1, 2.5, or 5 μg/mouse (9 mice/group) twice at a two-week interval. On day 7 from the second immunization, sera were harvested to determine whether vaccine-specific IgG was induced. Immunoassay results showed that compound **31** induced mouse IgG specific for PR8 virus; 1 μg compound **31** per mouse produced the highest vaccine-specific IgG level (Fig. 3A).

We further tested the HI assay using the same sera and chicken RBCs to determine whether the generated antibodies can neutralize virus infection. Consistent with the results of IgG measurement, prime/boost immunization of vaccine alone (0.5 μg/mouse) was not sufficient to generate antibodies that blocked HA-mediated blood agglutination; the results obtained were similar to those seen with the mock control (Fig. 3B). By contrast, all vaccinations combined with compound **31** improved HI titers. HI titers ranged from 8 to 32, but there were differences in sensitivity between the treatment groups. Quantitatively, after vaccination with inactivated PR8 virus and 0.2 μg compound **31**, three mice had neutralizing antibodies with HI titers of 18.7 ± 12.2; eight mice had HI titers of 28.0 ± 7.4 in the 1 μg compound **31** group; three mice had HI titers of 21.3 ± 9.2 in the 2.5 μg compound **31** group; and only one had a HI titer of 16 HI in the 5 μg compound **31** group (n = 9 each group). This result suggests that 1 μg per mouse of compound **31** is the optimal dose for use as a vaccine adjuvant to enrich antibody production with statistical significance.

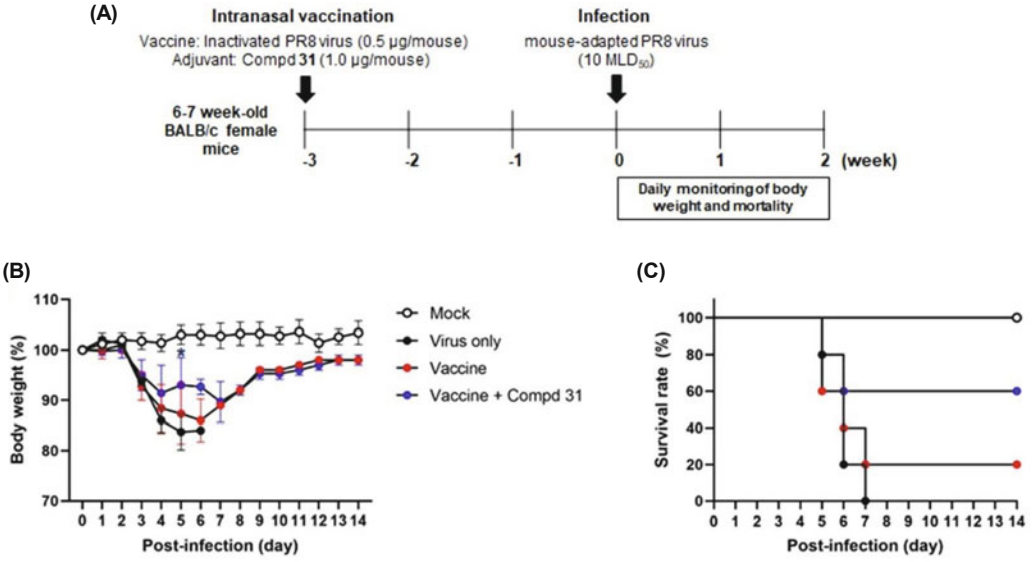

Fig. 4. The prophylactic activity of compound 31-adjuvanted vaccine in an influenza A virus-challenge model. (A) A schematic presentation of the *in vivo* challenge protocol. BALB/c mice were immunized with PBS or vaccine (inactivated PR8 virus; 0.5 μg/mouse) alone or in combination with compound **31** (Compd **31**; 1 μg/mouse) given intranasally. Three weeks after the immunization, mice were challenged by infection with maPR8 at 10× MLD_{50}. Body weight changes (B) and survival rates (C) were recorded every day after the virus challenge for 14 days. The percent values are expressed as mean ± standard deviation. Statistical analyses of body weight changes on day 5 were performed by unpaired t-test. Significance refers to the 'virus only' group. *$P < 0.05$. Mock, unvaccinated non-infected mice.

