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Intramolecular ion-pair prodrugs of zanamivir and guanidino-oseltamivir

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ABSTRACT

Zanamivir (ZA) is a potent anti-influenza drug, but it cannot be administrated orally because of the hydrophilic carboxylate and guanidinium groups. Guanidino-oseltamivir (GO) is another effective neuraminidase inhibitor with polar guanidinium group under physiological conditions. The ester prodrugs ZA–HNAP (**5**) and GO–HNAP (**6**) were prepared to incorporate a 1-hydroxy-2-naphthoic (HNAP) moiety to attain good lipophilicity in the intramolecular ion-pairing forms. ZA–HNAP resumed high anti-influenza activity (EC_{50} = 48 nM), in cell-based anti-influenza assays, by releasing zanamivir along with nontoxic HNAP. Under similar conditions, the hydrolysis of the GO–HNAP ester was too sluggish to show the desired anti-influenza activity.

1. Introduction

Influenza is a respiratory infection that causes severe health problem. The worldwide spread of the H5N1 avian flu and the outbreak of the new type H1N1 human flu in 2009 have increased public awareness of the potential for global influenza pandemics. Zanamivir (ZA, **1a**) is a potent neuraminidase (NA) inhibitor against influenza virus, known as Relenza™ on market.[1] However, ZA containing carboxylate and guanidinium groups of high hydrophilicity, with calculated distribution coefficient *c* log *P* = −4.13, greatly reduces its oral bioavailability (estimated to be 5% in humans).[2] As a result, ZA is administered intranasally, which increases risk of bronchospasms in patients having asthma and chronic obstructive pulmonary disease.[3] Oseltamivir carboxylic acid (OC, **2a**) is another potent neuraminidase inhibitor against both influenza A and B viruses.[4] Oseltamivir is the ethyl ester (OC–Et, **2b**); its phosphate salt is an orally administered prodrug known as Tamiflu™.[5,6] However, the extensive use of Tamiflu in treatment of influenza has caused serious problem due to the rapidly developed oseltamivir-resistant virus strains.[7,8] The resistance of influenza virus to ZA is relatively rare in comparison with Tamiflu.[7,9] By replacement of the amino group at C-5 position with a more basic guanidine group, the guanidino oseltamivir carboxylic acid (GOA, **3a**) exerts stronger electrostatic interactions with the residues of Glu119, Asp151, and Glu227 in the active site of neuraminidase to show better inhibitory activity than OC.[10] Modification of ZA and GOA to attain good oral bioavailability is required to advance their therapeutic uses.

Like Tamiflu, many ester-containing prodrugs have been utilized to improve the intestinal permeability and oral bioavailability.[11] The ester groups in prodrugs would undergo enzymatic hydrolysis in vivo to release the corresponding carboxylic acids as the active drug. The oral treatment of a ZA derivative containing a lipophilic alkoxyalkyl ester, in lieu of the carboxylic acid group, did show significant protective effect in the mice infected by influenza virus.[12]

The prodrugs of ZA and GOA with modification at the guanidinium groups are not yet available. In another approach, 1-hydroxy-2-naphthoic acid (HNAP, **4**) is used as a counterion of the guanidinium group in zanamivir heptyl ester (ZA–Hept, **1c**) and guanidino oseltamivir (GO–Et, **3b**) to enhance the lipophilicity of these antiviral agents.[13] This ion-pairing approach is successful to enhance the effective permeability of ZA–Hept by the addition of counterion HNAP in a concentration-dependent manner, presumably because the ion-pair of ZA–Hept and HNAP remains intact during membrane permeation. However, the addition of HNAP failed to increase the effective permeability of GO–Et, presumably due to ion exchange with the competing endogenous anions.

In this study, we devised compounds **5** (ZA–HNAP) and **6** (GO–HNAP) as possible ester prodrugs containing the 1-hydroxy-2-naphthoic moiety to increase lipophilicity. Figure 1 demonstrates this strategy comprising two concepts of ester prodrug and intramolecular ion-pairing. HNAP is a FDA-approved compound for the formation of salts as active pharmaceutical ingredients.[14,15] In addition, the phenol group in HNAP is considered to be negatively charged in physiologic condition to neutralize the

