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A novel C-3-substituted oleanolic acid benzyl amide derivative exhibits therapeutic potential against influenza A by targeting PA–PB1 interactions and modulating host macrophage inflammation



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Abstract The influenza A virus (IAV), renowned for its high contagiousness and potential to catalyze global pandemics, poses significant challenges due to the emergence of drug-resistant strains. Given the critical role of RNA polymerase in IAV replication, it stands out as a promising target for anti-IAV therapies. In this study, we identified a novel C-3-substituted oleanolic acid benzyl amide derivative, **A5**, as a potent inhibitor of the PA_C–PB1_N polymerase subunit interaction, with an IC₅₀ value of 0.96 ± 0.21 μmol/L. **A5** specifically targets the highly conserved PA_C domain and demonstrates

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Pentacyclic triterpenoids;
Oleanolic acid amide
derivatives;
Drug-resistant strains;
Inflammation;
Toll-like receptor 4

remarkable efficacy against both laboratory-adapted and clinically isolated IAV strains, including multidrug-resistant strains, with EC₅₀ values ranging from 0.60 to 1.83 $\mu\text{mol/L}$. Notably, when combined with oseltamivir, **A5** exhibits synergistic effects both *in vitro* and *in vivo*. In a murine model, dose-dependent administration of **A5** leads to a significant reduction in IAV titers, resulting in a high survival rate among treated mice. Additionally, **A5** treatment inhibits virus-induced Toll-like receptor 4 activation, attenuates cytokine responses, and protects against IAV-induced inflammatory responses in macrophages. In summary, **A5** emerges as a novel inhibitor with high efficiency and broad-spectrum anti-influenza activity.

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1. Introduction

Influenza A virus (IAV) causes annual epidemics and occasional pandemics, leading to significant global mortality and substantial economic losses¹. IAV-induced pulmonary inflammation aggravates the symptoms of influenza and induces complications^{2,3}. The World Health Organization (WHO) estimates that seasonal influenza outbreaks result in approximately 1 billion symptomatic infections globally each year, leading to 3 to 5 million cases of severe illness and causing 290,000 to 650,000 respiratory-related deaths annually⁴. Children and the elderly are recognized as vulnerable and high-risk populations for influenza virus infection⁵. Additionally, the rise in obstetric complications and morbidity among pregnant women with influenza has garnered significant attention⁶. More critically, the interaction between different respiratory viruses may exacerbate symptom transmission⁷, underscoring the urgent need for timely and effective containment of influenza viruses.

Medications targeting influenza are crucial for the prevention and treatment of influenza A infections. These drugs include neuraminidase (NA) inhibitors such as oseltamivir, RNA polymerase inhibitors like baloxavir, and M2 ion channel blockers such as amantadine and rimantadine^{8,9}. Nevertheless, the significantly diminished efficacy of oseltamivir against the influenza virus due to critical amino acid mutations in NA underscores the pressing concern of drug resistance¹⁰. The prevalent M2-S31N mutation has rendered amantadine and rimantadine no longer advisable¹¹, while the PA-I38T mutation poses a challenge for the use of baloxavir¹². The emergence of drug-resistant viruses, which are evolving with enhanced transmission capabilities, underscores the urgent need for innovative antivirals that possess a novel mechanism of action and a strong resistance barrier.

The IAV genome comprises eight RNA gene segments upon entering the host cell. Among these segments, the initial three genes encode the RNA-dependent RNA polymerase (RdRp), which controls the virus's RNA synthesis, and include polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA)¹³. PA forms a heterodimer with PB1 in the cytoplasm, while PB2 dissociates from its import factors. Subsequently, the PA–PB1 heterodimer and PB2 shuttle to the nucleus, where they assemble to form a heterotrimer¹⁴, which makes influenza RdRP itself particularly suitable for inhibitors of protein–protein interaction. A novel strategy for developing antiviral agents focuses on inhibiting the functions of RdRp by disrupting protein–protein interactions between its subunits. Such inhibitors hold promise for broad-spectrum

efficacy against influenza viruses and may provide a robust defense against drug resistance, thereby addressing key challenges associated with current treatments¹⁵. X-ray crystal structures reveal a significant interaction between the N-terminal tail of PB1 (PB1_N) and the C-terminal domain of PA (PA_C)¹⁶. The N-terminus of PB1 protein interacts with the C-terminal hydrophobic core N412, Q670, and W706 of PA to establish hydrophobic interactions, and PA-E623 has been reported to form a firm hydrogen bond with the N-terminus of PB1 protein¹⁷. The relatively small hydrophobic interface at the C-terminus of PA proteins with a small number of but highly conserved residues means that it is likely to be suitable for small molecularly mediated inhibition. The distinctive concave structure of the PB1_N-binding site in PA_C offers a promising focal point for strategic drug development efforts¹⁸.

Oleanolic acid, chemically identified as 3 β -hydroxyolean-12-en-28-oic acid, is a pentacyclic triterpene that is abundantly present in foods, medicinal plants, and nutritional supplements. It has demonstrated a range of antiviral activities against infections caused by influenza virus (IFV), human immunodeficiency virus (HIV), and hepatitis C virus¹⁹. Notably, oleanolic acid and its derivatives have been shown to bind to the hemagglutinin (HA) protein of the influenza virus, thereby inhibiting viral entry into host cells²⁰. Our recent research has identified a novel class of novel 3-*O*- β -chacotriosyl pentacyclic triterpene amide derivatives as potential H5N1 entry inhibitors²¹, exemplified by the oleanolic acid saponins **T-1** and **T-2** (Fig. 1), possessing efficient anti-H5N1 pseudo particles potency and excellent target specificity. However, **T-1** and **T-2** exhibited only moderate anti-H1N1 activity in the cell-based assay and had a low selectivity index, likely attributable to the presence of a double-branched trisaccharide moiety at the C-3 position of oleanolic acid, specifically chacotrioside. This suggests that further optimization of their pharmacophore structures is necessary.

In this study, we designed and synthesized a series of novel C-3-substituted oleanolic acid amide derivatives (**A1**–**A12**) using a scaffold hopping strategy. Notably, these new derivatives not only exhibited remarkable anti-IAV activity but also demonstrated alternative mechanisms of action compared to existing anti-influenza medications. Among these compounds, **A5** showed the most potent anti-IAV activity in both *in vitro* and *in vivo* assays. Mechanistic studies revealed that **A5** effectively inhibited the PA–PB1 interaction and displayed significant anti-inflammatory effects, thereby exhibiting broad-spectrum anti-IAV activities. This strategic approach aims to address the challenge posed by influenza strains that have developed resistance to conventional treatments.

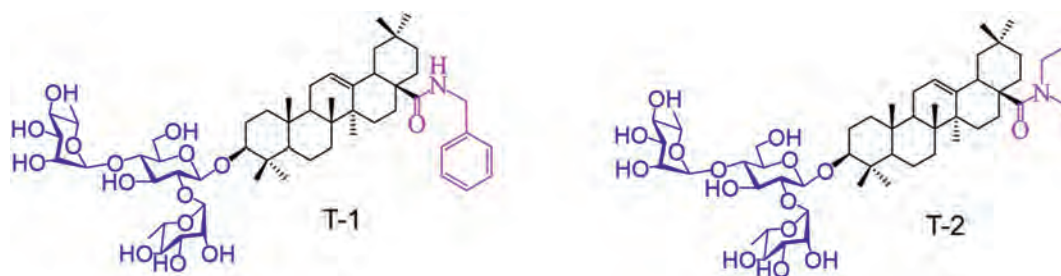


Figure 1 The chemical structures of hit compounds **T-1** and **T-2**.

2. Results

2.1. Rational design of novel oleanolic acid benzyl amide derivatives **A1–A12** based on a scaffold hopping strategy

Structurally, the top hit **T-1** comprises a hydrophobic oleanolic acid benzyl amide aglycone and a hydrophilic chacotrioside residue, which is a double-branched trisaccharide moiety. Given that the oleanolic acid benzyl aglycone has been identified as a privileged scaffold in our previous studies, we focused on derivatization by replacing the chacotrioside residue while preserving the core structure of the oleanolic acid benzyl amide. As illustrated in Fig. 2, inspired by the L-hydroxyproline skeleton, which contains three functional groups (carboxyl, hydroxyl, and secondary amine) within a five-membered ring, we hypothesized that substituting the stereochemically complex chacotrioside sugar with simpler, commercially available L-hydroxyproline-based double-branched chains could facilitate scaffold hopping from the trisaccharide moiety to a 2,4-disubstituted L-hydroxyproline-based pharmacophore. This approach may lead to novel discoveries. Consequently, a series of C-3-substituted oleanolic acid benzyl amide derivatives (**A1–A12**), incorporating sugar surrogates at the C-3 position of oleanolic acid, were designed and synthesized with the aim of developing more efficacious anti-IAV inhibitors.

2.2. Synthesis, biological evaluation, and preliminary structure–activity relationships (SARs) of oleanolic acid derivatives **A1–A12**

Starting from commercially available *trans*-N-Boc-4-*tert*-butyldimethylsilyloxy-L-proline, the target oleanolic acid derivatives **A1–A12** were synthesized *via* a concise and well-established route as outlined in Scheme 1. Initially, the ester coupling reaction between *trans*-N-Boc-4-*tert*-butyldimethylsilyloxy-L-proline and the known oleanolic acid benzyl amide **1**, catalyzed by EDC·HCl and DMAP, yielded compound **2**. Compound

2 was then hydrolyzed to produce intermediate **3**. Subsequently, condensation of various organic carboxylic acids with intermediate **3**, following similar procedures used for compound **2**, generated intermediates **4–11**. After deprotection of the Boc group using trifluoroacetic acid in CH₂Cl₂, an amidation reaction with 3,4-*bis*-(benzyloxy)benzoic acid, facilitated by EDCI, HOAt, and NMM, produced compound **12**. Removal of the TBDMS protecting group from compound **12** followed by hydrogenolysis over palladium/carbon in MeOH-THF afforded the final product **A1**. Similarly, compound **13** was synthesized from intermediate **5** through TBDMS deprotection and reduction, leading to the formation of **A2**. For compounds **A3–A5**, after removal of the Boc group from intermediates **5–7** using trifluoroacetic acid in CH₂Cl₂, direct amide coupling with appropriate carboxylic acids in the presence of EDCI, HOAt, and NMM yielded intermediates **14** and **15**. Hydrogenolysis of these intermediates over palladium/carbon in MeOH-THF produced the final products **A3–A5**. Finally, compounds **A6** and **A8–A12** were synthesized by removing the Boc group from their respective intermediates and subsequent condensation with different organic carboxylic acids, following the procedure used for compound **13**.

All the title compounds **A1–A12** and two reference compounds **T-1** and **T-2** were evaluated for their inhibitory effects against the A/WSN/1933 virus in infected MDCK cells using a cytopathic effect assay. As shown in Supporting Information Table S1, most of the derivatives exhibited good to excellent inhibitory activities (EC₅₀ range: 0.40–12.47 μmol/L), demonstrating significantly enhanced potency compared to the reference compounds **T-1** and **T-2**. This supports our scaffold hopping strategy, where the incorporation of L-hydroxyproline residues with double-branched chains as sugar replacements played a crucial role. Notably, derivative **A5** stood out for its potent antiviral activity against the A/WSN/1933 virus, achieving an EC₅₀ value of 0.76 μmol/L and the highest selective index (SI = 146) among this set.