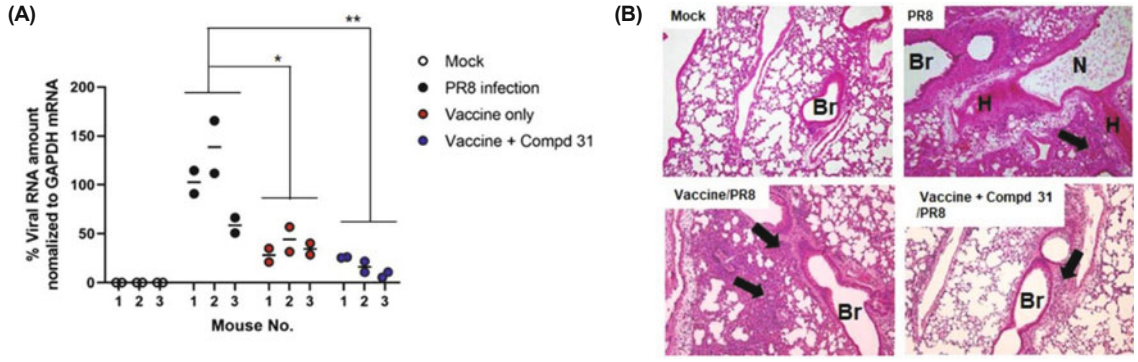

Fig. 5. Analysis of viral RNA expression in lung and histopathology. (A) Quantitative RT-PCR was used to quantify viral RNA copies in lung. BALB/c mice were immunized with PBS or vaccine (inactivated PR8 virus; 0.5 μg/mouse) alone or in combination with compound **31** (Compd **31**; 1 μg/mouse) given intranasally. Three weeks after the immunization, mice were challenged by infection with maPR8 at 10 × MLD_{50}. At day 5 post-infection, mice were sacrificed for total RNA preparation. cDNA was subjected to quantitative RT-PCR with influenza A virus NS2 gene-specific primers. The relative viral RNA copies are expressed as percentages after normalization to mouse GAPDH mRNA. Statistical analyses were performed by ordinary one-way ANOVA with the Tukey's multiple comparisons test. Significances refer to the unvaccinated, PR8 virus-infected group. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Histopathology images acquired by H&E staining of lung sections from representative mice. Samples are labeled on the top left corner of each image. Br, bronchiole; H, hemorrhage; N, necrosis. Black arrows indicate acute inflammation. Original magnification, ×100.

It was wondered whether this chemical can enhance mucosal immunity in the respiratory tract, antigen-specific IgA levels were measured in the nasal cavity and trachea as well as in the serum after second immunization of vaccine alone or in the presence of compound **31** (1 or 5 μg/mouse) (n = 8 each group). In all measurements, highest amounts of IgA were detected when the vaccine (0.5 μg/mouse) was intranasally administered together with 1 μg compound **31** rather than vaccine alone or with the compound at a higher dose (5 μg) (Fig. 3C). The results clearly exhibited that compound **31** is able to enhance both mucosal and serological antibody responses.

Improved intranasal vaccine efficacy by compound 31 in a virus-challenge model

To verify if the immune response elicited by compound **31** as shown in Fig. 3 could protect mice from influenza virus infection, the prophylactic effects of vaccine alone with a minimal dose (0.5 μg) and vaccine in combination with the optimized amount of compound **31** (1 μg) were tested in a virus-challenge model. Immunocompetent mice were intranasally administered PBS, inactivated PR8 virus alone, or PR8 together with compound **31**. Three weeks later, mice were infected with 10× MLD_{50} of its mouse-adapted strain, maPR8. Body weight changes and mortality were recorded every day for 2 weeks (Fig. 4A). The results showed that body weight was drastically lower in maPR8-challenged mice than in mock-infected mice; all maPR8-challenged mice had died by day 7 (Fig. 4B and C). The inactivated vaccine, which produced little or marginal humoral and mucosal immune responses (Fig. 3), failed to alleviate infection-mediated body weight loss except one of five (20%), which was survived (Fig. 4B and C). Importantly, co-administration of the vaccine and compound **31** significantly attenuated the infection-mediated decrease in body weight at day 5, when 60% of mice in all groups were alive, enabling statistical analysis (Fig. 4B). In addition, this combination protected 60% of virus-infected mice from death (Fig. 4C). The *in vivo* challenge study suggested that compound **31** when administered together with a vaccine has adjuvant efficacy by stimulating mTLR7-driven protective immunity.

