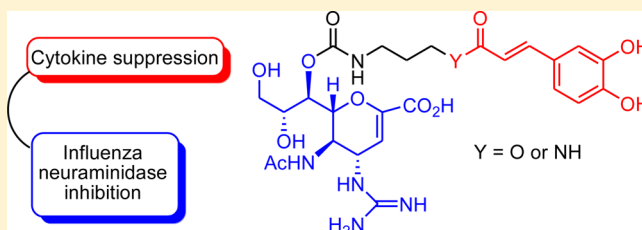


## Enhanced Anti-influenza Agents Conjugated with Anti-inflammatory Activity

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## S Supporting Information

**ABSTRACT:** Influenza therapy with a single targeted compound is often limited in efficacy due to the rapidly developed drug resistance. Moreover, the uncontrolled virus-induced cytokines could cause the high mortality of human infected by H5N1 avian influenza virus. In this study, we explored the novel dual-targeted bifunctional anti-influenza drugs formed by conjugation with anti-inflammatory agents. In particular, the caffeic acid (CA)-bearing zanamivir (ZA) conjugates ZA-7-CA (1) and ZA-7-CA-amide (7) showed simultaneous inhibition of influenza virus neuraminidase and suppression of pro-inflammatory cytokines. These ZA conjugates provided remarkable protection of cells and mice against influenza infections. Intranasal administration of low dosage (<1.2  $\mu\text{mol/kg/day}$ ) of ZA conjugates exhibited much greater effect than the combination therapy with ZA and the anti-inflammatory agents in protection of the lethally infected mice by H1N1 or H5N1 influenza viruses.



## INTRODUCTION

Influenza is a respiratory infection that causes severe health problems. The worldwide spread of the A/H5N1 avian flu and the outbreak of the new type A/H1N1 human influenza in 2009 have increased public awareness of the potential for global influenza pandemics. The H5N1 virus infection of macrophages also causes strong expression of pro-inflammatory cytokines and chemokines, namely cytokine storm.<sup>1,2</sup> The high mortality of human infected by H5N1 virus has been attributed to poor response of virus to neuraminidase (NA) inhibitor, e.g. oseltamivir, and the excessive induction of a severe cytokine storm.<sup>3,4</sup>

In the patients of H5N1 virus infection, induction of tumor necrosis factor (TNF)- $\alpha$  and interferons (IFN)- $\beta/\gamma$  have been observed in alveolar and bronchial epithelial cells.<sup>5</sup> Other cytokines, such as interleukins (e.g., IL-6 and IL-10), IFN-induced chemokines (e.g., IP-10, MIG, and MIG-1), are also found at high levels in the H5N1 virus infected patients.<sup>6</sup> Although treatment of the H5N1 virus infected mice with anti-inflammatory agent alone can inhibit pro-inflammatory cytokines,<sup>7</sup> the mortality of infected mice is not reduced.<sup>8,9</sup>

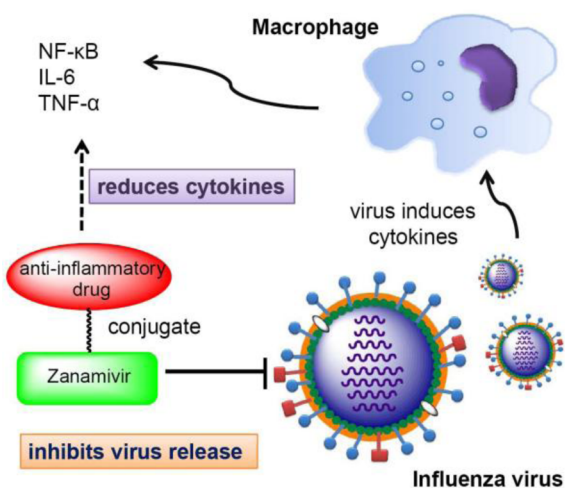
Monotherapy with a single antiviral drug for influenza may be limited in efficacy due to the rapidly developed drug resistance.<sup>10</sup> Moreover, the uncontrolled virus-induced cytokines could cause the high mortality of human infected by H5N1 avian influenza virus. In a different approach, combination chemotherapy consisting of two or more drugs that target different viral proteins or host immune response may provide additive or synergistic antiviral activities<sup>11</sup> and thus reduce the risk of drug resistance with monotherapy.

Zanamivir is a potent influenza virus NA inhibitor;<sup>12</sup> viral resistance to ZA is rare.<sup>13</sup> ZA combined with triamcinolone, an anti-inflammatory corticosteroid, has been applied to treat H3N2 virus infection.<sup>14</sup> In another study,<sup>15</sup> the mice receiving a triple combination of ZA with immunomodulating drugs celastrol and mesalazine after infection with highly virulent H5N1 viruses (1000 LD<sub>50</sub>) showed better survival rate in comparison with the treatment with ZA alone. The enhanced therapeutic effect may be attributable to the combination therapy for antiviral and cytokine suppressors.

Therapy with designed multiple ligands (DMLs) may have advantage of better pharmacological effect and improved safety over combination of individual drugs in treatment of multigenic and multitarget diseases such as cancer, diabetes, and infectious diseases.<sup>16–18</sup> A bifunctional conjugate compound is expected to yield a greater effect than the sum of single components.<sup>19</sup> It is possible to develop effective bifunctional drugs for the prophylaxis, treatment, and therapy of influenza virus infection where the drugs are formed by conjugation of an anti-influenza moiety with a chemical moiety that suppresses pro-inflammatory cytokines. We report herein the novel dual-targeted bifunctional ZA anti-influenza drugs formed by conjugation of ZA with anti-inflammatory agents for simultaneous inhibition of influenza virus NA and suppression of pro-inflammatory cytokines. Figure 1 shows the putative working mechanism of such dual-targeted ZA conjugates. Because of high affinity of ZA with NA, the ZA conjugates would be brought to the

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**Figure 1.** Working mechanism of the dual-targeted anti-influenza drugs for virus inactivation and cytokine suppression. The conjugate drug is brought to the virus infection sites via strong affinity of the NA inhibitor (e.g., ZA), so that the infection-mediated cytokine induction can be effectively suppressed by the anti-inflammatory component of the conjugate drug.