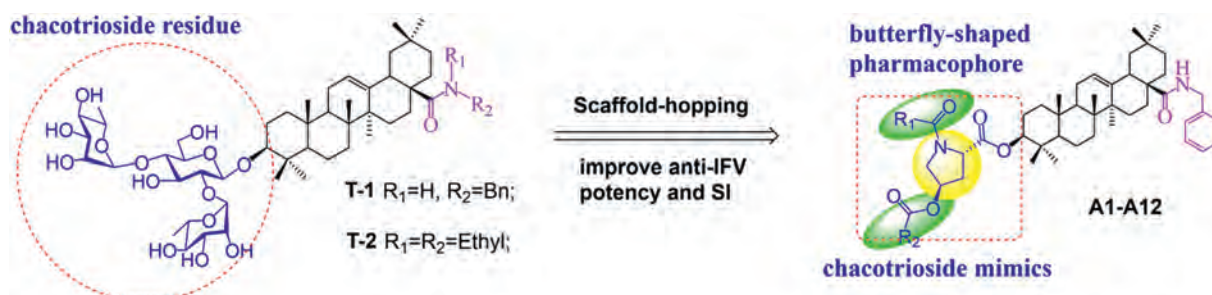
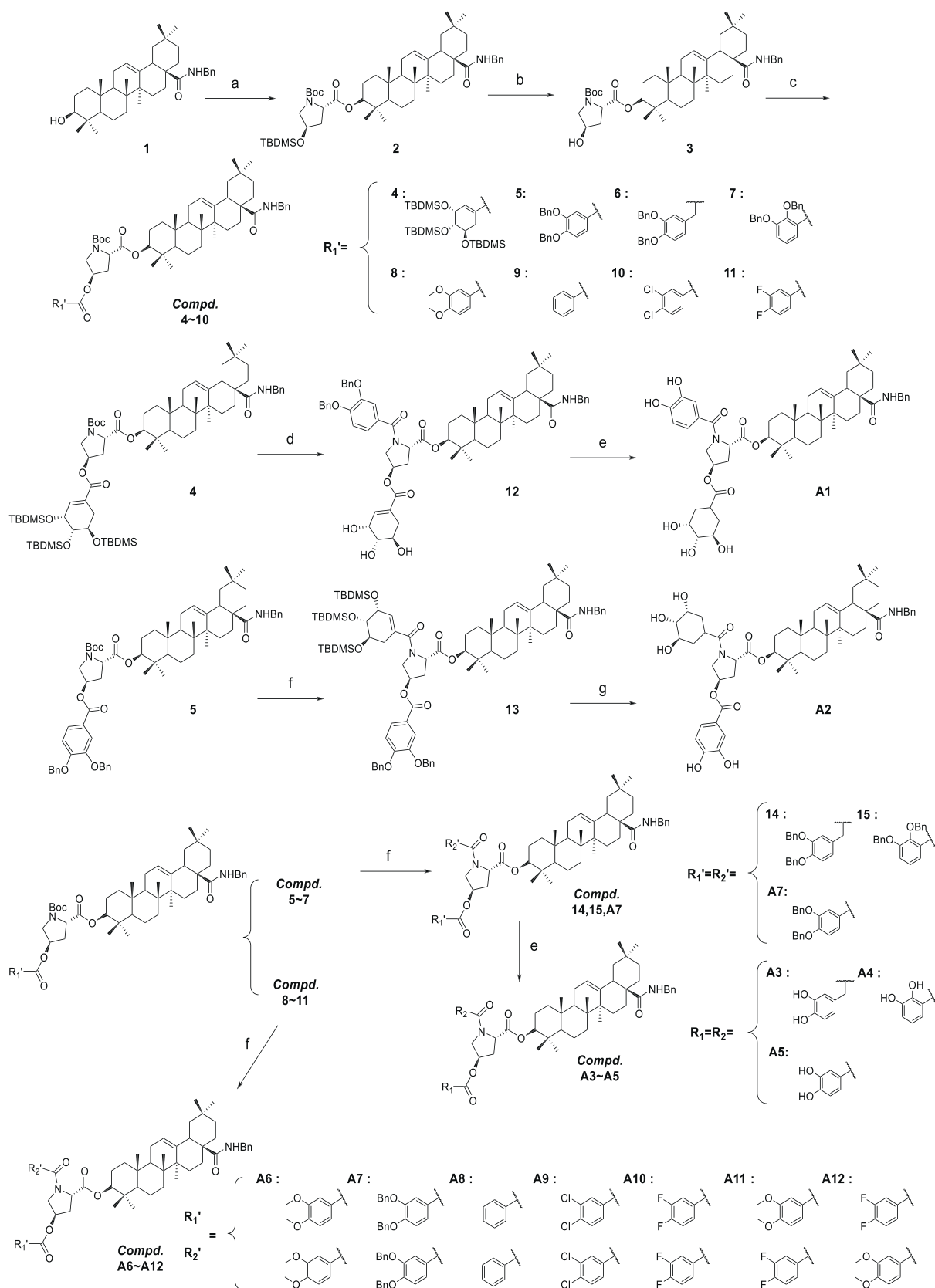


Figure 2 Design of title compounds **A1–A12** based on scaffold hopping strategy.



Scheme 1 Reagents and conditions: a. EDC·HCl, DMAP, DCM; b. TBAF·3H₂O, THF; c. EDC·HCl, DMAP, DCM, different acid; d. (i). CF₃COOH, DCM; (ii). EDC·HCl, HOAt, NMM, DCM, 3,4-bis(benzyloxy)benzoic acid; (iii). TBAF·3H₂O, THF; e. 10% Pd/C, H₂, MeOH-THF; f. (i). CF₃COOH, DCM; (ii). EDC·HCl, HOAt, NMM, DCM, different acid; g. (i). TBAF·3H₂O, THF; (ii). 10% Pd/C, H₂, MeOH-THF.

Title compounds **A1** and **A2**, featuring a hexacyclic fatty ring with multiple hydroxyl groups attached to C-4-OH or NH in an L-hydroxyproline ring, respectively, exhibited potent inhibitory effects against the A/WSN/1933 virus. However, their selective index was limited due to significant cytotoxicity. Compound **A3**, containing two phenolic hydroxyl groups on aralkyl rings, showed appreciable cytotoxicity ($CC_{50} > 100 \mu\text{mol/L}$) but lacked measurable anti-H1N1 activity. In contrast, compounds **A4** and **A5**, which incorporated two phenolic hydroxyl groups substituted benzoyl moieties at the 2-position and C-4-OH of the L-hydroxyproline residue, regained substantial antiviral activity while maintaining safety. This improvement can be attributed to a more balanced profile between inhibitory potency and cytotoxicity against MDCK cells. These findings suggest that free rotation around the methylene unit in the ester or amide linker between double-branched aromatic chains and the L-hydroxyproline ring is detrimental to achieving a favorable conformation. Compounds **A4** and **A5**, being conformationally rigid, lack this free rotation compared to **A3**. Notably, **A4** and **A5**, which contain catechol fragments as double-branched aromatic chains, demonstrated excellent antiviral activities at submicromolar levels and high selective indices, highlighting the critical role of catechol fragments in ligand–receptor interactions. Subsequent modifications to the catechol moiety in **A5** were conducted to evaluate the impact of different substituents on anti-IAV activity. The evaluation of analogs **A6–A8** revealed that removing the phenolic hydroxyl group (**A8**) resulted in a loss of antiviral activity against A/WSN/1933, while alkoxy-substituted derivatives **A6** and **A7** exhibited 5.3- and 20.2-fold reduced inhibitory potencies, respectively.

2.3. Anti-influenza A virus activity of oleanolic acid derivatives *in vitro* and *in vivo*

The *in vitro* anti-IAV activities of oleanolic acid derivatives were evaluated using a cytopathic effect-based cell culture model. The EC_{50} and CC_{50} values are summarized in Table S1, where compound **A5** exhibited the highest safety index. Specifically, **A5** demonstrated low cytotoxicity, suggesting that its potent antiviral activity is not attributable to cytotoxic effects. Subsequently, cytopathic effect protection assays were performed to evaluate the inhibitory effects of **A5** against H1N1, H3N2, and type B influenza viruses in MDCK cells. As shown in Table 1, all tested strains were sensitive to **A5**, with EC_{50} values ranging from 0.60 to $1.83 \mu\text{mol/L}$. These results indicate that **A5** has broad-spectrum anti-influenza properties.

The plaque reduction assay demonstrated that compound **A5** inhibited the release of progeny virions in a dose-dependent manner, as shown in Fig. 3A. Additionally, **A5** dose-dependently suppressed the expression of viral nucleoproteins

(NP), PA, and PB1 at both transcriptional and translational levels (Fig. 3B and C). Furthermore, **A5** effectively mitigated the IAV-induced overexpression of inflammation-related genes TNF- α and IFN- β (Fig. 3D). Subsequently, we observed a significant reduction in IL-6 and TNF- α concentrations in the supernatant of H1N1-infected A549 cells with increasing doses of **A5**, while zanamivir exhibited only a modest effect (Fig. 3E). These findings suggest that compound **A5** may exert distinct anti-inflammatory effects beyond its antiviral activity. Collectively, these results indicate that **A5** possesses potent *in vitro* anti-influenza and anti-inflammatory properties. To further evaluate its therapeutic potential, we conducted *in vivo* studies as illustrated in Fig. 3F. Mice ($n = 8$) infected with A/WSN/1933 began to lose significant amounts of weight from Day 3 post-infection. However, weight loss was markedly alleviated in mice treated with 50 mg/kg of **A5** (Fig. 3G). The survival rates for treatment doses of 12.5, 25, and 50 mg/kg were 12.5%, 62.5%, and 87.5%, respectively (Fig. 3H). Compared to the vehicle group, all **A5**-treated groups showed a dose-dependent increase in survival duration during otherwise lethal IAV infection.

2.4. **A5** targets the PA protein of the influenza A virus RdRp

Previous research has shown that oleanolic acid inhibits the replication of IAV strains by specifically binding to the viral HA and preventing its maturation²². In our experiments evaluating various treatment modes (Fig. 4A), **A5** demonstrated significant inhibitory effects on influenza virus production when administered either simultaneously with IAV infection or post-infection (Fig. 4B). Notably, **A5** was most effective during the first 2 h of the viral invasion, followed by a moderate effect between 2 and 5 h (Fig. 4C), suggesting its involvement in both the entry and early replication stages of the IAV infection cycle. However, within the concentration range of 0.78–100 $\mu\text{mol/L}$, **A5** showed no inhibitory activity against the H5N1 pseudovirus (Fig. 4D). Hemagglutination inhibition, hemolysis inhibition, and neuraminidase inhibition assays further confirmed that **A5** did not interfere with the functions of HA or NA (Supporting Information Fig. S1A–S1C). These data suggest that **A5** might be ineffective in inhibiting the viral entry phase. Molecular docking studies indicated that **A5**'s large molecular structure prevented stable interactions with the “head” and “neck” pockets of HA, as evidenced by comparisons with oleanolic acid (Fig. S1D and S1E). Further investigations revealed that **A5** directly combatted IAV rather than impeding adsorption and internalization processes (Fig. 4E). Subsequent RNase digestion assays demonstrated **A5**'s destructive effects on the viral envelope (Fig. 4F). To validate the viral envelope as the target during the first 2 h of the viral invasion, we tested **A5** against the non-enveloped enterovirus 71 (EV71); as expected, **A5** incubation with EV71 did not reduce

Table 1 *In vitro* activity of compound **A5** against multiple influenza virus strains.