Reduction of viral RNA titers in lungs and histopathology analysis

Viral titers in lungs and lung histopathology are crucial parameters to predict infection-associated lung disease, such as severe pneumonia and acute respiratory distress syndrome. We measured viral RNA levels from lung samples. At day 5 after infection, three mice from the mock-immunized and vaccine-immunized groups were sacrificed, and lungs were harvested for RNA preparation in parallel with histopathology analysis. Quantitative RT-PCR data showed that copies of influenza A virus NS2 genome-specific RNA were reduced by 64.4% ($P < 0.05$) in the vaccine-only group and by 83.4% ($P < 0.01$) in the vaccine plus compound **31**-treated group than in the unvaccinated control group (Fig. 5A). We assessed lung histopathology by H&E staining in the same lung samples (Fig. 5B). Microscopic images visualized normal lung sections in mock-treated mice with well-shaped alveoli surrounding bronchi (Fig. 5B, upper left panel). However, alveoli were damaged overall in virus-infected lung sections, and severe cellular inflammation, necrosis, and hemorrhage were observed (Fig. 5B, upper right panel). It was notable that this acute lung damage caused by influenza A virus infection was attenuated by intranasal immunization either with vaccine or with vaccine plus adjuvant (Fig. 5B, lower panels). When compared to the non-adjuvanted vaccine, the lung histology images from the adjuvanted vaccine were more similar to that observed in mock-treated mice. Importantly, in spite of a concern that the compound **31** could cause mTLR7-dependent systemic or local inflammation *in vivo*, its amount used as an adjuvant (1 μg/mouse) did not induce lung toxicity. Together, these results described that the optimized dose of compound **31** (1 μg/mouse) enhances the protective efficacy of a less immunogenic, single-dose vaccine by reducing infection-mediated pulmonary pathogenesis (Figs. 4 and 5).

Discussion

Live-attenuated vaccines are regarded to elicit more robust immunogenicity than inactivated or recombinant vaccines, because those acquire more potent innate immune responses through activation of PRRs during virus entry or virus genome replication. The yellow fever virus vaccine is an example of a clinically successful live-attenuated vaccine. Its immunization activates both conventional dendritic cell (DC) and plasmacytoid DC subsets by stimulating broad-spectrum PRRs, such as TLR2, TLR7, TLR8, and TLR9, and their downstream immune modulatory cytokines, such as IL-12, IL-6, and IFN-α (Querec *et al.*, 2006; Watson *et al.*, 2016; Bovay *et al.*, 2021). In addition, live-attenuated intranasal vaccines against influenza virus or yellow fever virus provide long-term and robust mucosal immunity with increased expression of T-helper type 1 (Th1) intracellular cytokines (Lanthier *et al.*, 2011; Mohn *et al.*, 2015). Despite high protective efficacy, safety issues associated with the potential for live-attenuated vaccines to mutate back to wild-type or pathogenic virus and to cause severe immune responses exist. For these reasons, live-attenuated vaccines are not recommended for infants, pregnant women, or immunocompromised people unless unavoidable, such as travel to an endemic area (de Menezes Martins *et al.*, 2015; Gemmill *et al.*, 2016).