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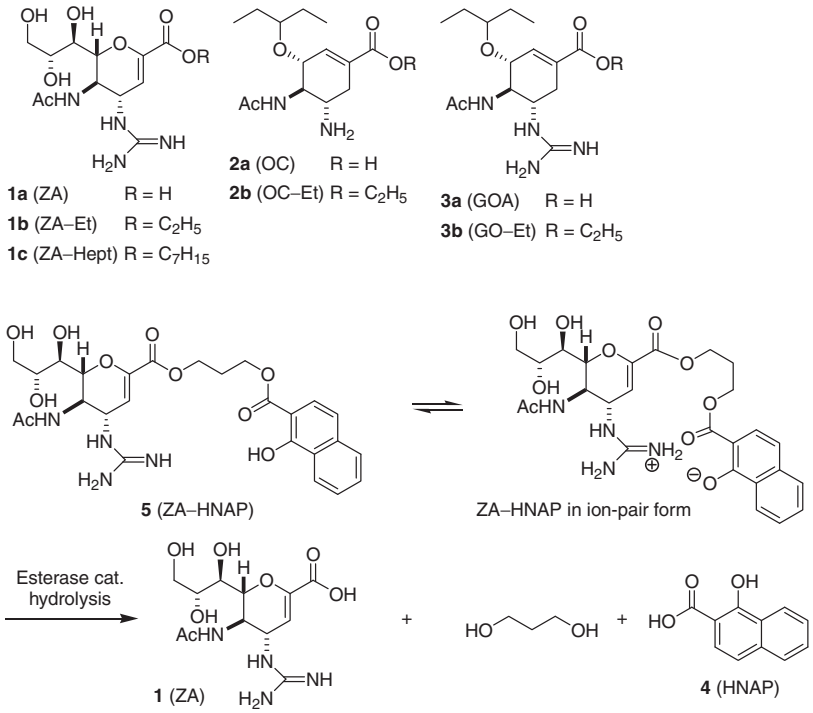
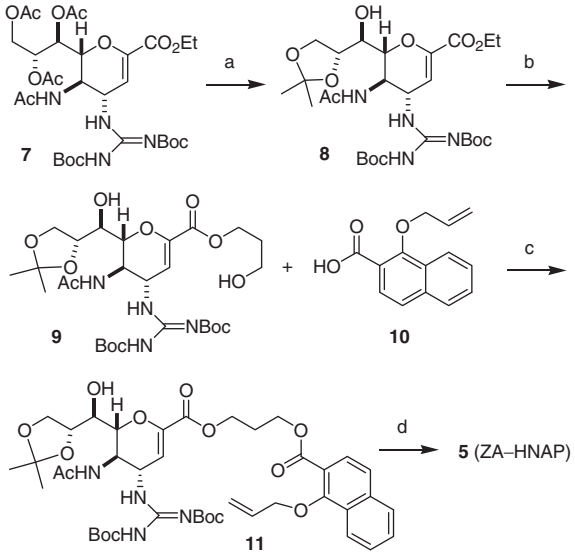


Figure 1. A strategy of using the zanamivir prodrug ZA–HNAP (**5**) that may exist as an intramolecular ion-pair to improve lipophilicity and cell membrane permeability. HNAP (1-hydroxy-2-naphthoic acid) is a FDA-approved compound for the formation of salts as active pharmaceutical ingredients. The anti-influenza drug zanamivir (**1**) will be released through esterase catalyzed hydrolysis.

positively charged guanidinium group, so that compounds **5** and **6** can form intramolecular ion-pairs to improve intestinal membrane permeation.

2. Results and discussion

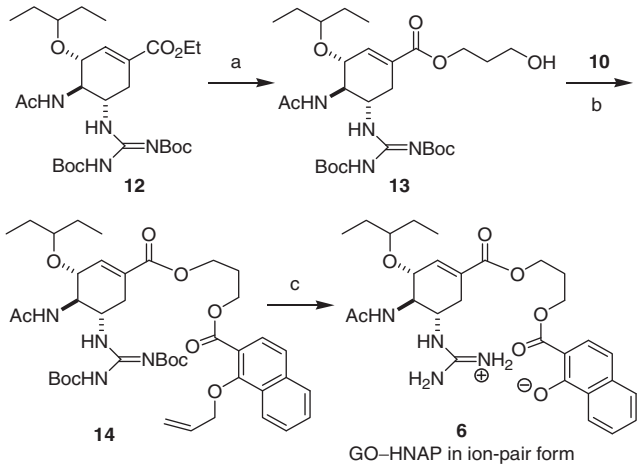
The synthesis of zanamivir–hydroxynaphthoate conjugate **5** is illustrated in Scheme 1. The free aromatic hydroxyl group in HNAP was protected by treatment with allyl bromide in the presence of anhydrous K_2CO_3, followed by saponification to give acid **10**. The protected ZA derivative **7** was prepared from sialic acid according to the previously reported method.[16] The protecting acetyl groups in **7** were removed by EtONa in EtOH, and the subsequent treatment with 2,2-dimethoxypropane selectively protected the 8,9-dihydroxy groups, giving acetonide **8**. After saponification, the potassium salt of carboxylate was reacted with 3-iodo-1-propanol to provide ester **9**. The coupling reaction of **9** and **10** culminated in ZA–HNAP (**5**) after removal of the protecting groups. GO–HNAP conjugate **6** was similarly synthesized from ethyl ester **12**[17] by a sequence of saponification with KOH, alkylation with 3-iodo-1-propanol, coupling with allyl HNAP, and removal of the allyl group (Scheme 2).