Flu strains	EC_{50} ($\mu\text{mol/L}$)	CC_{50} ($\mu\text{mol/L}$)	Selective index
A/WSN/1933 (H1N1)	0.76 ± 0.10	111.1 ± 19.05	146.18
A/Puerto Rico/8/34 (H1N1)	1.15 ± 0.54		96.61
A/Aichi/2/68 (H3N2)	1.6 ± 0.50		69.44
A/Fort Monmouth/1/1947 (H1N1)	0.60 ± 0.12		185.17
A/Dongguan/2/2023 (H3N2)	1.50 ± 0.45		74.07
BV/Dongguan/8/2023	1.27 ± 0.31		87.48
A/PR/8/34 with NA-H274Y	1.83 ± 0.88		60.71

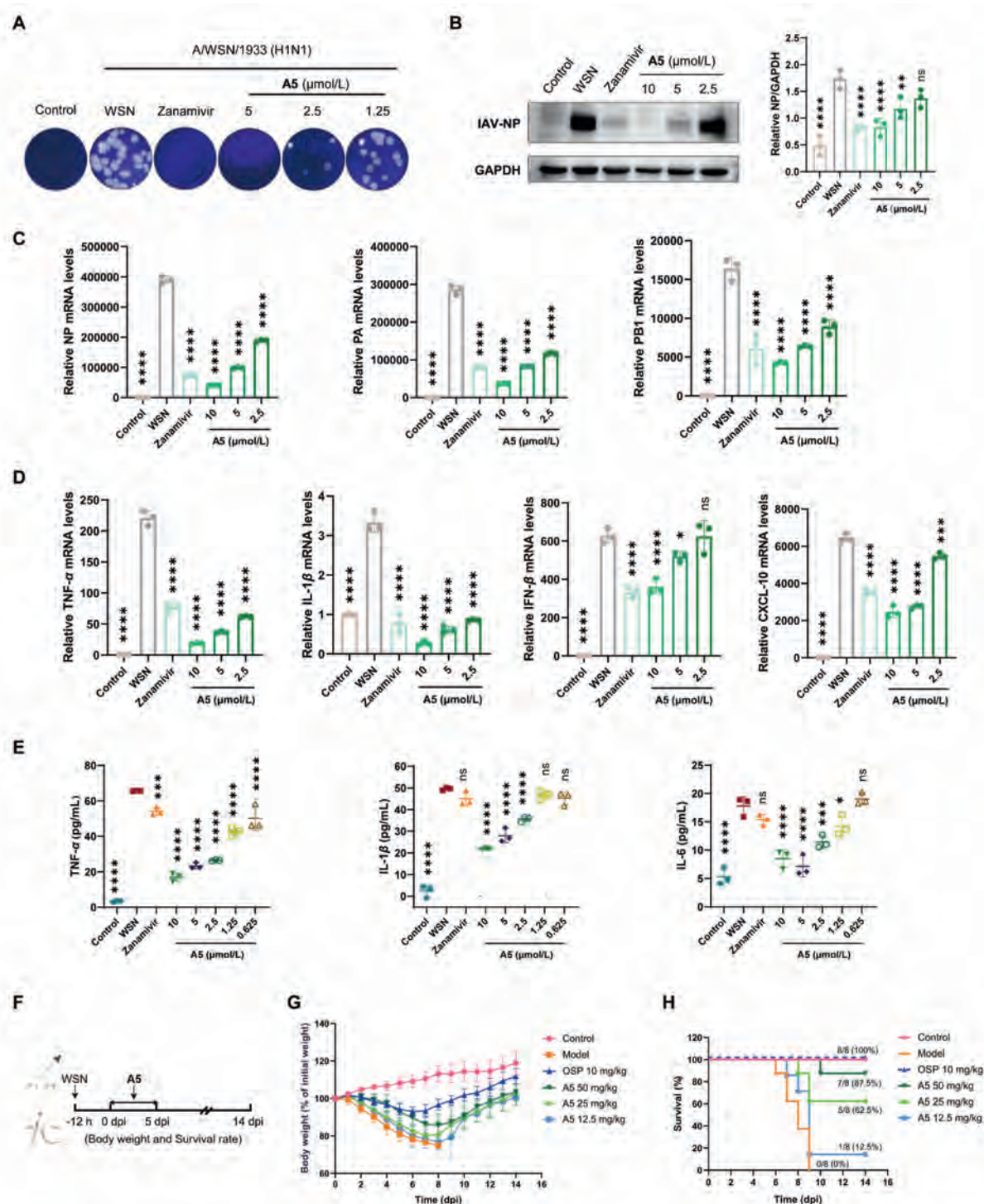


Figure 3 Anti-IAV activities of the compound **A5** *in vitro* and *in vivo*. (A) Shows that compound **A5** effectively reduced the viral plaque formation of A/WSN/1933. (B) Displays the impact of **A5** on the expression of viral NP protein as measured by Western blotting. (C) Examines the influence of **A5** on the expression of viral NP, PA, and PB1 mRNA. (D) Is the effect of **A5** on the expression levels of virus-induced TNF- α , IL-1 β , IFN- β , and CXCL-10 mRNA. Data are represented as mean $\pm$ SD. Statistical analyses employed ordinary one-way ANOVA, with significance levels indicated as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, ns indicates no significant difference. Three cell experiments were performed for each index. (E) Is the effect of **A5** on the content of TNF- α , IL-1 β , and IL-6 in the cellular supernatant detected by ELISA. Data are represented as mean $\pm$ SD, $n = 3$. Statistical analyses employed ordinary one-way ANOVA. (F) Is a schematic of infecting BALB/c mice with A/WSN/1933 by Figdraw. Six-week-old BALB/c mice were intranasally administered a 5-fold 50% lethal dose (LD_{50}) of A/WSN/1933 and were treated with **A5** or oseltamivir phosphate (OSP) or PBS intraperitoneally 12 h later. The intraperitoneal treatments were given once daily for 5 days. (G) Is a graph of body weight curves of infected mice for 14 days. (H) Is a graph of Kaplan–Meier survival curves of infected mice for 14 days. Mice were considered dead if they experienced a weight loss of 25% or more from their initial weight.

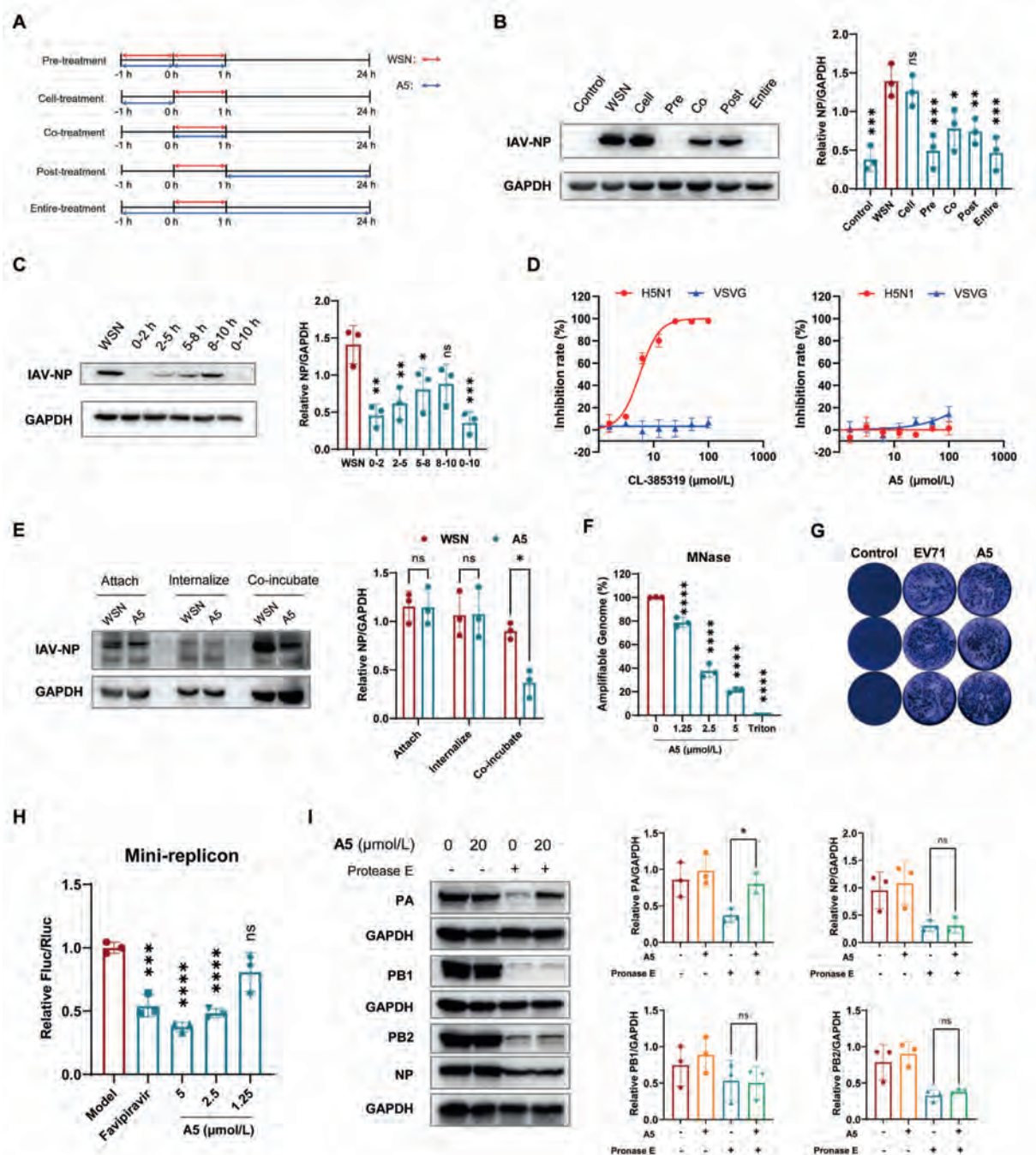


Figure 4 **A5** targets the PA protein of the influenza A virus RdRp. (A) Is a schematic diagram of five different addition modes of the experiment. The time intervals of -1 h–0 h refer to the incubation of either the individual **A5** or the **A5**-virus mixture with the cells for 1 h. The interval from 0 to 1 h indicates the time during which the virus is adsorbed onto the cells. The red line segments represent the contact between the virus and the cells, while the blue line segments indicate the contact between compound **A5** and the cells. (B) Shows the expression of NP protein in the experiment of five different addition modes. (C) Shows the expression of the NP protein at various exposures of **A5** times following viral infection. The different time intervals represent the duration of **A5** exposure to A549 cells during various infections. (D) Indicates the ineffective inhibition of **A5** to H5N1 pseudo-virus from entering cells with CL-385319 serving as a positive control, which was determined by luciferase assay. Values are mean $\pm$ SD ($n = 3$). The curves were created with nonlinear regression analysis. (E) Shows the expression of NP protein at viral adsorption and internalization or co-incubation phases. (F) Shows that **A5**-treated A/WSN/1933 was digested by micrococcal nuclease and then cleaved to extract RNA for RT-qPCR. The destruction of the viral envelope by **A5** exposed its nucleic acid to the micrococcal nuclease for degradation, resulting in a decrease in the amount of viral RNA extracted and detected. (G) Indicates compound **A5** did not affect the EV71 viral plaque formation in Vero cells regardless of dose. (H) Shows the effect of **A5** on the luciferase activity of the viral polymerase. Polymerase activity was measured as the Firefly/*Renilla* ratio. Values are mean $\pm$ SD ($n = 3$). (I) Is a Western blot analysis showing that **A5** prevents the PA protein degradation induced by protease E. The grayscale analysis of protein expression ($n = 3$) was performed in ImageJ. Values are mean $\pm$ SD. *P* values were determined using Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant.

virion counts (Fig. 4G). Within 2–5 h post-infection with influenza A virus, RdRp plays a critical role in early viral replication. The mini-replicon assay showed that compound **A5** exerted a dose-dependent inhibitory effect on RdRp-induced luciferase expression (Fig. 4H). DARTS analysis suggested that **A5** might bind to the viral PA protein, enhancing its structural stability and reducing protease E-mediated degradation, rather than targeting PB1, PB2, or NP proteins (Fig. 4I).