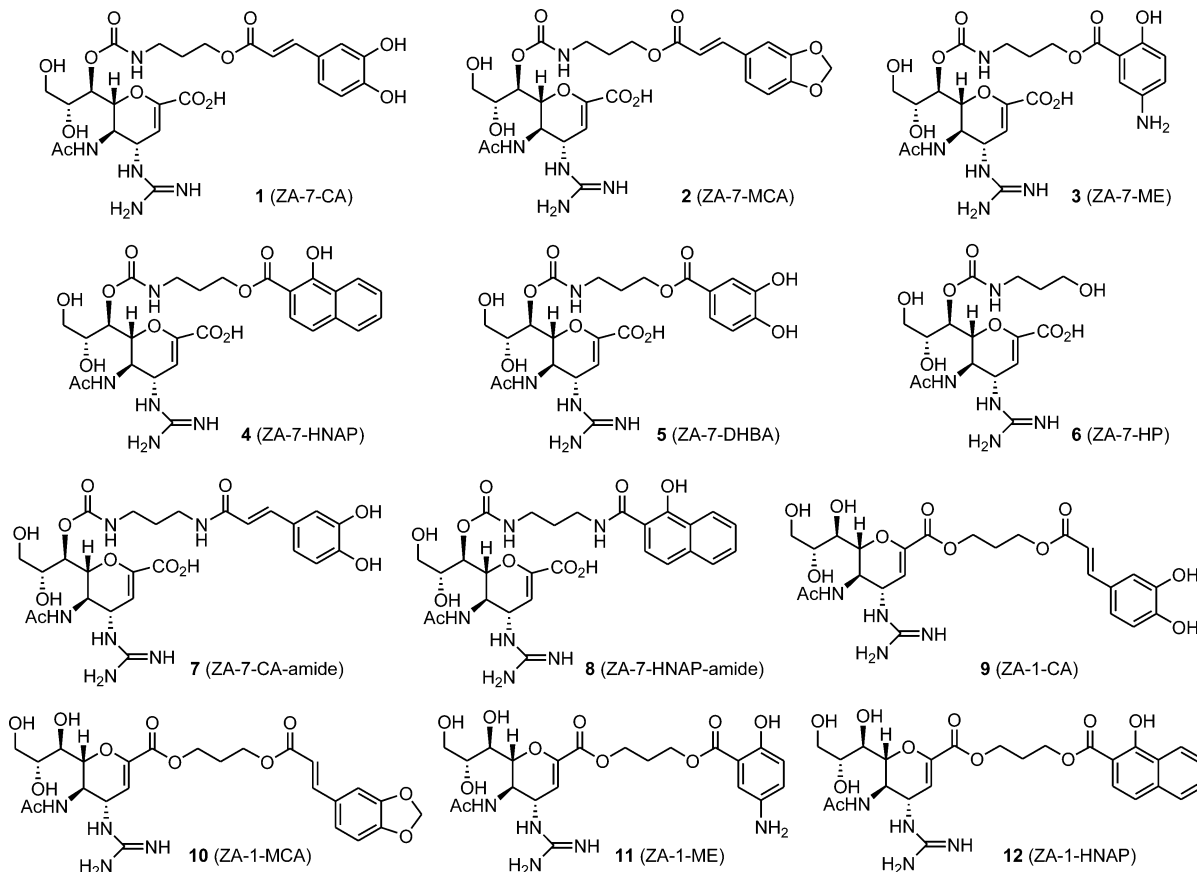
influenza infected tissues, allowing the anti-inflammatory moiety of the conjugates to suppress the induction of pro-inflammatory cytokines in a highly effective manner. Synthetic methods for preparation of these enhanced anti-influenza conjugate drugs are described. This study also demonstrates that the synthetic bifunctional ZA conjugates act synergistically

toward protection of mice lethally infected by H1N1 or H5N1 influenza viruses.

## RESULTS

Three ZA conjugates ZA-7-CA (1), ZA-7-ME (3), and ZA-7-CA-amide (7) that have the linkage at the C-7 position of ZA to caffeic acid (CA) or mesalazine (ME) via ester or amide bonds (Figure 2) were synthesized. CA and its ester derivatives are known to exhibit anti-inflammatory effect in addition to other biological activities.<sup>20–22</sup> Caffeic esters can suppress NF-κB and its downstream mediators, iNOS and COX-2, by inhibiting the formation of NF-κB with DNA complex.<sup>23,24</sup> ME is an anti-inflammatory drug used to treat ulcerative colitis and Crohn's disease, which are two major human chronic inflammatory bowel diseases.<sup>25–27</sup> ME is also known as an agonist of peroxisome proliferator-activator receptor (PPAR-γ) for induction of anti-inflammatory effects.<sup>28</sup>

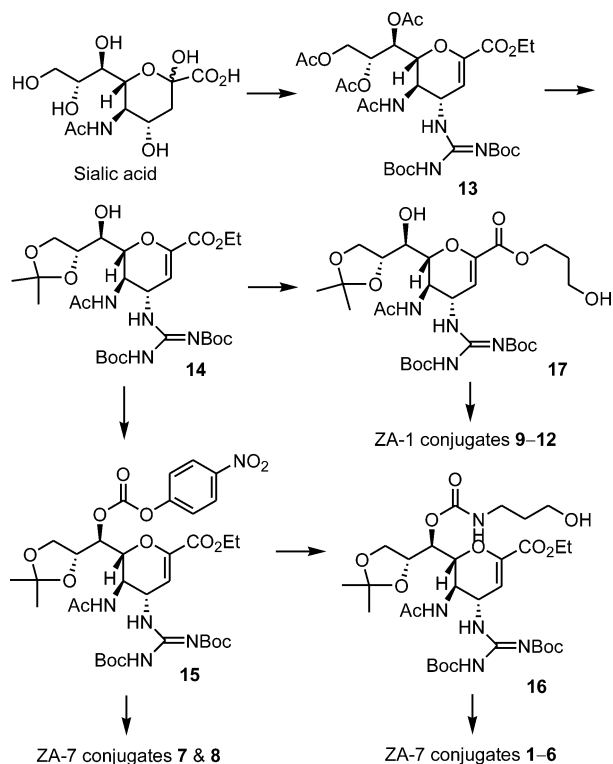
For comparison, other C-7 conjugates ZA-7-MCA (2), ZA-7-HNAP (4), ZA-7-DHBA (5), ZA-7-HP (6), and ZA-7-HNAP-amide (8) as well as C-1 conjugates ZA-1-CA (9), ZA-1-MCA (10), ZA-1-ME (11), and ZA-1-HNAP (12) were also prepared. According to the structural analysis of the ZA–NA complex,<sup>29</sup> the 7-OH group of ZA is exposed to water and makes no direct interaction with the active site of influenza NA protein. It has been shown that the ZA derivatives with modification at the C7 hydroxyl group retain good NA inhibitory activity.<sup>30–32</sup> Thus, the conjugates 1–8 with intact ZA structural features except modifications at the 7-OH for improved lipophilicity are expected to exhibit good anti-influenza activity. The derivatives of ZA with modification at



**Figure 2.** Chemical structures of anti-influenza conjugate drugs used in this study.

the 1-carboxy and 9-hydroxyl groups have also been explored.<sup>33–36</sup> The alkoxyalkyl esters of ZA are effective drugs for inhibition of influenza virus in cell culture and in mice.<sup>33</sup> The 7-methoxy-ZA analogues with esterification of the 9-OH group act as long-acting influenza NA inhibitors.<sup>34–36</sup> Likewise, the conjugates 9–12 with ester linkages are expected to undergo enzymatic hydrolysis in the cellular systems to release the active ZA drug.