Scheme 1. Synthesis of zanamivir–hydroxynaphthoate conjugate ZA–HNAP (**5**). Reagents and conditions: (a) NaOEt, EtOH, rt, 1 h; then 2,2-dimethoxypropane, *p*-TsOH, acetone, rt, 12 h; 66%; (b) KOH(aq), MeOH, rt, 0.5 h; then 3-iodo-1-propanol, DMF, 50 °C, 4 h; 57%; (c) EDCI, DMAP, CH_2Cl_2, rt, 1.5 h; 68%; (d) $Pd(PPh_3)_4$, morpholine, THF, rt, 4 h; then $CF3CO_2H$, CH_2Cl_2, rt, 3 h; 75%.

The structures of ZA–HNAP and GO–HNAP conjugates were fully characterized by their physical and spectroscopic properties (mp, $[\alpha]_D$, UV–vis, IR, HRMS, ^1H and ^{13}C NMR). No trifluoroacetic acid could be found in these samples as shown by the absence of any fluorine signal in their ^{19}F NMR spectra. The elemental analysis of ZA–HNAP fits a molecular formula $C_{26}H_{32}N_4O_{10}\cdot H_2O$ for its monohydrate. For comparison, the ethyl esters ZA–Et (**1b**)[16,18] and GO–Et (**3b**)[17,19] were also prepared by slightly modified procedures as those reported previously. The presence of CF_3CO_2H in **1b** and **3b** was confirmed by their ^{19}F and ^{13}C NMR spectra, showing the fluorine signal at δ_F ~77 as well as the carbon signals at δ_C ~160 and ~116. Thus, ZA–Et and GO–Et existed as the salts of trifluoroacetic acid, whereas ZA–HNAP and GO–HNAP conjugates might exist as the form of intramolecular ion-pairs.

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. For an ionized compound, the distribution coefficient ($\log D$) is appropriate to represent the partition of drug between octanol and PBS buffer (pH 7.4). In general speaking, most oral drugs have the $\log P$ values between −1 and 5.[20] For example, oseltamivir



Scheme 2. Synthesis of (guanidino-oseltamivir)–hydroxynaphthoate conjugate GO–HNAP (**6**). Reagents and conditions: (a) KOH(aq), THF, rt, 0.5 h; then 3-iodo-1-propanol, DMF, rt, 5 h; 55%; (b) EDCI, DMAP, CH_2Cl_2, rt, 12 h; 60%; (c) $Pd(PPh_3)_4$, morpholine, THF, rt, 12 h; then CF_3CO_2H, CH_2Cl_2, rt, 4 h; 56%.

carboxylic acid (**2a**) exhibits a high polarity with log *D* value of –1.50 at pH 7.4 (entry 1 in Table 1). Having the carboxylic group changed to ethyl ester, oseltamivir (**2b**) becomes an oral drug with an improved lipophilicity with log *D* value of 0.36 (entry 6).[21]

In order to test our hypothesis, a series of zanamivir and guanidino oseltamivir derivatives were also subjected to log *P* calculation and log *D* determination (Table 1). The parent carboxylic acids ZA (**1a**) and GOA (**3a**) exhibited high polarities with log *D* values of −1.00 and −0.13 at pH 7.4, respectively (entries 1 and 7). The lipophilicity of ethyl esters **1b** and **3b** increased, showing log *D* values of −0.28 and 0.31 at pH 7.4, respectively (entries 2 and 8). In mild acidic condition (pH 6.5), the lipophilicity of ZA ester would be decreased, as shown in the low log *D* value (−1.31) for ZA heptyl ester (**1c** in entry 3), presumably due to protonation of its guanidine moiety. HNAP had an aromatic moiety to impart high lipophilicity to the ZA–HNAP and GO–HNAP conjugates as supported by their relatively high log *D* values of 0.75 and 0.82 (entries 4 and 9). The intramolecular pairing effect of phenoxide with guanidinium might also partially contribute to the high lipophilicity of ZA–HNAP and GO–HNAP with good log *D* values required for the development of oral drugs.