2.5. The disruption effect of **A5** on the interaction of PA_C–PB1_N

Our subsequent research focused on elucidating how **A5** influences viral replication through its interaction with PA proteins. Molecular dynamics simulations revealed the formation of a stable complex between the **A5** and the C-terminal region of the PA protein (Supporting Information Fig. S2A and S2B), as evidenced by low root mean square deviation (RMSD), steady radius of gyration and solvent accessible surface area (SASA), small root mean square fluctuation (RMSF) values, persistent hydrogen bonding and smooth free energy landscape (FEL), indicating high stability throughout the simulation period (Fig. S2C–S2H). The calculated total binding free energy between the PA protein and the **A5** was -207.297 kJ/mol (Supporting Information Table S2). Confocal fluorescence microscopy confirmed that **A5** effectively prevents PA from entering the nucleus (Supporting Information Fig. S3A). Surface plasmon resonance (SPR) experiments demonstrated a high affinity of 7.05×10^{-8} M between compound **A5** and the PA protein (Fig. 5A). Given that **A5** binds to the C-terminal region of the PA protein, where the N-terminal region of PB1 protein interacts with PA, we hypothesized that **A5** targets PA to disrupt the protein–protein interaction between PA and PB1. This hypothesis was supported by co-immunoprecipitation (Co-IP) experiments, which confirmed that **A5** significantly reduced the interaction between PB1 and PA proteins (Fig. 5B). The inhibitory effect of **A5** on the PA_C–PB1_N polymerase subunit interaction was further validated by ELISA assays (Fig. 5C), with an IC₅₀ value of 0.96 ± 0.21 μ mol/L (Fig. 5D). Notably, confocal fluorescence microscopy studies indicated that **A5** did not affect the nuclear entry of PB1 (Fig. 5E).

To identify the key binding sites of **A5** to the PA protein, molecular docking simulations were conducted using the X-ray crystal structure of the PA subunit. Fig. S2I demonstrates that **A5** occupies the C-cavity of PA protein and establishes hydrogen bonds as well as hydrophobic interactions with PA residues N412, E416, E623, Q670, and W706. Furthermore, the minimal distance between **A5** and the PA residues at the predicted binding site were calculated (Fig. S2J). Notably, these five residues are conserved across various strains of influenza A viruses (Fig. S3B). We introduced specific mutations at five sites into the PA expression plasmid and examined their effects on the **A5** interaction. In the DARTS assay, mutations at PA residues N412, E623, and Q670 prevent **A5** from rescuing PA protein degradation, while mutations at E416 and W706 also significantly impair binding (Fig. 5F). SPR experiments were conducted using PA proteins with specific mutations, and the results are presented in Fig. 5G. Following mutations at N412, E623, or Q670, **A5** failed to form stable interactions with PA proteins. Meanwhile, mutations at E416 or W706 resulted in markedly weakened binding between **A5** and PA proteins. The N412 and E416 sites are crucial for polymerase formation, whose mutations significantly diminished the activity of the recombinant polymerase. Correspondingly, mutations at any of the five sites reduced the inhibitory effect of **A5** on RdRp to

varying extents (Fig. S3C). In addition, Co-IP experiments were conducted using the PB1 plasmid and the mutant PA plasmids to validate the interaction. The PA proteins failed to bind to the PB1 protein when mutations were introduced at N412, E623, or Q670 (Fig. 5H). Collectively, these experiments demonstrated that the N412, E416, E623, Q670, and W706 residues of the PA protein interact with compound **A5**, thereby enhancing the stable binding of **A5** to PA. Specifically, the N412, E623, Q670, and W706 residues of the PA protein engage in hydrophobic interactions and hydrogen bonding with the PB1 protein. When these residues are occupied by **A5**, the PB1 protein is unable to form a heterodimer with PA, thus preventing the assembly of an active RdRp trimer in the nucleus. This disruption ultimately leads to the failure of influenza virus replication.

2.6. High resistance barrier of compound **A5** *in vitro*

Drug resistance represents a substantial challenge for antiviral therapies. Developing anti-influenza virus drugs with a high genetic barrier to resistance is both critically important and urgently needed. The hydrophobic interface between PA and PB1, which contains highly conserved amino acids, presents a promising target for inhibitor development²³. In our study, we performed serial viral passage experiments to evaluate the resistance potential of compound **A5** (Fig. 6A). Notably, viruses treated with **A5** for up to 40 passages retained their sensitivity to the compound, with no significant increase in EC₅₀ values (Table 2). In contrast, the EC₅₀ of oseltamivir increased by 22-fold after only 12 passages, while that of baloxavir increased by 78-fold after 40 passages (Fig. 6B). These results suggest that compound **A5** is a promising candidate for further drug development, demonstrating a superior resistance profile compared to oseltamivir and baloxavir. Additionally, **A5** remained equally effective against oseltamivir-resistant WSN strains that had been passaged 12 times in the presence of oseltamivir (Fig. 6C), as well as baloxavir-resistant WSN strains that had been passaged 40 times in the presence of baloxavir, with an EC₅₀ of 1.04 μ mol/L (Fig. 6D).

2.7. **A5**'s synergistic effect when combined with oseltamivir *in vitro* and *in vivo*

Neuraminidase inhibitors, such as oseltamivir, are widely utilized in the clinical management of influenza infections. However, prolonged use of these drugs has led to the emergence of drug resistance. Combination therapies present a promising strategy to mitigate resistance and reduce antiviral dosages. In this study, we evaluated the efficacy of combining **A5** with oseltamivir against A/WSN/1933 both *in vitro* and *in vivo*. Using the SynergyFinder software, we calculated the drug interaction potency scores and obtained a zero interaction potency (ZIP) synergy score of 12.78 for the **A5** and oseltamivir combination in MDCK cells, which supports their synergistic application *in vitro* (Fig. 7A and B). Additionally, mice infected with $5 \times \text{LD}_{50}$ of A/WSN/1933 were treated with each antiviral agent either alone or in combination. The treatment was administered once daily for five consecutive days. Over a 14-day monitoring period, it was observed that combination therapy resulted in significantly reduced weight loss compared to monotherapy (Fig. 7C). At doses of 1.25 and 2.5 mg/kg/day, oseltamivir phosphate (OSP) alone achieved survival rates of 25% and 75%, respectively, while **A5** at 25 mg/kg/day resulted in a 50% survival rate (Fig. 7D). Notably, the combination of **A5** with OSP significantly enhanced survival