Figure 3 shows the general synthetic strategy for ZA conjugates 1–12. Sialic acid was converted to ethyl ester

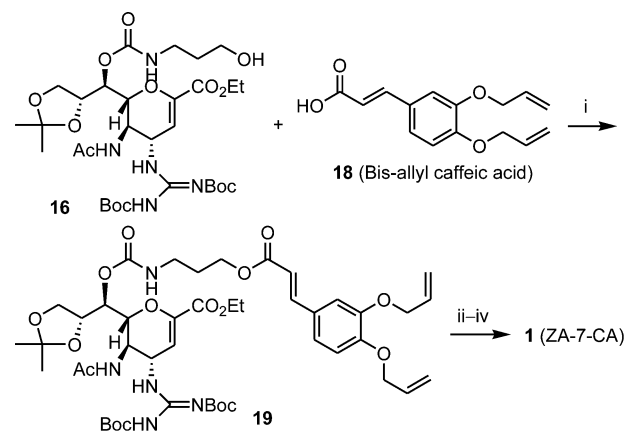


**Figure 3.** Synthetic design for ZA conjugates containing anti-inflammatory agents.

derivative 13 by a slight modification of the reported method for the corresponding methyl ester.<sup>37</sup> Compound 13 was then transformed to 14, which has a free 7-OH group readily for further linkage with appropriate anti-inflammation drugs, via an active carbonate 15<sup>38,39</sup> or a carbamate derivative 16, to form conjugates 1–8. For example, compound 16 was subject to saponification, followed by removal of the *tert*-butoxycarbonyl (Boc) groups with trifluoroacetic acid (TFA) to afford the conjugate 6 bearing an *N*-(3-hydroxypropyl)carbamate moiety at the C-7 position of ZA. Alternatively, ester 14 was hydrolyzed to give the corresponding acid, which could react with 3-iodo-1-propanol to provide compound 17. The conjugates 9–12 were then obtained by the coupling reactions of 17 with appropriate carboxylic acids.

In another approach, the condensation reaction of 16 with the bis-allyl ether of caffeic acid (18) was carried out by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and 4-dimethylaminopyridine (DMAP) as the promoters to yield the desired coupling product 19 with an ester linkage (Scheme 1). Compound 19 was treated with KOH (1 M aqueous solution) at room temperature for a selective saponification of the ethyl ester at C-1 position without cleavage of the caffeic ester linked

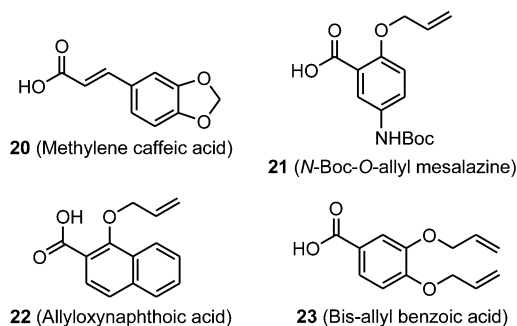
### Scheme 1. Synthesis of Conjugate 1<sup>a</sup>



<sup>a</sup>Reagents and conditions: (i) EDCI, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, 25 °C, 1.5 h; 78%; (ii) KOH (1 M aqueous solution), THF, 25 °C, 1.5 h; (iii) Pd(PPh<sub>3</sub>)<sub>4</sub>, morpholine, THF, 25 °C, 4 h; (iv) TFA, CH<sub>2</sub>Cl<sub>2</sub>, 25 °C, 3 h; 56% overall yield for steps ii–iv.

to ZA. Palladium-catalyzed deallylation was carried out, and the intermediate was subsequently treated with TFA to remove the acetonide and Boc protecting groups, giving conjugate 1 in a reasonable yield.

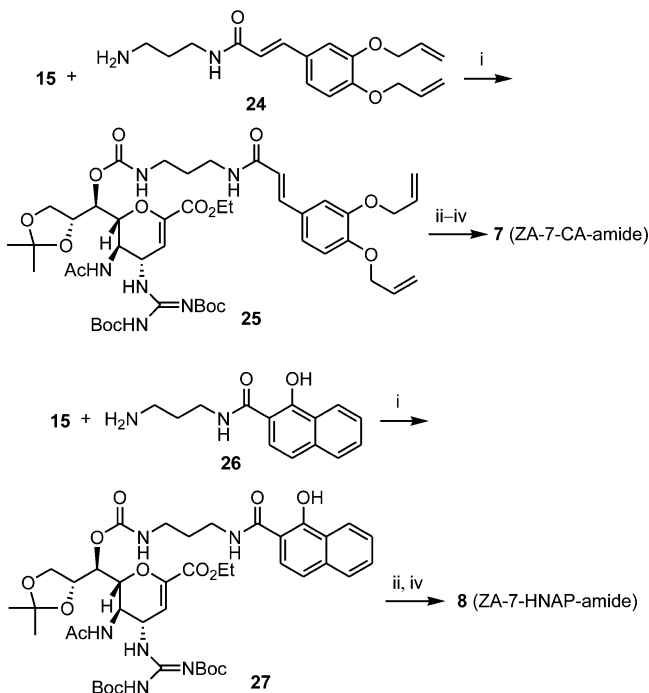
By similar procedures, compound 16 was coupled with 3,4-(methylenedioxy)cinnamic acid (MCA, 20), ME allyl ether (21), 1-hydroxy-2-naphthoic acid (HNAP) allyl ether (22), and 3,4-dihydroxybenzoic acid (DHBA) bis-allyl ether (23), respectively, and eventually led to the corresponding conjugates 2–5.



In another approach, the CA derivative 24 bearing a terminal amino group was prepared and then reacted with carbonate 15 to give a carbamate product 25 (Scheme 2). Conjugate 7 was obtained after all the protecting groups were removed. Conjugate 8 was similarly synthesized via the coupling reaction of carbonate 15 with the amine-annexed HNAP derivative 26, followed by removal of the protecting groups (Scheme 2). The conjugates 7 and 8 with amide linkage are considered more stable in a biological system than the conjugates 1–5 with ester linkage.

To prepare conjugates 9–12, alcohol 17 was subject to the coupling reactions with appropriate acids 18, 20, 21, and 22, respectively, followed by removal of the protecting groups.

**Inhibition of Influenza Virus Neuraminidase Activities by ZA Conjugates.** The NA inhibition activities of ZA and its conjugates 1–12 were evaluated using a fluorogenic substrate 2'-(4-methyl-umbelliferyl)- $\alpha$ -D-*N*-acetylneuraminic acid (MUNANA) against the NA of influenza A/WSN/1933 (H1N1) viruses (Table 1). The ZA-7-conjugates 1–8 exhibited NA

Scheme 2. Synthesis of Conjugates 7 and 8<sup>a</sup>

<sup>a</sup>Reagents and conditions: (i) Et<sub>3</sub>N, CH<sub>3</sub>CN, 25 °C, 2 h; (ii) KOH (1 M aqueous solution), THF, 25 °C, 1.5 h; (iii) Pd(PPh<sub>3</sub>)<sub>4</sub>, morpholine, THF, 25 °C, 4 h; (iv) TFA, CH<sub>2</sub>Cl<sub>2</sub>, 25 °C, 4 h. Overall yield: 39% for conjugate 7, 38% for conjugate 8.