The potency of the new ZA–HNAP and GO–HNAP conjugates against the N1 neuraminidases from influenza A/WSN/1933 (H1N1) virus and their anti-influenza activity using cytopathic

Table 1
c log *P* and log *D* of zanamivir and oseltamivir derivatives

Entry	Compound	*c* Log *P*[a]	Log *D* (pH 7.4)[b]
1	1a, ZA	−4.13	−1.00 ± 0.23
2	1b, ZA–Et	−3.83	−0.28 ± 0.01
3	1c, ZA–Hept	0.69	ND[c] (−1.31)[d]
4	5, ZA–HNAP	−0.59	0.75 ± 0.08
5	2a, OC	0.45	ND[c] (−1.50)[e]
6	2b, OC–Et	1.49	ND[c] (0.36)[e]
7	3a, GOA	−0.22	−0.13 ± 0.05
8	3b, GO–Et	0.83	0.31 ± 0.05 (−1.17)[d]
9	6, GO–HNAP	4.07	0.82 ± 0.13
10	4, HNAP	3.29	0.16 ± 0.03 (0.25)[f]

[a] Calculated values using Advanced Chemistry Development (ACD/Labs) Software V12.01.
[b] Octanol–water partition coefficient of five repeated experiments.
[c] Not determined in this study.
[d] Log *D* at pH 6.5 (adapted from Ref. 13).
[e] Log *D* at pH 7.4 (adapted from Ref. 26).
[f] Log *D* at pH 6.5 (adapted from Ref. 27).

Table 2
Neuraminidase inhibition and anti-influenza activities of zanamivir and guanidino-oseltamivir derivatives

Entry	Compound	NA inhibition IC_{50}[a] (nM)	Anti-influenza EC_{50}[b] (nM)
1	ZA	2.7	34
2	ZA–Et	1952	106
3	ZA–Et + HNAP	1761	241
4	ZA–HNAP	590	48
5	GOA	1.4	8.0
6	GO–Et	5158	1170
7	GO–Et + HNAP	4876	1626
8	GO–HNAP	23,367	1465
9	HNAP	$>10^5$	$>10^5$

[a] Neuraminidase inhibition against influenza virus A/WSN/1933 (H1N1).
[b] Concentration of NA inhibitors for 50% protection of the cytopathic effects due to influenza (A/WSN/1933) infection.

prevention assays are summarized in Table 2. In comparison with the active zanamivir (entry 1) and guanidino oseltamivir carboxylic acid (entry 5), the esters ZA–Et, ZA–HNAP, GO–Et and GO–HNAP all showed inferior NA inhibition (entries 2, 4, 6 and 8) presumably due to loss of the electrostatic interactions with the three arginine residues (Arg118, Arg292 and Arg371) in the active site of NA. Though the anti-influenza activity of GO–HNAP conjugate (EC_{50} = 1.5 μM) was still inferior to its parental carboxylic acid (GOA), we found that the ZA–HNAP conjugate resumed a high activity (EC_{50} = 48 nM) in protection of MDCK cells from infection by H1N1 influenza virus. This result suggested the potential use of ZA–HNAP as a prodrug of zanamivir.

The ester bonds could be hydrolyzed by esterases in the blood, liver and kidney.[11] To demonstrate the esterase catalyzed hydrolysis, compound **5** (ZA–HNAP) was incubated in rat plasma at 37 °C for 24 h. The mixture was extracted by methanol and analyzed by MALDI–TOF mass spectrometry (Fig. S1 in Supplementary data). The protonated ions $[M+H]^+$ for the degradative products ZA (*m/z* 333) and HNAP (*m/z* 189) were observed along with disappearance of the parental ZA–HNAP. This result was also confirmed by LC–QTOF MS analysis (Fig. S2 in Supplementary data). The anti-influenza activity of ZA–HNAP was thus attributable to the efficient release of the active ZA drug during incubation in the cells. The hydrolysis of GO–HNAP was slow as shown by the MALDI–TOF and LC–QTOF mass analyses (Figs. S3 and S4 in Supplementary data). After 24 h incubation, a considerable content of GO–HNAP still remained along with the degradative products of GOA and HNAP. This phenomenon might partially explain why the anti-influenza activity of GO–HNAP was weak under the indicated incubation conditions.

3. Conclusion

Zanamivir is a potent anti-influenza drug with poor oral bioavailability due to its low lipophilicity caused by the polar carboxylic acid and guanidine groups. In search of a way to circumvent this problem, a hydroxynaphthoate conjugate ZA–HNAP (**5**) was considered to act as an ester prodrug having an additional advantage by intramolecular ion-pairing between the phenoxide and guanidinium groups to increase the lipophilicity. ZA–HNAP was prepared and characterized by physical and spectral methods. Unlike the corresponding ethyl ester (ZA–Et, **1b**) prepared as the TFA salt, ZA–HNAP existed as an intramolecular ion-pair without complexation with external acid. Our preliminary study showed that the ZA–HNAP conjugate underwent esterase catalyzed hydrolysis to release nontoxic HNAP compound and the active anti-influenza drug zanamivir. The ZA–HNAP conjugate appeared to have a suitable lipophilicity (log *D* = 0.75) for oral drug development.