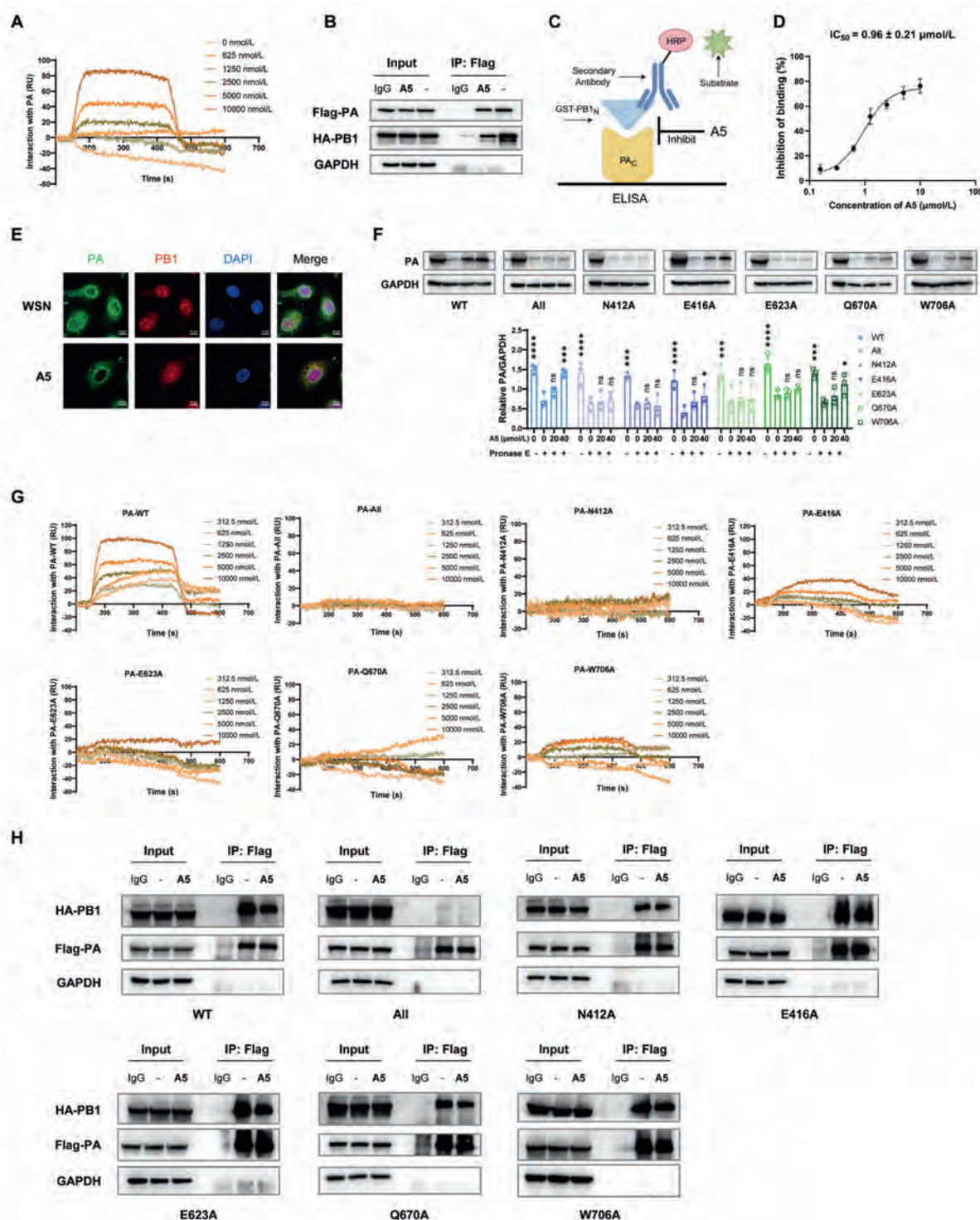


Figure 5 The disruption effect of A5 on the interaction of PA_C–PB1_N. (A) Indicates the characterization of the affinity between A5 and the PA protein. The equilibrium dissociation constant (K_D) was determined by fitting the dose–response curve using BIAcore evaluation software, calculated from the association (k_a) and dissociation (k_d) rates, with $K_D = k_d/k_a$. (B) Presents a Co-IP analysis conducted on HEK-293T cells transfected with expression plasmids of PA and PB1. (C) Is a cartoon representation of the PA_C–PB1_N ELISA assay by Figdraw. The PA protein was pre-coated in a high-adsorption 96-well plate, and A5 was added to the plate along with GST-PB1. The GST content was measured at 450 nm to quantify the interaction of PB1 binding to PA protein. (D) Shows the binding curve of the PB1_N protein at different concentrations to 400 ng of PA_C protein and the IC₅₀ curve of compound A5 in the PA_C–PB1_N ELISA assay. The curves were created with nonlinear regression analysis. Values are mean ± SD ($n = 3$). (E) Is a confocal microscopy analysis of A5's effect on PA and PB1 proteins in A549 cells. Scale bar = 10 μm. (F) Is the Western blot analysis showing A5 prevents the degradation of the PA mutational protein induced by protease E. The data of grayscale analysis was performed in ImageJ. Values are mean ± SD ($n = 3$). P values were determined using ordinary one-way ANOVA. (G) Indicates the binding curves between A5 and the mutated PA protein. (H) Presents a Co-IP analysis conducted on HEK-293T cells transfected with expression plasmids of PB1 and mutational PA.

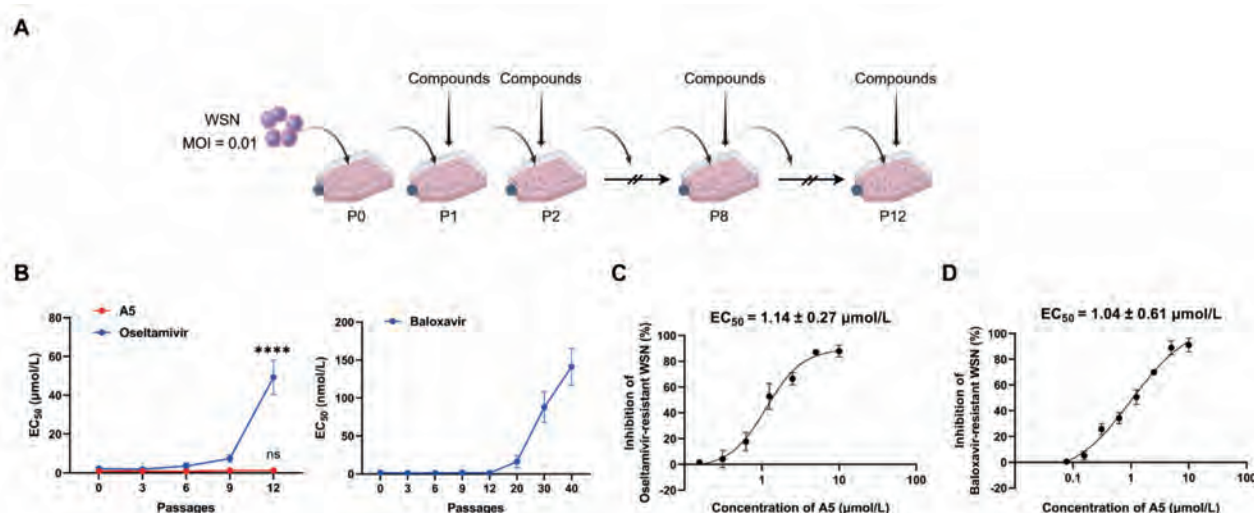


Figure 6 High resistance barrier of compound **A5** *in vitro*. (A) Is a diagrammatic drawing representing the serial viral passage experiment by Figdraw. P0 refers to infecting MDCK cells with A/WSN/1933 at a multiplicity of infection (MOI) of 0.01. P (*n*+1) refers to the infection of cells with the supernatant collected from P *n*, followed by the addition of the compound at its half-maximal effective concentration (EC₅₀) of P *n*. The EC₅₀ values were tested by cell counting kit 8 assay. (B) Shows the resistance curves of **A5**, oseltamivir, and baloxavir. Data are mean ± SD, *n* = 3. (C) Is an antiviral curve of **A5** against oseltamivir-resistant WSN. (D) Is an antiviral curve of **A5** against baloxavir-resistant WSN. The curves were created with nonlinear regression analysis. Data are mean ± SD, *n* = 3.

rates, achieving 100% survival with 2.5 mg/kg of OSP. These findings underscore the superior protective effect of the drug combination of **A5** and OSP over monotherapy. The observed recovery trends during viral infection strongly support the synergistic antiviral effect of **A5** and oseltamivir.

One significant limitation of neuraminidase inhibitors is their reduced efficacy when administered in the later stages of infection. To investigate the therapeutic potential of **A5** for advanced infections, mice were administered a lethal dose of IAV and subsequently treated with 50 mg/kg of **A5** at intervals of 48, 72, and 96 h post-inoculation. Treatment was administered once daily for five consecutive days. As shown in Fig. 7E, weight loss due to viral infection was minimal in the group that received treatment 48 h post-infection. Additionally, survival rates were 87.5% in the 48-h treatment group and 37.5% in the 72-h group (Fig. 7F). In contrast, similar experiments with OSP revealed that 87.5% of mice in the 48-h treatment group either succumbed to the infection or required euthanasia due to severe weight loss. These data

suggest that **A5** is more effective than OSP for treating IAV infection at later stages.

2.8. **A5** reduced viral load and improved lung pathology in IAV-infected mice

To evaluate the efficacy of antiviral therapeutics *in vivo*, BALB/c mice (*n* = 6 per group) were intranasally infected with 5 × LD₅₀ of A/WSN/1933. Due to the negligible levels of **A5** detected in rat plasma following oral administration (data not shown), mice were treated with **A5** at doses of 12.5, 25, or 50 mg/kg/day *via* intraperitoneal injection (IP) starting 12 h post-infection. A positive control group received 10 mg/kg oseltamivir phosphate (OSP) daily (Fig. 8A). Infected mice exhibited weight loss; however, all animals survived three days post-viral exposure. Lung tissues were collected at this time point for further biochemical analysis. Both OSP and **A5** treatment groups showed reduced pulmonary edema and spleen index compared to the untreated group

Table 2 Higher resistance barrier of compound **A5** than oseltamivir *in vitro*.

Passages of WSN	Viral susceptibility to A5 EC ₅₀ (μmol/L)	Viral susceptibility to oseltamivir EC ₅₀ (μmol/L)	Viral susceptibility to baloxavir EC ₅₀ (nmol/L)
0	0.94 ± 0.23	2.17 ± 0.24	1.80 ± 0.37
3	0.92 ± 0.14	1.93 ± 0.46	1.31 ± 0.34
6	0.74 ± 0.21	3.53 ± 1.58	1.34 ± 0.28
9	1.21 ± 0.32	9.63 ± 2.32	1.51 ± 0.28
12	1.24 ± 0.32	49.24 ± 8.87	1.37 ± 0.33
20	1.48 ± 0.37	ND	16.25 ± 7.97
30	1.62 ± 0.19	ND	87.75 ± 19.76
40	2.47 ± 0.46	ND	141.2 ± 24.05

ND, not detected.

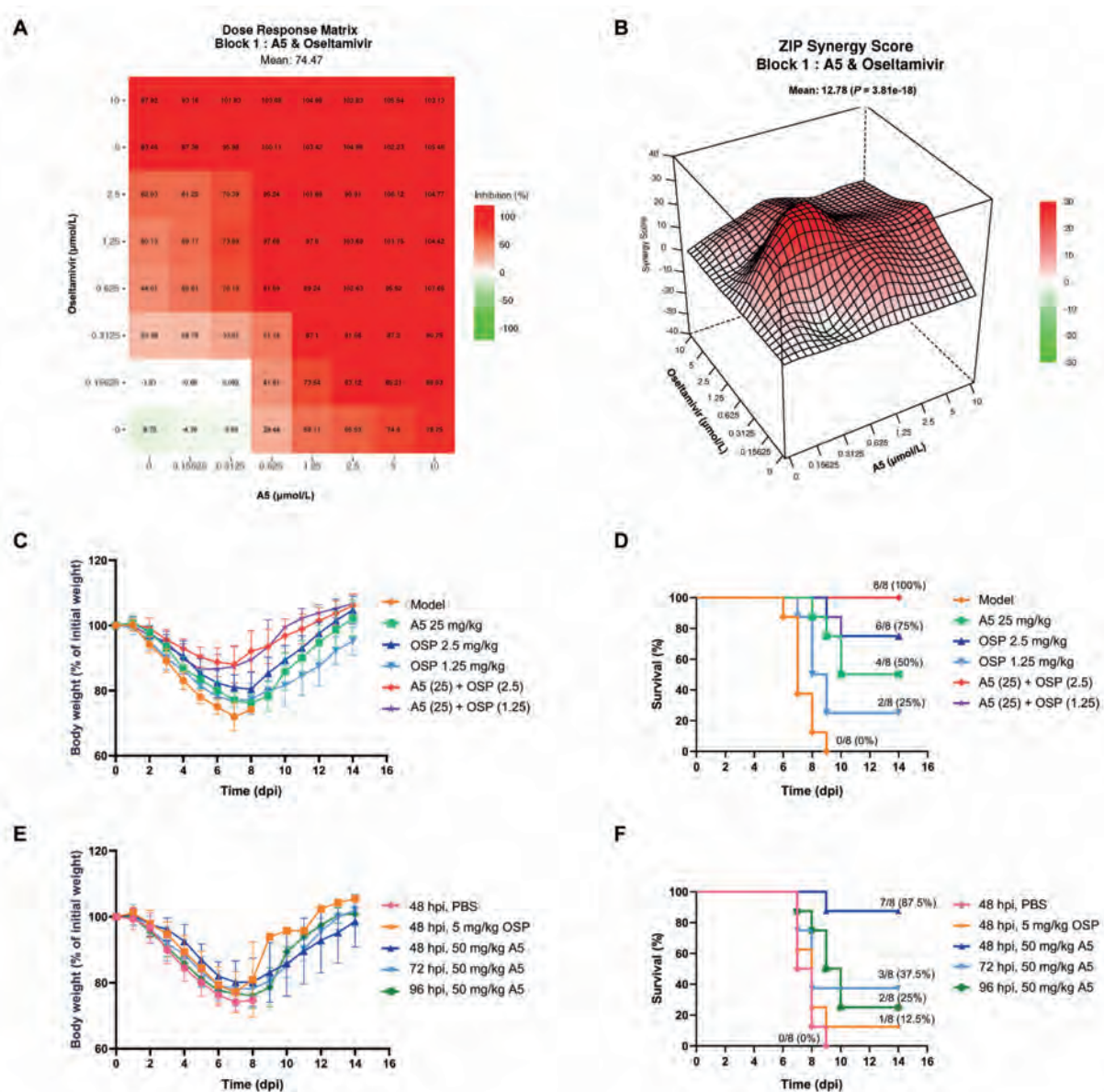


Figure 7 A5's synergistic effect when combined with oseltamivir *in vitro* and *in vivo*. (A) Is the heatmap of drug combination responses. (B) Displays the heatmap of ZIP Synergy scores generated using SynergyFinder Plus. Scores greater than 0 indicate synergistic effects, while scores above 10 are regarded as strong synergy. The varying shades of red represent the degree of synergism. (C) Indicates the body weights in the combined application of A5 and OSP *in vivo*. (D) Shows the survival ratio for mice treated with a combination of A5 and OSP *in vivo*. (E) Indicates the weight change of infected mice with treatment of A5 at varying time intervals. (F) Illustrates the survival rates of infected mice receiving A5 treatment at different time intervals. Mice were euthanized when they reached 75% of their initial body weight. The survival curves were analyzed with the Kaplan–Meier method.