**Table 1. Influenza NA Inhibition (IC<sub>50</sub>) and Anti-influenza Activity (EC<sub>50</sub>) against A/WSN/1933 (H1N1) Virus**

compd <sup>a</sup>	IC <sub>50</sub> (nM) <sup>b</sup>	EC <sub>50</sub> (nM) <sup>b</sup>	cLogP <sup>c</sup>
ZA	2.4–7.0 (8)	3.5–20.2 (8)	-4.13
CA	>10 <sup>5</sup>	>10 <sup>5</sup>	1.42
MCA	>10 <sup>5</sup>	>10 <sup>5</sup>	2.53
ME	>10 <sup>5</sup>	>10 <sup>5</sup>	0.46
HNAP	>10 <sup>5</sup>	>10 <sup>5</sup>	3.29
ZA-7-CA (1)	2.9–7.4 (8)	1.4–10.6 (8)	-1.63
ZA-7-MCA (2)	5.4–7.9 (3)	66, 146	-0.52
ZA-7-ME (3)	8.6	3.3, 7.9	-2.26
ZA-7-HNAP (4)	0.7–3.6 (3)	50–168 (3)	0.65
ZA-7-DHBA (5)	2.9	53	-1.13
ZA-7-HP (6)	11.7, 19.8	105, 371	-3.54
ZA-7-CA-amide (7)	41.3–60.3 (3)	5.1, 5.9	-2.36
ZA-7-HNAP-amide (8)	3.1	6.6	-0.39
ZA-1-CA (9)	2853	25	-3.60
ZA-1-MCA (10)	3986	28	-2.49
ZA-1-ME (11)	996	16	-3.49
ZA-1-HNAP (12)	590	48	-0.59

<sup>a</sup>All the test compounds are nontoxic to MDCK cells at the highest testing concentrations (100 μM). <sup>b</sup>IC<sub>50</sub> and EC<sub>50</sub> values were determined by nonparametric curve fitting of assay results. If more than two assays were conducted, the data of assay results are shown in ranges with number of assays in parentheses. <sup>c</sup>Calculated values of octanol–water partition coefficients using Advanced Chemistry Development (ACD/Laboratories) software V12.01.

inhibitory activities with IC<sub>50</sub> values in nanomolar range comparable to ZA. In contrast, the NA inhibitory activity of ZA-1-conjugates 9–12 deteriorated, supporting that the C-1 group of carboxylic acid played an essential role in electrostatic

interactions with the three arginine (Arg118, Arg292, and Arg371) residues in the active site of NA.<sup>40–44</sup> The anti-inflammation agents CA, MCA, and ME alone did not exhibit any NA inhibitory activity.

**In Vitro Anti-influenza Activity of ZA Conjugates.** The cytopathic prevention assays in Madin–Darby canine kidney (MDCK) cells were conducted to evaluate the anti-influenza activity of ZA conjugates. To our anticipation, the ZA-7-conjugates were potent inhibitors against human H1N1 virus, while CA, MCA, ME, and HNAP are inactive (Table 1). The ZA-1-conjugates also resumed anti-influenza activity with EC<sub>50</sub> values of 16–48 nM in the cell-based assays. In sharp contrast, our assays indicated that 3-hydroxypropyl caffeate, 3-(*tert*-butoxyamido)propyl caffeate, and chlorogenic acid (a caffeic ester formed with *L*-quinic acid) were inactive to H1N1 influenza viruses (data not shown).<sup>9</sup> Thus, the observed potent anti-influenza activity of conjugates 1 and 9 may be related to the enzymatic cleavage of the ester linkages to release the active ZA drug (see Figure S1 in Supporting Information).

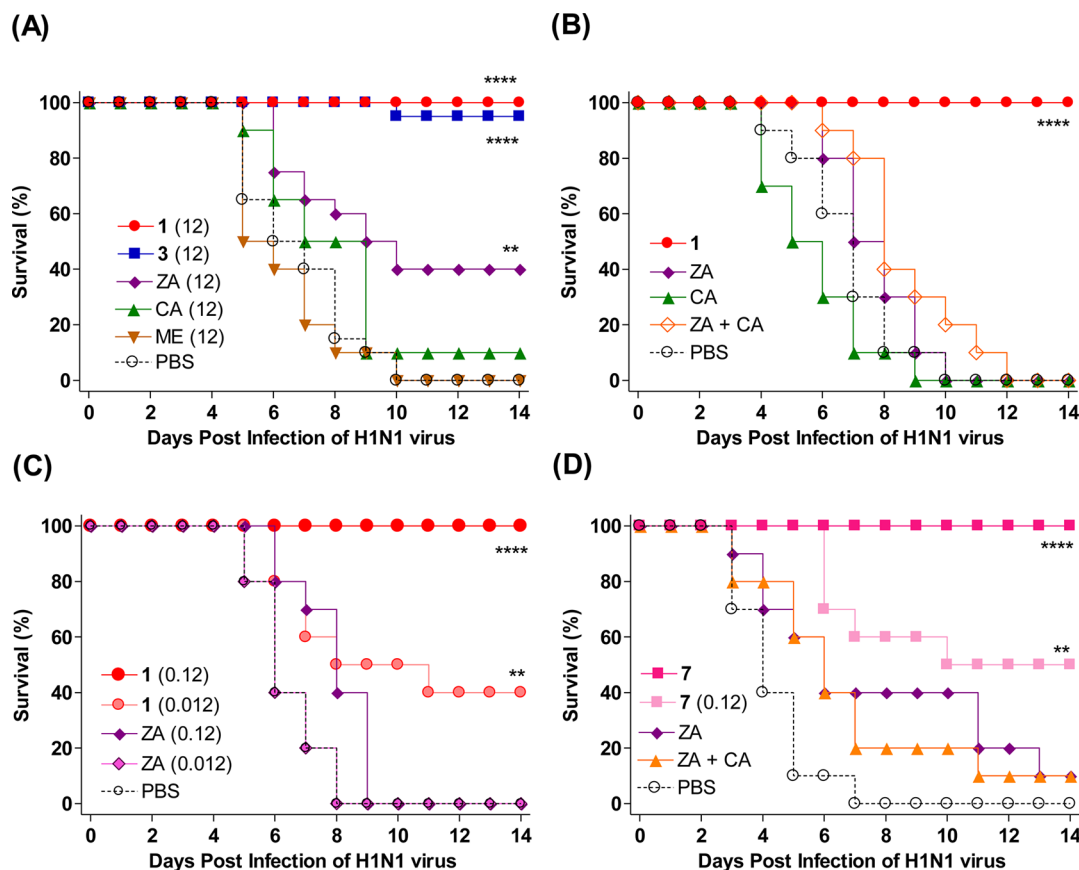
The activity against avian H5N1 influenza virus was also evaluated by the cytopathic prevention assays using the recombinant NIBRG-14 (A/VietNam/1194/2004) virus. In particular, conjugate 1 showed very high activity against the avian H5N1 virus with an EC<sub>50</sub> value of 50 nM, about 13-fold greater potency than ZA (EC<sub>50</sub> = 680 nM), implying that the inhibition may also partly come from the intact conjugate itself. Conjugates 3 and 6 showed the inhibitory activities against H5N1 virus with the EC<sub>50</sub> values of 430 and 820 nM, similar to the potency of ZA.