4. Experimental section

4.1. General

Melting points were recorded on a Yanaco or Electrothermal MEL-TEMP 1101D apparatus in open capillaries and are not corrected. Optical rotations were measured on digital polarimeter of Japan JASCO Co. DIP-1000; $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}. Infrared (IR) spectra were recorded on Nicolet Magna 550-II or Thermo Nicolet 380 FT-IR spectrometers. UV–vis spectra were measured on a Perkin Elmer Lambda 35 spectrophotometer; extinction coefficients (ε) are given in units of M^{-1} cm^{-1}. Nuclear magnetic resonance (NMR) spectra were obtained on Bruker Advance-400 (400 MHz) spectrometer. Chemical shifts (δ) are given in parts per million (ppm) relative to δ_H 7.24/δ_C 77.0 (central line of *t*) for $CHCl_3/CDCl_3$, δ_H 4.80 for H_2O/D_2O, δ_H 3.31/δ_C 48.2 for CD_3OD, or δ_H 2.49/δ_C 39.5 for DMSO-d_6. The splitting patterns are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (double of doublets) and br (broad). Coupling constants (*J*) are given in Hz. Distortionless enhancement polarization transfer (DEPT) spectra were taken to determine the types of carbon signals. The ESI-MS experiments were conducted on a Bruker Daltonics BioTOF III high-resolution mass spectrometer. The MALDI-TOFMS measurements were performed on a Bruker Daltonics Ultraflez II MALDI-TOF/TOF 2000 mass spectrometer. The MALDI matrix 2,5-dihydroxybenzoic acid (DHB) was photoionized at different irradiances of a UV laser with λ_{max} at 337 or 355 nm. The LC–QTOF MS analyses were performed using a nanoACQUITY UltraPerformance LC system coupled to a Q-TOF Premier (both from Waters Corp., Milford, MA). Element analysis data were acquired using HERAEUS VarioEL-III.z

4.2. Materials

All reagents were reagent grade and used without further purification unless otherwise specified. All solvents were anhydrous grade unless indicated otherwise. CH_2Cl_2 was distilled from CaH_2. All non-aqueous reactions were performed in oven-dried glassware under a slight positive pressure of argon unless otherwise noted. Reactions were magnetically stirred and monitored by thin-layer chromatography on silica gel using aqueous *p*-anisaldehyde as visualizing agent. Silica gel (0.040–0.063 mm particle sizes) and LiChroprep RP-18 (0.040–0.063 mm particle sizes) were used for column chromatography. Flash chromatography was performed on silica gel of 60–200 μm particle size.

Influenza A/WSN/1933 (H1N1) virus was obtained from Dr. Shin-Ru Shih at Chang Gung University in Taiwan. All Viruses were cultured in the allantoic cavities of 10-day-old embryonated chicken eggs for 72 h, and purified by sucrose gradient centrifugation. Madin-Darby canine kidney (MDCK) cells were obtained from American Type Culture Collection (Manassas, Va), and were grown in DMEM (Dulbecco's modified Eagle medium, GibcoBRL) containing 10% fetal bovine serum (GibcoBRL) and penicillin-streptomycin (GibcoBRL) at 37 °C under 5% CO_2. Sprague Dawley® rat plasma was obtained from the Development Center for Biotechnology in Taiwan.

4.3. Synthetic procedure and compound characterization

4.3.1. 1-(2-Propenoxy)-2-naphthoic acid (10)

To a solution of 1-hydroxy-2-napthoic acid **4** (1.04 g, 5.5 mmol) and allyl bromide (1.4 mL, 16.5 mmol) in anhydrous acetone (12 mL) was added finely powdered K_2CO_3 (2.28 g, 16.5 mmol). The mixture was stirred for 4 h at 60 °C, and then concentrated under reduced pressure. The residue was partitioned between EtOAc and 1 M HCl. The organic layer was washed with saturated $NaHCO_3$ and brine, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation under reduced pressure. The crude material in MeOH (10 mL) was added 1 M NaOH (1 mL), stirred for 4 h at 60 °C, and then concentrated under reduced pressure. The mixture was diluted with water (20 mL), washed with ether, acidified with 1 M HCl, and then extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation under reduced pressure. The residue was purified by silica gel column chromatography (EtOAc/hexane = 3:7) to yield the allyl ether **10** (942 mg, 75%). $C_{14}H_{12}O_3$; yellow solid, mp 99–101 °C; TLC (EtOAc/hexane = 3:7) R_f = 0.18; IR ν_{max} (neat) 3455, 1738, 1365, 1232, 1228 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (1H, d, *J* = 9.6 Hz), 8.03 (1H, d, *J* = 8.4 Hz), 7.83 (1H, dd, *J* = 8.4, 1.2 Hz), 7.63 (1H, d, *J* = 8.8 Hz), 7.60–7.52 (2H, m), 6.28–6.17 (1H, m), 5.50 (1H, dd, *J* = 17.2, 1.0 Hz), 5.37 (1H, dd, *J* = 10.4, 1.0 Hz), 4.71 (2H, d, *J* = 6.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.2, 157.1, 137.2, 132.5, 128.8, 128.0, 127.8, 126.7, 126.6, 124.3, 123.3, 119.2, 118.2, 77.4; ESI–HRMS (negative mode) calcd for $C_{14}H_{11}O_3$: 227.0708, found: *m/z* 227.0715 $[M-H]^-$.