As an approach to improving the protective efficacy of relatively safe, inactivated and recombinant vaccines in a controllable manner but to securing broad cross-protectivity and extended longevity, various adjuvant modalities have been developed. For instance, rabies and influenza vaccines have been formulated with single-stranded RNA, which is a natural TLR7/8 ligand (Luo *et al.*, 2019; Kim *et al.*, 2020). HBV and HPV vaccines have been combined with monophosphoryl lipid A, which is a synthetic TLR4 activator (Giannini *et al.*, 2006; Moon *et al.*, 2015). Along with development of small molecular TLR7 or TLR8 agonists instead of polymeric, unstable ssRNA, several researchers examined the feasibility of imiquimod and its derivatives as vaccine adjuvants targeting HBV, HIV, or norovirus in mice (Otero *et al.*, 2004; Thomsen *et al.*, 2004; Wille-Reece *et al.*, 2005; Du *et al.*, 2010; Velasquez *et al.*, 2010). Nevertheless, the efficacy of imiquimod and its derivatives was limitedly proved in an influenza A virus (H3N2) challenge model, in which a lipidated imidazoquinoline compound enhanced the efficacy of an intramuscular vaccine (Miller *et al.*, 2020), or in a HIV-1 p24-expressing vaccinia virus challenge model, in which a TLR7/NOD2 dual agonist increased the efficacy of an intranasally administered HIV-1 p24 vaccine (Gutjahr *et al.*, 2020). Moreover, the reliability and dose-optimization of TLR7/8 agonists have not yet been fully investigated.

Here, we explored whether a recently identified small molecule TLR7/8 agonist, compound **31**, is safe and has sufficient immunogenicity to enhance the protective efficacy of an inactivated influenza A virus vaccine. Intranasal, single-dose administration of vaccine in the presence of compound **31** induced adaptive humoral immune responses in mice by increasing levels of antigen-specific neutralizing antibody (Fig. 3). Intriguingly, the optimal dose of compound **31** was 1 μg per mouse when used with 0.5 μg of an inactivated vaccine. On the basis of the Th1/Th2 paradigm, Th1 cells dominate cell-mediated immunity, whereas Th2 cells potentiate humoral immunity (Netea *et al.*, 2005). It has been reported that several TLR7/8 agonists, when used as a vaccine adjuvant, could prompt Th1-polarized immune responses *in vivo* (Wille-Reece *et al.*, 2005; Miller *et al.*, 2020). Considering these findings, we assume that compound **31** could support Th1/Th2 balance to elicit robust adaptive immune response at an optimized concentration, but its higher concentrations might lead to Th1-biased proinflammatory cytokines, possibly skewing antigen-specific immune responses by repressing Th2 responses (Morrow *et al.*, 2010). Thus, dose-optimization of a TLR7/8 agonist, with variable factors such as the antigen amount or its immunogenicity as well as the vaccination route and frequency, seems to be necessary before adopting it as a vaccine adjuvant.

We proved that compound **31** enhances the efficacy of an influenza A virus vaccine in a mouse challenge model (Fig. 4). The results showed that the combination reduced body weight loss compared with the vaccine only, and also increased the survival rate from 20% to 60%. Notably, a minimum amount of inactivated vaccine (0.5 μg/mouse) was used in our infection model to reflect the poor immunogenic conditions that can be caused by antigen loss during intranasal delivery or by the circulation of antigenically mismatched vaccine strains. In the present study, vaccine plus compound **31** failed to fully protect against death. This insufficient effect might result from the inability of compound **31** to stimulate mTLR8 in the mouse system (Fig. 1 and Table 1). To overcome the limitation, we are genetically engineering BALB/c mice to stably express hTLR8 under a mouse macrophage-specific synthetic promoter (Tang *et al.*, 2018). It is expected that compound **31** will have improved adjuvant efficacy in these transgenic mice by triggering both mTLR7- and hTLR8-mediated mucosal immunity.

To the best of our knowledge, this is a first report suggesting that a 2,4-diaminoquinazoline compound containing a chemical core structure that is different from well-investigated imidazoquinoline derivatives, such as imiquimod and resiquimod, has immunogenic potential as a viral vaccine adjuvant. Our study could provide insights useful for the development of novel intranasal vaccine adjuvants that are based on the structure of TLR7/8 dual agonists and protect against diverse respiratory viral infections, such as influenza viruses and coronaviruses.

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Conflict of Interest

The authors have no conflict of interest to report.

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