**Protection of Mice from Virus-Induced Death by ZA Conjugates in Animal Experiments.** ZA is an inhaled drug requiring a dose of 20 mg/50 kg/day (1.2 μmol/kg/day) by intranasal administrations twice (with half of the dosage at each administration) for treatment of influenza infected patients. Accordingly, the test compounds at the indicated dosages (12, 1.2, 0.12, 0.012, or 0.0012 μmol/kg/day) were intranasally administered to mice twice daily on days 1–4 and day 0 before infection with lethal doses (10 LD<sub>50</sub>) of influenza A/Cal/07/2009 H1N1 (Figures 4A–D) or NIBRG-14 (A/VietNam/1194/2004) H5N1 (Figure 5A,B) viruses. The survival of mice was monitored for 14 days. The synthetic conjugates 1 and 3 at a dose of 12 μmol/kg/day showed greater protection than ZA, CA, or ME in H1N1 virus infected mice (Figure 4A). The combination therapy with ZA + CA showed slight increase in survival time compared to those treated with ZA or CA alone at a dose of 1.2 μmol/kg/day, but all the mice died at day 12 except for those treated with conjugate 1 (Figure 4B). The group of mice treated with conjugate 1 at a low dose of 0.12 μmol/kg/day all survived at day 14 (Figure 4C). The survival rate of mice decreased to 40% at day 14 on treatment with an even lower dose (0.012 μmol/kg/day) of 1. However, 0.0012 μmol/kg/day of 1 did not show significant protection of mice from influenza virus infection (data not shown).

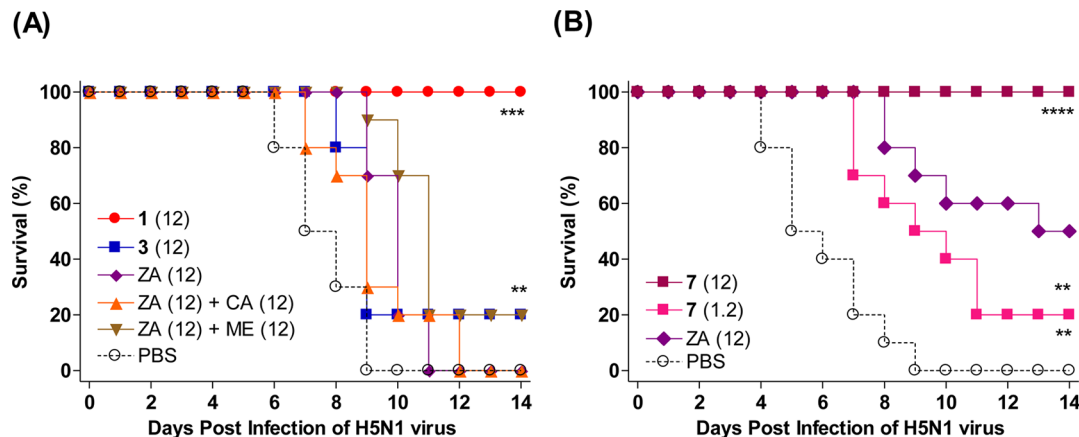
The conjugate 7 with amide linkage at a dose of 1.2 μmol/kg/day also showed 100% protection from virus-induced death, greater protection than ZA or the combination therapy of ZA + CA in treatment of the H1N1 virus infected mice (both showed 10% survival at day 14) (Figure 4D). Treatment with 0.12 μmol/kg/day of conjugate 7 led to 50% survival at day 14, but treatment with a lower dose at 0.012 μmol/kg/day did not improve the survival rate (data not shown).

The mice infected by NIBRG-14 H5N1 virus all survived by treatment with intranasal administration of conjugate 1 at 12





**Figure 4.** Survival rates of virus-infected mice on treatment with conjugates **1**, **3**, and **7**. BALB/c mice were intranasally challenged with 10 LD<sub>50</sub> of A/Cal/07/2009 (H1N1) viruses. The animal experiments were conducted with at least 10 mice per group. Compounds were administered by intranasal instillation and continued twice daily for 5 days. The administered dosages of each compound are shown in parentheses in unit of  $\mu\text{mol}/\text{kg}/\text{day}$  if the human-equivalent dose ( $1.2 \mu\text{mol}/\text{kg}/\text{day}$ ) is not used. (A)  $12 \mu\text{mol}/\text{kg}/\text{day}$  dosage, (B)  $1.2 \mu\text{mol}/\text{kg}/\text{day}$  dosage, (C) various dosages, (D) various dosages. \*\*:  $p < 0.05$ . \*\*\*:  $p < 0.001$ . \*\*\*\*:  $p < 0.0001$ .



**Figure 5.** Survival rates of virus-infected mice on treatment with conjugates **1**, **3**, and **7**. BALB/c mice were intranasally challenged with 10 LD<sub>50</sub> of NIBRG-14 (A/VietNam/1194/2004) (H5N1) viruses. The animal experiments were conducted with 10 mice per group. Compounds were administered by intranasal instillation and continued twice daily for 5 days. (A)  $12 \mu\text{mol}/\text{kg}/\text{day}$  dosage and (B)  $12$  or  $1.2 \mu\text{mol}/\text{kg}/\text{day}$  dosage. \*\*:  $p < 0.05$ . \*\*\*:  $p < 0.001$ . \*\*\*\*:  $p < 0.0001$ .

$\mu\text{mol}/\text{kg}/\text{day}$ , whereas the combination therapy ZA + CA or ZA + ME at the same dose did not improve the survival rate nor did the treatment with ZA alone (Figure 5A). Conjugate **3** did not provide better protection effect than ZA against avian H5N1 virus (Figure 5A), although this conjugate showed good activity in treatment of human H1N1 influenza (Figure 4A). Conjugate **7** at  $12 \mu\text{mol}/\text{kg}/\text{day}$  showed greater potency

against H5N1 virus in comparison with ZA alone or the combination therapy of ZA + CA (Figure 5B). On treatment with conjugate **7** at a dose of  $1.2 \mu\text{mol}/\text{kg}/\text{day}$ , improved survival rate was still noted (Figure 5B).