4.3.2. Ethyl ester of zanamivir as the salt of trifluoroacetic acid (1b)

Ethyl ester **7** was prepared from sialic acid by a slightly modified procedure as that reported for the corresponding methyl ester.[16,22,23] A solution of ester **7** (33 mg, 0.06 mmol) and NaOEt (2 mg, 0.03 mmol) in anhydrous ethanol (3 mL) was stirred at room temperature for 1 h to remove the acetyl protecting groups. The mixture was neutralized by Dowex 50W × 8 (H^+), filtered, and concentrated under reduced pressure to afford a crude product. The yellow solid crude product was dissolved in CH_2Cl_2 (2 mL) and treated with TFA (2 mL). The mixture was stirred at room temperature for 1 h, and then evaporated under reduced pressure. The residue was triturated with Et_2O, and the precipitate was collected by centrifugation to give **1b** (18 mg, 91%). $C_{14}H_{24}N_4O_7$; colorless solid, mp 130–132 °C; UV–vis (MeOH) λ_{max} 260 nm (ε = 30,990); UV–vis (PBS) λ_{max} 220 nm (ε = 7320); IR ν_{max} (neat) 3405, 1673, 1377, 1280, 1202, 1138 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 5.86 (1H, d, *J* = 2.0 Hz), 4.51 (1H, d, *J* = 8.4 Hz), 4.41 (1H, d, *J* = 10.0 Hz), 4.28–4.17 (3H, m), 3.87–3.81 (2H, m), 3.70–3.66 (2H, m), 2.01 (3H, s), 1.30 (3H, t, *J* = 5.6 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 173.5, 162.5, 159.9 (CO_2 of TFA, q, *J* = 36.2 Hz), 157.9, 145.6, 116.1 (CF_3 of TFA, q, *J* = 285.8 Hz), 107.7, 77.0, 70.3, 69.2, 63.7, 61.8, 50.4, 47.0, 21.6, 13.4; ^{19}F NMR (400 MHz, CD_3OD) δ −77.5; ESI-HRMS calcd for $C_{14}H_{25}N_4O_7$: 361.1723, found: *m/z* 361.1725 $[M+H]^+$.

4.3.3. 5-Acetylamino-4-[N^2,N^3-bis(*tert*-butoxycarbonyl) guanidino]-6-[(2,2-dimethyl-1,3-dioxolan-4-yl)-hydroxymethyl]-5,6-dihydro-4*H*-pyran-2-carboxylic acid ethyl ester (8)

To a solution of **7** (1100 mg, 1.6 mmol) in anhydrous ethanol (10 mL) was added NaOEt (54.4 mg, 0.8 mmol). The mixture was stirred at room temperature for 1 h, neutralized by Dowex 50W×8 (H^+), filtered, and concentrated under reduced pressure. The residue was dissolved in anhydrous acetone (10 mL), and *p*-toluenesulfonic acid (28 mg, 0.16 mmol) and 2,2-dimethoxypropane (10 mL) were added. The mixture was stirred at room temperature for 12 h. After concentration, the residue was purified by silica gel column chromatography (EtOAc/hexane = 2:3) to afford the product **8** (635 mg, 66%). $C_{27}H_{44}N_4O_{11}$; white solid, mp 118–120 °C; TLC (EtOAc/hexane = 2:3) R_f = 0.22; $[\alpha]_D^{20}$ −33.4 (*c* 1.0, $CHCl_3$); IR ν_{max} (neat) 3309, 2981, 1725, 1647, 1609, 1559, 1369, 1251, 1151 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.30 (1H, s), 8.54 (1H, d, *J* = 7.2 Hz), 7.91 (1H, d, *J* = 4.4 Hz), 5.71 (1H, d, *J* = 2.4 Hz), 5.21 (1H, br s), 5.08–5.05 (1H, m), 4.33–4.30 (1H, m), 4.19–4.13 (2H, m), 4.11–4.00 (2H, m), 3.98–3.88 (1H, m), 3.45