(Fig. 8B). Additionally, these treatment groups demonstrated decreased viral protein and mRNA levels (Fig. 8C and E), significantly lowering the viral load relative to the vehicle group (Fig. 8D). Histopathological examination on Day 3 post-infection revealed that untreated infected mice exhibited diffuse pneumonia characterized by interstitial edema, inflammatory cell infiltration in thickened alveolar walls, and interstitial fibrosis. In contrast, OSP and A5 treatments mitigated these pathological features, including reducing the thickness of alveolar walls and interstitial fibrosis (Fig. 8F). In summary, A5 exhibited a significant dose-dependent anti-influenza virus effect *in vivo*.

2.9. A5 reduced proinflammatory cytokines and attenuated activation of the NF- κ B pathway *in vivo*

Treatment with A5 significantly reduced the serum and mRNA expression levels of pro-inflammatory cytokines and interferon, as shown in Fig. 9A and B. Western blot analysis of lung tissue revealed that A5 effectively decreased the phosphorylation of NF- κ B and p38 (Fig. 9C). In the immunohistochemical study, A5 exhibited marked inhibition of PA protein expression and NF- κ B nuclear translocation compared to the model group (Supporting Information Fig. S4). Viral pneumonia induced by IAV is typically

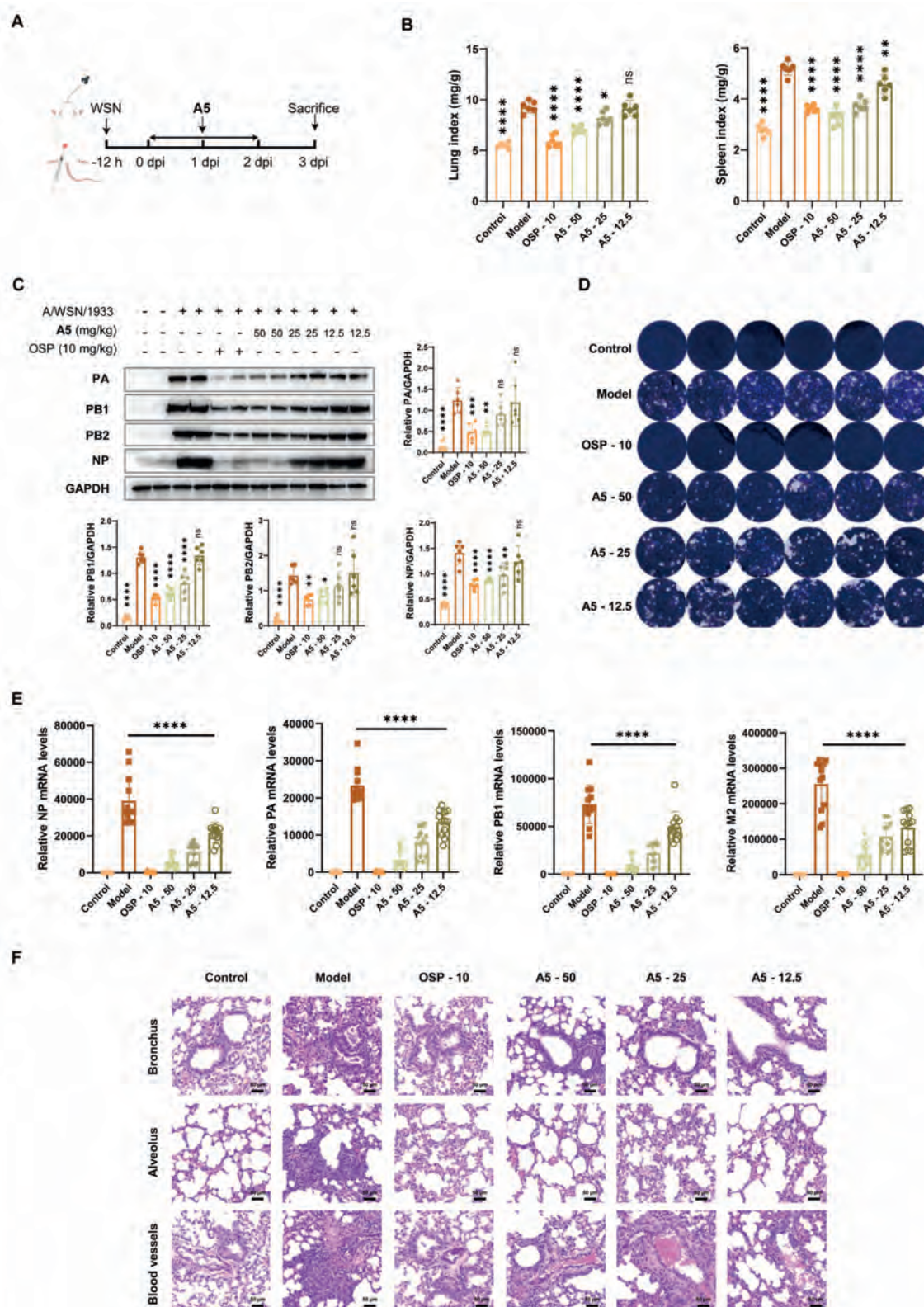


Figure 8 A5 reduced viral load and improved lung pathology in IAV-infected mice. (A) Is a schematic of the therapeutic effects of A5 by Figdraw. 12 h after intranasal inoculation with a 5-fold 50% lethal dose (LD_{50}) of A/WSN/1933 virus, the mice ($n = 6$) received an intraperitoneal injection of A5 for continuous three days. Mice were euthanized on Day 3 post-infection with their lungs removed for the next biochemical experiment. (B) Shows the lung index and spleen index respectively by dividing the weight of the whole lung or spleen tissues by the weight of the mice. Values are mean $\pm$ SD ($n = 6$). P values were determined using ordinary one-way ANOVA. (C) Indicates the expression of viral protein by Western blotting. (D) Presents the viral load measured by the number of plaques. (E) Indicate the expression of viral genes by RT-qPCR. Data are mean $\pm$ SD. P values were determined using ordinary one-way ANOVA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant. (F) Displays the lung histopathology shown by HE staining. Scale bar = 50 μ m.

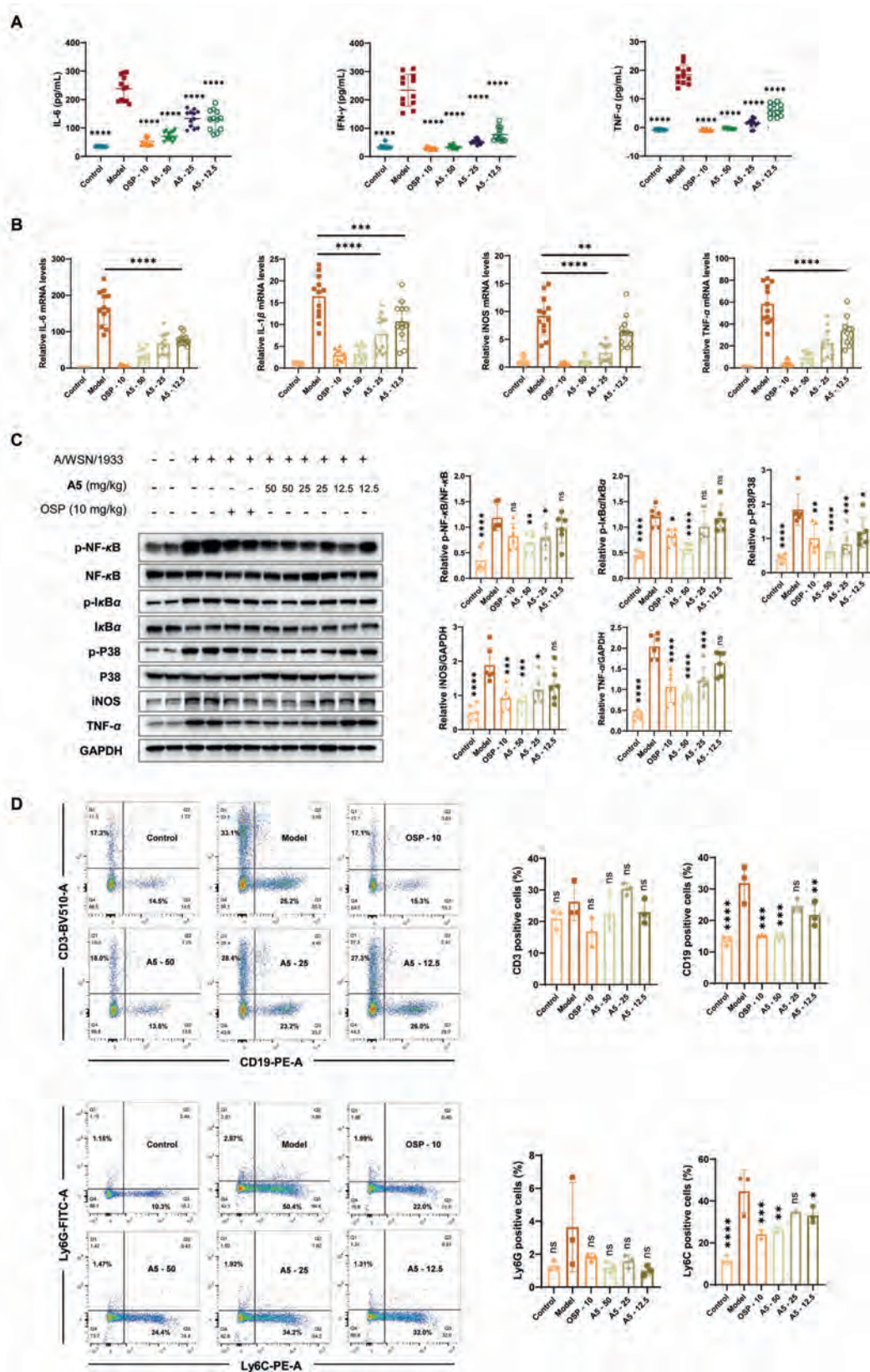


Figure 9 A5 reduced proinflammatory cytokine and attenuated activation of the NF-κB pathway *in vivo*. (A) Illustrates the serum concentrations of IL-6, IFN-γ, and TNF-α measured by ELISA. Data are mean ± SD. *P* values were determined using ordinary one-way ANOVA. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; ns, not significant. (B) and (C) display the expression levels of inflammatory cytokines and associated signaling pathway proteins as assessed by RT-qPCR and Western blotting. (D) Shows the cytokines in the spleen by staining different flow antibodies. Data are mean ± SD (*n* = 3).

associated with the infiltration of macrophages and peripheral neutrophils, along with the overactivation of pro-inflammatory and antiviral mediators²⁴. IAV also triggers the influx of Ly6G⁺ and Ly6C⁺ monocytes²⁵. The polymerases (PB1, PB2, PA) are crucial targets for CD4⁺ and CD8⁺ T cells²⁶. The number of CD11c⁺ plasmacytoid dendritic cells and Ly6G⁺ monocytes in the spleens of **A5**-treated mice was lower than that in the model group, although the difference did not reach statistical significance (Supporting Information Fig. S5A). Additionally, **A5** treatment decreased the proportion of CD19-positive neutrophils and Ly6C⁺ monocytes in the spleen (Fig. 9D), suggesting its efficacy in mitigating systemic immune overactivation. Furthermore, **A5** did not alter the expression of specific immunosuppressive CD11b⁺ macrophage populations or the numbers of CD4⁺, CD8 α ⁺, and CD3⁺ cells.