**Cytokine Suppression Effects of ZA Conjugates.** The potential effects of synthetic conjugates on inhibition of the production of pro-inflammatory cytokines were determined in

lipopolysaccharide (LPS)-activated RAW264.7 cells (Figure 6A). Apigenin, a flavone known to possess anti-inflammatory activity, was used in 20 or 50  $\mu\text{M}$  as a positive control (Figure 6A, lanes 3 and 4). The anti-inflammatory drugs (CA, MCA, and ME in lanes 5, 6, and 11–14) and their ZA conjugates 1 and 3 (lanes 7–10) did cause dose-dependent decreases in the mRNAs encoding IL-6 and TNF- $\alpha$ .

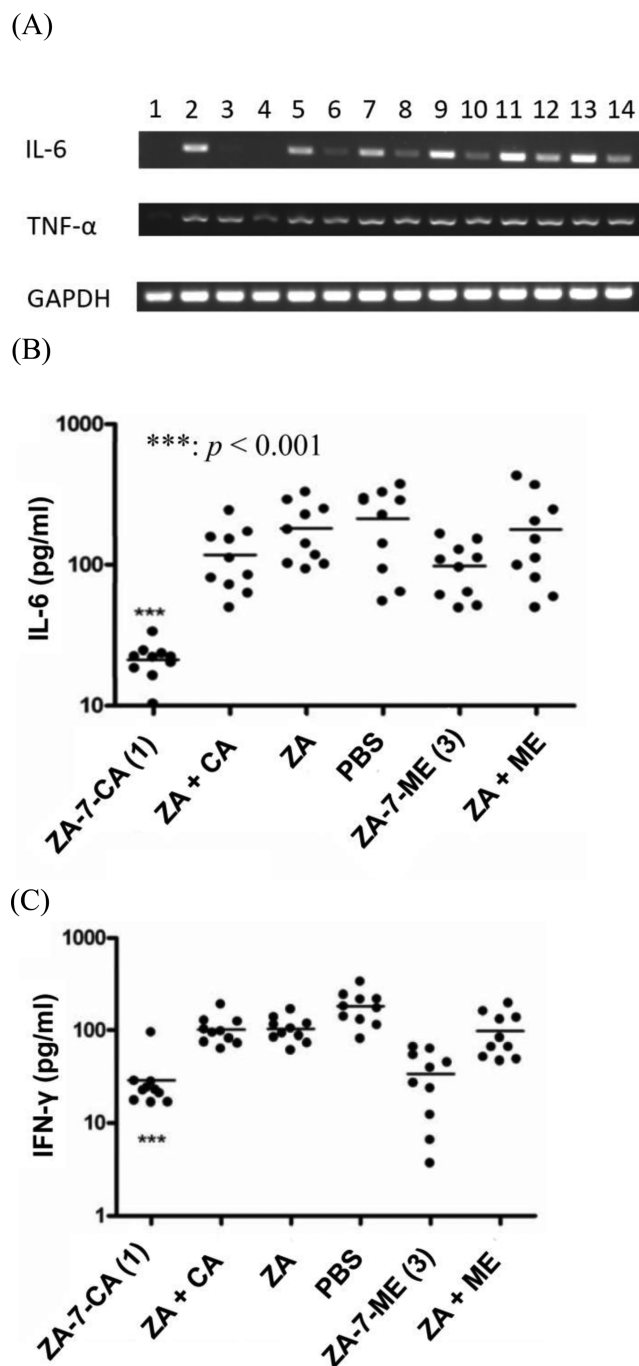
For the H5N1 virus infected mice, the test compounds at 12  $\mu\text{mol/kg/day}$  were intranasally administered and the sera samples were collected at 72 h to determine the levels of IL-6 and INF- $\gamma$ . The results indicated that the cytokine levels in the sera were lower in the groups treated respectively with conjugates 1 and 3 (Figure 6B,C) by comparison with the groups treated with the combination of ZA + CA and ZA + ME or ZA alone. These preliminary results suggested that the ZA conjugates acted to prevent the accumulation of pro-inflammatory cytokines at the transcriptional level.

## DISCUSSION

A series of ZA conjugates linked with anti-inflammatory agents were synthesized. Biological tests clearly indicated that the survival rates in the mice challenged with H1N1 or H5N1 viruses were greatly improved by treatment with the ZA conjugates, such as 1, 3, and 7, in comparison with the combination treatments with ZA and anti-inflammatory drugs. Although the detailed processes for such synergistic effect on influenza treatment is not fully resolved, some observations from our tests may give insights into the working mechanism. These dual-targeted conjugates not only inactivated influenza viruses but also decreased pro-inflammatory cytokines, as shown by the cell-based assays and mice challenge experiments. It was suggested that the ZA component in the conjugates might act as a specific binder to influenza virus and brought the anti-inflammatory component to the location where the virus-induced cytokine storm might be effectively suppressed.

The ester bonds could be hydrolyzed by endogenous esterases.<sup>45</sup> As shown in Table 1, the ZA-1-conjugates did not exhibit good NA inhibition; however, they resumed anti-influenza activity ( $\text{EC}_{50} = 16\text{--}48\text{ nM}$ ) in the cell-based assays, likely due to enzymatic cleavage of the C-1 ester to release the active ZA drug. Indeed, incubation of conjugate 12 in rat plasma at 37  $^{\circ}\text{C}$  for 24 h gave the expected degradative products of ZA and HNAP, as evidenced by the MALDI-TOF and LC-QTOF MS analyses (see Figure S1 in Supporting Information). Under the similar incubation conditions, the ester linkages in conjugate 9 were also cleaved by plasma esterases to release the components ZA and CA. The enzymatic cleavage of conjugate 1 in plasma also released a degradation product 6, showing the protonation signal at  $m/z$  434. However, conjugate 7 with amide linkage was relatively stable in plasma. Because both conjugates 1 and 7 exhibited good anti-influenza activity, enzymatic cleavage of the ester linkage in ZA-7-conjugates seemed not to be a prerequisite for the synergistic dual-targeted functions in NA inhibition and cytokine suppression.

In another aspect, ZA has poor bioavailability (estimated to be 5% in humans) due to its low lipophilicity and rapid metabolism.<sup>46</sup> Lipophilicity is a key determinant of the pharmacokinetic behavior of drugs. The partition coefficient ( $P$ ) between octanol and water is usually taken as a suitable measure of lipophilicity. The calculated value  $\text{cLogP}$  for ZA is  $-4.13$  (Table 1). The ZA-7-conjugates with CA, MCA, ME, and HNAP that contain aromatic moieties would have higher