(1H, d, J = 3.6 Hz), 1.93 (3H, s), 1.44 (9H, s), 1.41 (9H, s), 1.34 (3H, s), 1.28–1.22 (6H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.8, 162.2, 161.3, 157.5, 152.5, 147.0, 108.9, 106.3, 84.2, 79.9, 78.5, 74.1, 69.6, 67.3, 61.4, 51.9, 48.3, 28.1 (3×), 27.9 (3×), 26.9, 25.1, 22.8, 13.9; ESI–HRMS (negative mode) calcd for $C_{27}H_{43}N_4O_{11}$: 599.2928, found: m/z 599.2926 $[M-H]^-$.

4.3.4. 5-Acetylamino-4-[N^2,N^3-bis(*tert*-butoxycarbonyl) guanidino]-6-[(2,2-dimethyl-1,3-dioxolan-4-yl)-hydroxy-methyl]-5,6-dihydro-4*H*-pyran-2-carboxylic acid 3-hydroxypropyl ester (9)

To a solution of ester **8** (480 mg, 0.8 mmol) in MeOH (5 mL) was added KOH (90 mg, 1.6 mmol). The mixture was stirred at room temperature for 30 min, and then concentrated under reduced pressure to give a potassium salt which was then dissolved in DMF (5 mL) and treated with 3-iodo-1-propanol (93 μL, 1.0 mmol). After stirring at 50 °C for 4 h, the mixture was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (20 mL), and extracted with 1 M HCl and brine, dried over $MgSO_4$, concentrated under reduced pressure, and purified by flash chromatography on a silica gel column (EtOAc/hexane = 1:1 to 4:1) to afford ester **9** (287 mg, 57%). $C_{28}H_{46}N_4O_{12}$; colorless solid, mp 86–88 °C; TLC (EtOAc/hexane = 4:1) R_f = 0.48; $[\alpha]_D^{21}$ −23.1 (c 1.0, $CHCl_3$); IR ν_{max} (neat) 3275, 2931, 1727, 1646, 1611, 1558, 1150 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.32 (1H, s), 8.58 (1H, d, J = 7.6 Hz), 7.96 (1H, d, J = 0.8 Hz), 5.76 (1H, d, J = 2.0 Hz), 5.26 (1H, d, J = 4.4 Hz), 5.12–5.08 (1H, m), 4.34–4.28 (2H, m), 4.14–4.10 (1H, m), 4.07–3.91 (3H, m), 3.68 (2H, t, J = 6.0 Hz), 3.47–3.44 (1H, m), 1.96 (3H, s), 1.90–1.87 (2H, m), 1.46 (9H, s), 1.44 (9H, s), 1.37 (3H, s), 1.31 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.9, 162.2, 161.6, 157.5, 152.6, 146.7, 109.1, 107.0, 84.3, 80.0, 78.5, 74.0, 69.7, 67.4, 62.7, 59.1, 51.7, 48.3, 31.4, 28.1 (3×), 27.9 (3×), 26.9, 25.1, 22.9; ESI-HRMS calcd for $C_{28}H_{47}N_4O_{12}$: 631.3190, found: m/z 631.3187 $[M+H]^+$.

4.3.5. Compound 11

To a solution of ester **9** (117 mg, 0.19 mmol) in CH_2Cl_2 (3 mL) was added acid **10** (43 mg, 0.19 mmol), EDCI (36 mg, 0.19 mmol) and 4-dimethylaminopyridine (21 mg, 0.19 mmol). The mixture was stirred at room temperature for 1.5 h, was and extracted with 1 M HCl, saturated $NaHCO_3$ and brine. The organic phase was dried over $MgSO_4$, concentrated under reduced pressure, and purified by flash chromatography on a silica gel column (EtOAc/hexane = 1:4 to 2:3) to afford ester **11** (105 mg, 68%). $C_{42}H_{56}N_4O_{14}$; pale yellow foam; TLC (EtOAc/hexane = 2:3) R_f = 0.22; $[\alpha]_D^{219}$ −25.5 (c 1.0, $CHCl_3$); IR ν_{max} (neat) 2980, 2930, 1726, 1647, 1607, 1565, 1369, 1250, 1152, 1129 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.32 (1H, s), 8.57 (1H, d, J = 7.6 Hz), 8.22 (1H, d, J = 8.0 Hz), 7.94 (1H, d, J = 4.8 Hz), 7.81 (1H, d, J = 8.8 Hz), 7.58–7.47 (3H, m), 6.21–6.11 (1H, m), 5.75 (1H, d, J = 2.0 Hz), 5.45 (1H, dd, J = 17.2, 1.2 Hz), 5.27 (2H, d, J = 11.6 Hz), 5.09–5.04 (1H, m), 4.61 (1H, d, J = 5.6 Hz), 4.45–4.42 (2H, m), 4.38–4.33 (3H, m), 4.16–4.01 (3H, m), 3.97–3.91 (2H, m), 3.47–3.44 (1H, m), 2.18–2.14 (2H, m), 1.97 (3H, s), 1.46 (9H, s), 1.45 (9H, s), 1.37 (3H, s), 1.31 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.9, 165.9, 162.2, 161.2, 157.5, 156.9, 152.6, 146.7, 136.6, 133.6, 128.7, 128.3, 127.8, 126.5, 126.4, 123.6, 119.5, 117.6, 109.0, 106.8, 84.2, 80.0, 78.5, 76.5, 73.9, 69.7, 67.4, 62.3, 61.5, 60.3, 51.9, 48.3, 28.1 (3×), 27.9 (3×), 26.9, 25.0, 22.9, 14.1; ESI-HRMS calcd for $C_{42}H_{57}N_4O_{14}$: 841.3871, found: m/z 841.3907 $[M+H]^+$.