2.10. **A5** modulated host macrophage inflammation by targeting TLR4–MyD88–NF- κ B molecular pathways

It remained possible that the antiviral activity of **A5** was mediated through multiple mechanisms, particularly since the concentration required for **A5** to inhibit IAV infection was significantly lower than that needed to bind PA in the DARTS assay. Our previous findings demonstrated that **A5** markedly inhibits the release of inflammatory factors while suppressing the replication of IAV both *in vitro* and *in vivo*. To investigate whether **A5**'s anti-inflammatory properties are distinct from its antiviral effects, we exposed THP-1 cells to poly I:C and RAW 264.7 cells to lipopolysaccharide (LPS)²⁷. The RT-qPCR results demonstrated that **A5** significantly attenuated the aberrantly elevated pro-inflammatory cytokines and interferon-stimulated genes induced by non-viral factors (Fig. 10A and B) through modulation of the host immune response. The influenza matrix 1 (M1) protein triggers immune overstimulation *via* activation of Toll-like receptor 4 (TLR4) signaling²⁸. Previous studies have reported that oleanolic acid inhibits the TLR4/MyD88 pathway²⁹. Here, we infected mouse primary peritoneal macrophages with A/WSN/1933 and utilized a TLR4 activation model stimulated by LPS as the control³⁰. We demonstrated that compound **A5** inhibited the TLR4–MyD88 signaling pathways and markedly reduced the expression of inflammatory factors in both models (Fig. 10C, Fig. S5B). The inhibitory effect of **A5** on the phosphorylation of NF- κ B in the cytoplasm and its subsequent translocation into the nucleus was confirmed by detecting NF- κ B protein levels in both compartments (Fig. 10D). Molecular docking studies revealed that the parent nucleus of oleanolic acid in compound **A5** engaged in non-hydrogen bond interactions with the TLR4 protein. Furthermore, hydrogen bond and non-hydrogen bond interactions between the functional groups at positions 3 and 28 of oleanolic acid and the TLR4 protein contributed to the stability of the resulting complex (Fig. S5C and S5D). In the cellular thermal shift assay (CETSA), proteins typically undergo degradation at elevated temperatures. However, the thermostability of TLR4 protein in the **A5**-treated group was significantly enhanced compared to the DMSO control group, indicating a potential binding affinity of compound **A5** for TLR4 protein (Fig. 10E). This binding interaction was further validated by the DARTS assay, which showed a significant reduction in protease-induced degradation of TLR4 protein in the presence of compound **A5** (Fig. 10F).

2.11. **A5** was non-toxic when administered to mice intraperitoneally at 200 mg/kg

During a 7-day intraperitoneal administration of 200 mg/kg **A5** solution, the mice exhibited normal locomotor activity, with no significant changes in feeding or defecation patterns and no signs of distress. In the 14-day subacute toxicity study conducted in mice (Supporting Information Fig. S6A), no mortalities were observed, and all animals showed consistent weight gain. There were no statistically significant differences in body weight between the control and **A5**-treated groups (Table 3). To evaluate renal function, serum levels of urea and creatinine were measured, while AST and ALT levels were assessed to determine potential liver injury in the group receiving 200 mg/kg of **A5**. The serum biochemical results for the mice are summarized in Table 4, indicating no significant differences among the parameters measured. Furthermore, morphological and histopathological examinations revealed no remarkable changes in the heart, liver, spleen, lung, kidneys, or brain (Fig. S6B and S6C). These findings suggest that **A5** is non-toxic under the experimental conditions tested, highlighting its favorable safety profile and supporting its potential as a well-tolerated therapeutic agent.

3. Discussion

IAVs are prevalent pathogens responsible for causing severe respiratory illnesses. Owing to the continuous antigenic drift of the influenza virus, the influenza vaccine's efficacy in preventing viral invasion is significantly compromised³¹. A universal influenza vaccine that provides protection against various strains of influenza viruses represents a promising research direction, though it remains in the developmental stage³². Effective antiviral medications are pivotal in both preventing and managing influenza A infections³³. This research has identified compound **A5** as a promising antiviral candidate capable of inhibiting the replication of various influenza virus subtypes, including human H1N1, H3N2, influenza B virus, and oseltamivir-resistant and baloxavir-resistant H1N1 strains. During the structural optimization of the lead compound, we observed that oleanolic acid derivatives featuring branched aromatic chains with catechol fragments exhibited significant antiviral activity and favorable selectivity indices at submicromolar concentrations. These findings indicate that catechol fragments play a critical role in ligand–receptor interactions. Specifically, **A5** binds to the C-terminus of the viral polymerase subunit PA, thereby competitively disrupting the formation of the functional RdRp complex¹⁴. This interference results in a significant reduction in viral polymerase activity, thereby impeding IAV replication and reducing pathogenicity in mouse models. The PA_C domain is highly conserved across various types and subtypes of influenza viruses, which confers compound **A5** with broad-spectrum efficacy and poses a substantial barrier to the development of drug resistance^{13,16}. Given the distinct mechanism of anti-viral action, **A5**, an inhibitor of the PA–PB1 interaction, demonstrates a synergistic anti-influenza effect when used in conjunction with NA inhibitors. This approach not only enhances antiviral efficacy but also reduces the risk of developing drug resistance to compounds that have a lower resistance threshold³⁴. Our work confirms compound **A5** as a promising candidate for the treatment of IAV and IBV infections. Its efficacy against multi-drug-resistant strains further enhances its potential.

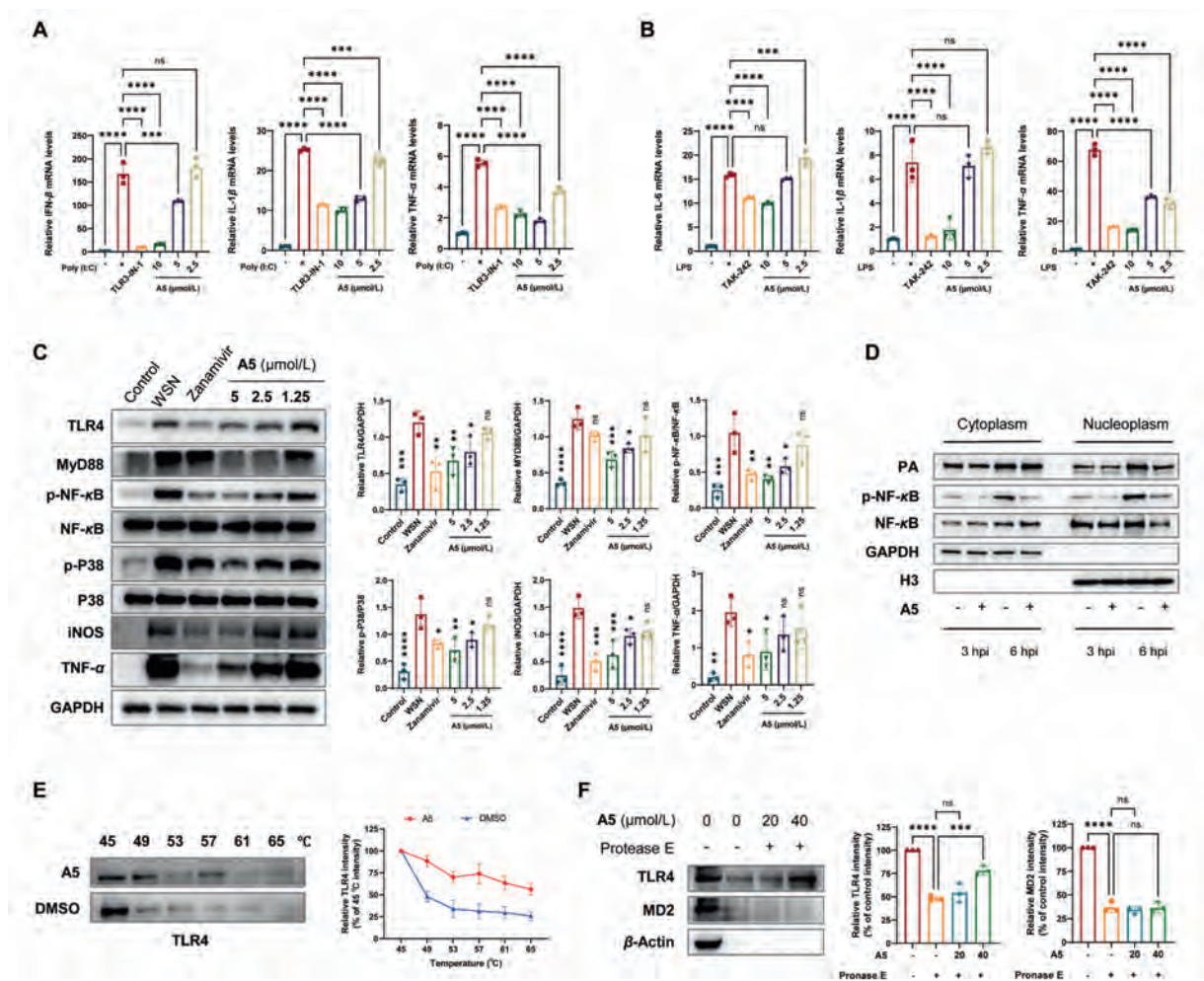


Figure 10 A5 modulated host macrophage inflammation by targeting TLR4–MyD88–NF-κB molecular pathways. (A) Indicates the mRNA levels of IFN-β, IL-1β, and TNF-α in THP-1 cells stimulated with 25 μg/mL of poly I:C for 4 h. (B) Illustrates the mRNA levels of IL-6, IL-1β, and TNF-α in RAW264.7 cells treated with 100 ng/mL of LPS for 6 h. Data are mean ± SD. *n* = 3. *P* values were determined using ordinary one-way ANOVA. (C) Displays the Western blot analysis of mouse primary peritoneal macrophages exposed to A/WSN/1933 for 6 h. (D) Indicates the protein level of PA and NF-κB in the cytoplasm and nucleus respectively in RAW264.7 cells infected with IAV for 3 and 6 h. (E) Is Western blot analysis showing that A5 prevents the TLR4 protein degradation induced by high temperature. Data are mean ± SD, *n* = 3. (F) Is Western blot analysis showing that A5 prevents the TLR4 protein degradation induced by protease E. Data are mean ± SD, *n* = 3. *P* values were determined using ordinary one-way ANOVA. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; ns, not significant.