**Figure 6.** Cytokine suppression in LPS-induced inflammation assay and in virus-infected mice. (A) RAW 264.7 cells were treated with different concentrations of anti-inflammatory drugs for 30 min before induction with lipopolysaccharide (LPS, 100 ng/mL): lane 1, negative control (no LPS); lane 2, no compound treatment; lane 3, apigenin (20  $\mu\text{M}$ ); lane 4, apigenin (50  $\mu\text{M}$ ) as a positive control; lane 5, CA (20  $\mu\text{M}$ ); lane 6, CA (50  $\mu\text{M}$ ); lane 7, conjugate 2 (20  $\mu\text{M}$ ); lane 8, conjugate 2 (50  $\mu\text{M}$ ); lane 9, conjugate 1 (20  $\mu\text{M}$ ); lane 10, conjugate 1 (50  $\mu\text{M}$ ); lane 11, MCA (20  $\mu\text{M}$ ); lane 12, MCA (50  $\mu\text{M}$ ); lane 13, ME (20  $\mu\text{M}$ ); and lane 14, ME (50  $\mu\text{M}$ ). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as an internal control. (B) The levels of cytokine IL-6 were detected in the sera collected at 72 h after the mice ( $n = 10$ ) were challenged by 10 LD<sub>50</sub> NIBRG14 (A/VietNam/1194/2004) H5N1 viruses and then treated with test compounds at a dose of 12  $\mu\text{mol/kg/day}$ . \*\*\* represents  $p < 0.001$ . (C) The levels of cytokine IFN- $\gamma$  were detected in the sera under the same conditions as those described in (B).

lipophilicity than ZA, as shown by the cLogP values of these conjugates in the range of  $-2.36$  to  $0.65$  (Table 1). The C-1 ester of ZA containing a lipophilic alkoxyalkyl moiety has been shown to exhibit significant protective effect in the mice infected by influenza virus.<sup>33</sup> In this study, the ZA-7-conjugates with increased lipophilicity may also improve pharmacokinetic properties for better anti-influenza activity.

## CONCLUSION

To treat influenza infection that is accompanied by the virus-induced cytokine storm, the drug comprising an anti-influenza moiety linked with an anti-inflammatory agent may provide a better therapy. Among the examined anti-inflammatory agents, caffeic acid is appropriate to conjugate with ZA to render potent anti-influenza activity. In this study, we found that the dual-targeted ZA conjugates **1** and **7** with covalent ester or amide linkages exhibited synergistic anti-influenza activity in comparison with ZA alone or the combination of ZA and anti-inflammatory agent.

## EXPERIMENTAL SECTION

**Compound Characterization.** New compounds were characterized by their physical and spectroscopic properties (mp, TLC,  $[\alpha]$ , IR, ESI-MS,  $^1\text{H}$  and  $^{13}\text{C}$  NMR). Purity of synthetic compounds was assessed to be  $\geq 95\%$  by HPLC analysis (Agilent HP-1100) on HC-C18 column (250 mm  $\times$  4.6 mm i.d., 5  $\mu\text{m}$  particle size) using gradients of mixed solvents A/B, whereas solvent A was  $\text{CH}_3\text{CN}$  containing 0.5% TFA and solvent B was  $\text{H}_2\text{O}$  containing 0.5% TFA, at a flow rate of 1.0 mL  $\text{min}^{-1}$  with detection at 360 or 214 nm wavelength. The gradient program was 0–20 min from A/B (5:95, v/v) to A/B (45:55, v/v) and 20–30 min from A/B (45:55, v/v) to A/B (5:95, v/v).

**Representative Procedure for the Synthesis of ZA-7-Conjugates.** The acetyl groups in compound **13** (1.60 mmol) were removed by treatment with EtONa (0.80 mmol) in EtOH (25  $^\circ\text{C}$ , 1 h). The intermediate triol compound was subject to acetalization by catalysis of *p*-toluenesulfonic acid to give compound **14** (1.06 mmol, 66%). The 7-OH group in **14** (0.8 mmol) was activated by treatment with 4-nitrophenyl chloroformate (5.6 mmol) and DMAP (5.6 mmol) in anhydrous pyridine (25  $^\circ\text{C}$ , 16 h) to give carbonate **15** (0.48 mmol, 60%), which reacted with 3-amino-1-propanol (1.02 mmol) in the presence of  $\text{Et}_3\text{N}$  (1.02 mmol) at 25  $^\circ\text{C}$  for 2 h to afford carbamate **16** (0.42 mmol, 88%).

To a solution of alcohol **16** (210 mg, 0.30 mmol) in  $\text{CH}_2\text{Cl}_2$  (10 mL) was added the allyl ether of caffeic acid **18** (86 mg, 0.33 mmol), EDCI (63 mg, 0.33 mmol), and DMAP (37 mg, 0.33 mmol). The mixture was stirred at room temperature for 1.5 h. The resulting solution was extracted with 1 M HCl, saturated  $\text{NaHCO}_3$ , and brine, dried over  $\text{MgSO}_4$ , concentrated under reduced pressure, and purified by flash chromatography on a silica gel column (EtOAc/hexane = 2:3 to EtOAc/hexane = 3:2) to afford ester **19** (240 mg, 85%).

To a solution of compound **19** (215 mg, 0.23 mmol) in THF (4 mL) was added 1 M KOH (4 mL). The solution was stirred at room temperature for 1.5 h, neutralized by Dowex 50W  $\times$  8 ( $\text{H}^+$ ), filtered, and concentrated under reduced pressure. The residue was dissolved in anhydrous THF (5 mL), and then  $\text{Pd}(\text{PPh}_3)_4$  (26 mg, 0.02 mmol) and morpholine (0.4 mL, 4.5 mmol) were added. The mixture was stirred at room temperature for 4 h. The resulting solution was extracted with 1 M HCl and brine, dried over  $\text{MgSO}_4$ , concentrated under reduced pressure, and purified by flash chromatography on a silica gel column (MeOH/ $\text{CH}_2\text{Cl}_2$  = 1:9 to MeOH/ $\text{CH}_2\text{Cl}_2$  = 1:4). The residue was dissolved in  $\text{CH}_2\text{Cl}_2$  (2 mL) and TFA (2 mL). After stirring at room temperature for 3 h, the mixture was evaporated under reduced pressure. The residue was triturated with  $\text{Et}_2\text{O}$  and centrifuged to give conjugate **1** (75 mg, 56%).  $\text{C}_{25}\text{H}_{33}\text{N}_5\text{O}_{12}$ ; yellow solid, mp 217–219  $^\circ\text{C}$ ;  $[\alpha]_D^{24} +144.9$  ( $c = 0.33$ ,  $\text{H}_2\text{O}$ ). IR  $\nu_{\text{max}}$  (neat) 3403, 1702, 1628, 1404, 1262  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$

7.55 (1 H, d,  $J = 16.0$  Hz), 7.04 (1 H, d,  $J = 1.6$  Hz), 6.94 (1 H, dd,  $J = 8.4$ , 2.0 Hz), 6.78 (1 H, d,  $J = 8.4$  Hz), 6.26 (1 H, d,  $J = 16.0$  Hz), 5.84 (1 H, d,  $J = 2.4$  Hz), 5.00 (1 H, dd,  $J = 8.8$ , 2.0 Hz), 4.57 (1 H, dd,  $J = 10.0$ , 2.0 Hz), 4.43 (1 H, dd,  $J = 6.8$ , 1.6 Hz), 4.25–4.16 (2 H, m), 4.04–3.99 (1 H, m), 3.89–3.86 (1 H, m), 3.66 (1 H, dd,  $J = 12.0$ , 3.2 Hz), 3.53–3.47 (1 H, m), 3.23–3.19 (2 H, m), 1.95 (3 H, s), 1.92–1.86 (2 H, m).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  172.3, 168.0, 163.7, 157.5, 156.6, 148.2, 145.8, 145.5, 145.4, 126.3, 121.6, 115.1, 113.7 (2 $\times$ ), 107.1, 75.9, 69.5, 69.1, 62.9, 61.6, 51.1, 48.5, 37.3, 28.6, 21.4. ESI-HRMS (negative mode) calcd for  $\text{C}_{25}\text{H}_{32}\text{N}_5\text{O}_{12}$ , 594.2047; found,  $m/z$  594.2051 [ $\text{M} - \text{H}$ ] $^-$ .

**Determination of Influenza Virus TCID<sub>50</sub>.** The TCID<sub>50</sub> (50% tissue culture infectious dose) was determined by serial dilution of the influenza virus stock onto 100  $\mu\text{L}$  of MDCK cells at  $1 \times 10^5$  cells/mL in 96-well microplates. The infected cells were incubated at 37  $^\circ\text{C}$  under 5%  $\text{CO}_2$  for 48 h and added to each wells with 100  $\mu\text{L}$  per well of CellTiter 96 AQueous Nonradioactive Cell Proliferation Assay reagent (Promega). After incubation at 37  $^\circ\text{C}$  for 15 min, absorbance at 490 nm was read on a plate reader. Influenza virus TCID<sub>50</sub> was determined using Reed–Muench method.<sup>47,48</sup>

**Determination of NA Activity by a Fluorescent Assay.** The neuraminidase (NA) activity was measured using diluted allantoic fluid harvested from influenza A/WSN/1933 (H1N1) infected embryonated eggs. A fluorometric assay was used to determine the NA activity with the fluorogenic substrate MUNANA (Sigma). The fluorescence of the released 4-methylumbelliferone was measured in a plate reader (Envision, Perkin-Elmer) using excitation and emission wavelengths of 365 and 460 nm, respectively. NA activity was determined at 200  $\mu\text{M}$  of MUNANA. Enzyme activity was expressed as the fluorescence increase during 15 min of incubation at room temperature.

**Determination of IC<sub>50</sub> of NA Inhibitor.** NA inhibition was determined by mixing inhibitor and neuraminidase for 10 min at room temperature followed by the addition of 200  $\mu\text{M}$  of substrate. Inhibitor IC<sub>50</sub> value were determined from the dose–response curves by plotting the percent inhibition of NA activity versus inhibitor concentrations using Graph Pad Prism 4.

**Determination of EC<sub>50</sub> of NA Inhibitor.** The anti-influenza activities of NA inhibitors were measured by the EC<sub>50</sub> values, the inhibitor concentrations for 50% protection of virus-induced cytopathic effects (CPE). First, 50  $\mu\text{L}$  of diluted H1N1 at 100 TCID<sub>50</sub> were mixed with equal volumes of NA inhibitors at varied concentrations. Then the mixtures were used to infect 100  $\mu\text{L}$  of MDCK cells at  $1 \times 10^5$  cells/mL in 96-wells. After 48 h incubation at 37  $^\circ\text{C}$  under 5.0%  $\text{CO}_2$ , the CPE was determined with CellTiter 96 AQueous Nonradioactive Cell Proliferation Assay reagent as described above. Inhibitor EC<sub>50</sub> value were determined by fitting the curve of percent CPE versus the concentrations of NA inhibitor using Graph Pad Prism 4.

**Animal Experiments.** Female BALB/c mice (18–20 g, 4–5 weeks old) were obtained from National Laboratory Animal Center (Taiwan). The mice were quarantined for 48–72 h before use. The mice were anesthetized by intraperitoneal injection of Zoletil before intranasal administration of drugs or inoculation of infectious influenza virus. Drugs in PBS solution (25  $\mu\text{L}$ ) were administered to groups of 10 or 20 mice by intranasal instillation twice daily for 5 days, each time at half amount of the indicated daily dosage (0.0012, 0.012, 0.12, 1.2, or 12  $\mu\text{mol}$  per kg of body weight per day). Control (placebo) mice received PBS on the same schedule. Four hours after the first dose of drug at day 0, mice were inoculated with 10 LD<sub>50</sub> of influenza virus [(A/Cal/07/2009 (H1N1)) or NIBRG-14 (A/VietNam/1194/2004 (H5N1))]. Mice were observed daily for the following 14 days for survival or bleed at 72 h post virus infection for cytokine determination.

**RNA Isolation and Real-Time Polymerase Chain Reaction (RT-PCR).** Raw264.7 cells were cultured in 6-well plates at  $3 \times 10^6$  cells per well for 24 h and treated with test compounds for 30 min, followed by addition of 100  $\text{ng mL}^{-1}$  LPS for another 6 h. The cells were collected and stored at  $-80$   $^\circ\text{C}$  until use. Total RNA was extracted using Trizol (Invitrogen) and processed for RT-PCR as described previously.<sup>49</sup>



**Cytokine Determination by Enzyme-Linked Immunosorbent Assay (ELISA).** The concentrations of IL-6 and IFN- $\gamma$  in the mice serum were assayed using the Quantikine mouse ELISA kits (R&D Systems) according to the manufacturer's protocol.

**Incubation of ZA Conjugates in Plasma and MS Analyses.** Sprague–Dawley rat plasma (550  $\mu$ L) and 50  $\mu$ L of analyte were mixed to yield a final concentration of 1 mM. The mixture was incubated at 37 °C for 24 h, extracted with methanol (3.6 mL) by vortex mixing at 4 °C for 2 h, and then subjected to centrifugation at 10000 rpm for 20 min. The precipitate was removed by filtration. The filtrate was concentrated under reduced pressure, and the content of enzymatic cleavage products was analyzed by MALDI-TOF MS or LC-QTOF MS.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Incubation of zanamivir conjugates in rat plasma and the mass spectrometric analyses, material and methods, synthetic procedures and compound characterization,  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### ■ Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

CA, caffeic acid; COX, cyclooxygenase; CPE, cytopathic effect; DHBA, 3,4-dihydroxybenzoic acid; DML, designed multiple ligands; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNAP, 1-hydroxy-2-naphthoic acid; HP, 3-hydroxypropyl; IFN, interferons; IL, interleukin; IP, interferon  $\gamma$ -induced protein; LPS, lipopolysaccharide; MCA, 3,4-(methylenedioxy)cinnamic acid; MDCK, Madin–Darby canine kidney; ME, mesalazine; MIG, monokine induced by interferon gamma; MUNANA, 2'-(4-methyl-umbelliferyl)- $\alpha$ -D-N-acetylneuraminic acid; NA, neuraminidase; PPAR, peroxisome proliferator-activator receptor; QTOF, quadrupole time-of-flight; RT-PCR, real-time polymerase chain reaction; TCID, tissue culture infectious dose; ZA, zanamivir

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