Table 3 Effect of A5 on the body weight and mortality.					
Animal	Gender	Dose (mg/kg)	Body weight (g)		Deaths
			Initial day	Fourteenth day	
BALB/c	Female	0	18.2 ± 0.7	20.3 ± 0.9	0
		200	18.0 ± 0.6	20.8 ± 1.3	0
	Male	0	20.2 ± 0.6	23.2 ± 0.9	0
		200	20.3 ± 0.9	23.0 ± 0.8	0

Values are presented as mean ± SD in grams for all weight measurements (*n* = 5).

The influenza virus triggers airway epithelial cells to produce an excessive amount of cytokines and chemokines, a phenomenon referred to as the cytokine storm. This response significantly contributes to the development of viral pneumonia in patients with

severe influenza³⁵. Uncontrolled inflammation may result in tissue injury and subsequent organ dysfunction in the host². While current antiviral compounds exhibit significant antiviral efficacy, they frequently lack anti-inflammatory properties. In contrast, the oleanolic acid derivative synthesized in this study not only effectively suppresses the inflammatory response induced by IAV but also protects the host from the harmful cytokine storm, thereby improving overall prognosis *in vivo*.

4. Conclusions

The C-terminus of the PA protein features a flat, highly hydrophobic interface that facilitates the effective binding of PA–PB1 interaction inhibitors primarily through hydrophobic interactions. This characteristic contributes to the poor water solubility of these small molecules. A common strategy to enhance the binding affinity of small molecules to the PA protein involves increasing

Table 4 Effect of **A5** on serum biochemical indices.

Gender	<i>n</i>	Parameters	Dose (mg/kg)	
			0	200
Female	5	ALT (U/L)	35.8 ± 6.1	29.1 ± 5.6
		AST (U/L)	53.3 ± 10.9	42.0 ± 7.7
		CRE (μmol/L)	4.7 ± 1.3	5.1 ± 0.5
		BUN (mmol/L)	5.2 ± 2.5	4.8 ± 1.1
		TG (mmol/L)	0.27 ± 0.09	0.34 ± 0.08
		TC (mmol/L)	1.52 ± 0.28	1.55 ± 0.26
		HDL-C (mmol/L)	1.42 ± 0.26	1.44 ± 0.26
		LDL-C (mmol/L)	0.04 ± 0.01	0.05 ± 0.02
Male	5	ALT (U/L)	52.0 ± 12.8	42.5 ± 8.3
		AST (U/L)	69.1 ± 19.5	56.3 ± 4.1
		CRE (μmol/L)	3.8 ± 0.6	4.2 ± 0.5
		BUN (mmol/L)	5.0 ± 0.3	4.4 ± 1.6
		TG (mmol/L)	0.3 ± 0.09	0.34 ± 0.15
		TC (mmol/L)	1.37 ± 0.36	1.42 ± 0.27
		HDL-C (mmol/L)	1.34 ± 0.23	1.28 ± 0.25
		LDL-C (mmol/L)	0.04 ± 0.01	0.06 ± 0.03

their hydrophobicity, which often compromises their solubility¹⁵. However, it is imperative to consider pharmacokinetics in this context. Consequently, enhancing the solubility of the PA–PB1 inhibitor **A5** through strategic optimization of the side chain at the carboxamide terminus represents a promising avenue for future research.

Given their broad spectrum of activity, high genetic barrier to resistance, and unique mechanisms that inhibit currently circulating drug-resistant influenza strains, protein–protein interaction inhibitors targeting the influenza RdRp represent a highly innovative and promising therapeutic strategy. While PA–PB1 protein–protein interaction inhibitors are still in the research phase and not yet available as marketed drugs, they constitute an emerging class of drug targets with significant potential. Although oral administration remains the standard route for anti-influenza medications, **A5** offers a promising avenue for research due to its novel antiviral mechanism, robust resistance barrier, and particularly its synergistic effect when combined with oseltamivir, the primary anti-influenza drug used in clinical settings⁹. Considering these, it is of great significance to develop new potent **A5** derivatives *via* contemporary medicinal chemistry strategies to overcome the above-mentioned bottleneck, thus improving their biopharmaceutical properties in our subsequent research. On the other hand, new approaches should be introduced to enhance drug delivery properties such as nanoemulsions, solid lipid nanoparticles, liposomes, liposomal gels, and solid dispersions, which are beneficial to increasing the pharmacokinetics and drug ability³⁶. Our team is actively engaged in structural modifications to enhance the therapeutic potential of this oleanolic acid benzyl amide derivative for development into a patent medicine. Our work establishes a robust foundation for the exploration of protein–protein interaction inhibitors in the development of anti-influenza virus drugs.

5. Experimental

5.1. Preparation of oleanolic acid derivatives

The detailed synthetic procedures for the C-3-substituted oleanolic acid benzyl amide derivatives **A1**–**A12** are provided in the Supporting Information.

5.2. Viral strains, reagents

Influenza strain A/WSN/1933 (H1N1), A/Puerto Rico/8/34 (H1N1), A/Aichi/2/68 (H3N2), A/Fort Monmouth/1/1947, A/PR/8/34 with NA-H274Y were purchased from ATCC. Clinically isolated influenza virus strains, including A/Dongguan/2/2023 (H3N2), and BV/Dongguan/8/2023 were isolated in Dongguan People's Hospital and the sequence data were supplied in appendix files. The laboratory-adapted oseltamivir-resistant H1N1 strain and baloxavir-resistant H1N1 were built in our laboratory.

Zanamivir, oseltamivir phosphate, favipiravir, and baloxavir were purchased from MedChemExpress (NJ, USA). TAK-242 (TLR4 inhibitor) was purchased from TargetMol (Boston, USA).

5.3. Plaque reduction assay

MDCK cells were seeded in 12-well plates at a density of 3×10^5 cells per well and incubated for 24 h prior to infection. The cells were then infected with cell or lung tissue homogenate supernatant for 1 h, followed by treatment with a maintenance medium consisting of $2 \times$ DMEM supplemented with 0.5 μg/mL TPCK-trypsin and 2% microcrystalline cellulose for 48 h at 37 °C. Meanwhile, confluent Vero cells were infected with the non-enveloped virus enterovirus 71 (EV71) and maintained in a medium composed of 2% carboxymethylcellulose and DMEM containing 2% fetal bovine serum (FBS). After removing the media, the cells were stained with crystal violet dissolved in 4% paraformaldehyde (Bio-Channel, Nanjing, China).

5.4. Micrococcal nuclease (MNase) assay

IAV at a concentration of 1000 PFU was incubated with or without the tested compound at room temperature for 1 h. Subsequently, MNase (Beyotime, Shanghai, China) was added and the mixture was incubated at 37 °C for 2 h. Viral RNA was then extracted using a viral nucleic acid (RNA/DNA) extraction kit (Takara, Japan) and reverse transcription was performed for detection on the Light Cycler 480 system. The viral HA primers are listed in Supporting Information Table S3.

5.5. Mini-replicon assay

The plasmids pHW2K-PB1, pHW2K-PB2, pHW2K-PA, pHW2K-NP, and a firefly luciferase reporter plasmid (pPoll-Fluc) were kindly provided by Professor Bojian Zheng from the University of Hong Kong. The *Renilla* luciferase plasmid (hRluc-TK) was obtained from Promega (WI, USA). These plasmids were co-transfected into HEK-293T cells, followed by the addition of related compounds 6 h post-transfection. After a 48-h incubation period, the cells were lysed using the reagent from the Dual-Glo Luciferase Assay Kit (Promega, WI, USA). Luminescence was measured from a white 96-well plate using a microplate luminometer (TECAN, Männedorf, Switzerland).

5.6. Drug affinity responsive target stability assay (DARTS)

MDCK cells infected with IAV at an MOI of 0.5 were lysed using NP-40 buffer, and the resulting protein supernatants were incubated with either the compound or DMSO at 4 °C for 16 h. After this incubation period, the samples were treated with Protease E (5 μg/mL) for 30 min at 37 °C³⁷. After the addition of $5 \times$ loading

buffer and boiling, the samples were subjected to Western blot analysis.

5.7. Flow cytometry

The spleens were rinsed with pre-chilled phosphate-buffered saline (PBS) and mechanically dissociated into a single-cell suspension using the rubber plunger of a 3-mL syringe. After filtration through a 200- μ m nylon mesh filter, the supernatant was discarded, and the cells were resuspended in PBS containing 1% FBS. Flow cytometry staining and analysis were performed using antibodies against CD11c, CD11b, Ly6G, Ly6C, CD3, CD19, CD4, and CD8 α , following the manufacturer's instructions.

5.8. Mouse model of IAV infection

Six-week-old BALB/c mice (equally divided by sex) were randomly allocated to different groups and inoculated intranasally with A/WSN/1933 (H1N1) in 45 μ L of PBS. Twelve hours post-inoculation, mice received an intraperitoneal injection of the test compound at doses of 12.5, 25, or 50 mg/kg/day, or a placebo as a negative control. Oseltamivir phosphate (OSP) was used as the positive control. Treatment continued for three consecutive days. The compounds were dissolved in DMSO and further diluted with PBS to achieve a final concentration of 8% DMSO. On Day 3 post-inoculation, mice were euthanized *via* spinal dislocation, and their lungs were harvested. In survival studies, mice were monitored daily for weight loss and other clinical signs. Mice that lost more than 25% of their pre-infection body weight were considered moribund and humanely euthanized immediately³⁸.

5.9. Ethics statement

Animal husbandry and care were performed in strict accordance with the guidelines established by the National Advisory Committee for Laboratory Animal Research (NACLAR). All animal experiments complied with the ethical regulations of Southern Medical University (Approval No. SMUL202311036), which are based on NACLAR standards.

5.10. Statistical analysis

Data are presented as the mean $\pm$ standard deviation (SD). Statistical analyses were conducted using GraphPad Prism 9.0, with a significance level set at $P < 0.05$. Protein expression levels were quantified *via* grayscale analysis using ImageJ software. Graphs were generated using Figdraw and GraphPad Prism 9.0. Comparisons between two groups were performed using Student's *t*-test, whereas comparisons among three or more groups were analyzed using one-way ANOVA. Survival distributions were compared using the Kaplan–Meier method.

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Author contributions

Kunyu Lu, Jianfu He, and Chongjun Hong designed the manuscript and contributed to original draft, data curation. Haowei Li, Jiaai Ruan, Jinshen Wang, Haoxing Yuan, Binhao Rong contributed to investigation, methodology, validation. Shuwen Liu, Gaopeng Song, Chan Yang critically reviewed the manuscript and provided supervision, conceptualization, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no competing interests.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2025.05.031>.

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