4.3.6. ZA–HNAP conjugate 5

To a solution of compound **11** (65 mg, 0.08 mmol) in anhydrous THF (4 mL) were added $Pd(PPh_3)_4$ (12 mg, 0.01 mmol) and morpholine (0.1 mL, 1.4 mmol). The mixture was stirred at room temperature for 4 h and extracted with 1 M HCl and brine. The organic phase was dried over $MgSO_4$, concentrated under reduced pressure, and purified by flash chromatography on a silica gel column (EtOAc/hexane = 1:4 to 1:1). The intermediate product was dissolved in CH_2Cl_2 (2 mL) and stirred with TFA (2 mL) at room temperature for 3 h. The mixture was evaporated under reduced pressure. The residue was triturated with Et_2O, and the precipitate was collected by centrifuge to give the ZA–HNAP conjugate **5** (33 mg, 75%). $C_{26}H_{32}N_4O_{10}$; pale yellow solid, mp 120–122 °C; $[\alpha]_D^{21}$ +19.3 (c 1.0, MeOH); UV–vis (MeOH) λ_{max} 316 nm (ε = 5340), 260 nm (ε = 28,990); UV–vis (PBS) λ_{max} 316 nm (ε = 4180), 260 nm (ε = 4420); IR ν_{max} (neat) 3422, 1719, 1663, 1638, 1253, 1203, 1139, 1090 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 8.35 (1H, d, J = 8.4 Hz), 7.81 (1H, d, J = 8.4 Hz), 7.77 (1H, d, J = 8.8 Hz), 7.63 (1H, m), 7.53 (1H, m), 7.33 (1H, d, J = 8.8 Hz), 5.89 (1H, d, J = 2.8 Hz), 4.54–4.51 (2H, m), 4.46–4.41 (4H, m), 4.23–4.18 (1H, m), 3.89–3.81 (2H, m), 3.72–3.67 (2H, m), 2.26–2.20 (2H, m), 2.00 (3H, s); ^{13}C NMR (100 MHz, CD_3OD) δ 173.5, 171.3, 162.4, 161.0, 158.0, 145.6, 137.8, 129.8, 127.8, 126.1, 125.0, 124.3, 123.6, 119.0, 108.0, 105.7, 77.1, 70.5, 69.3, 63.8, 62.5, 62.3, 50.4, 47.9, 28.1, 21.7; ESI–HRMS calcd for $C_{26}H_{33}N_4O_{10}$: 561.2197, found: m/z 561.2194 $[M+H]^+$. A sample of ZA–HNAP conjugate was recrystallized from EtOH/hexane for elemental analysis. Anal. Calcd for $C_{26}H_{32}N_4O_{10}\cdot H_2O$: C, 53.97; H, 5.92, N, 9.68. Found: C, 54.25; H, 5.77; N, 9.67.

4.3.7. Guanidino-oseltamivir as the salt of trifluoroacetic acid (3b)[13,19]

Compound **12** was prepared according to the previously described method.[17] A solution of compound **12** (50 mg, 0.09 mmol) in CH_2Cl_2 (3 mL) was stirred with TFA (0.1 mL) at room temperature for 4 h, was and then evaporated under reduced pressure to give guanidino-oseltamivir **3b** (40 mg, 99 %). $C_{17}H_{30}N_4O_4$; white solid; mp 187–189 °C; UV–vis (MeOH) λ_{max} 260 nm (ε = 50,630); UV–vis (PBS) λ_{max} 230 nm (ε = 8370); ^{1}H NMR (400 MHz, D_2O) δ 6.83 (1H, s); 4.36–4.34 (1H, d, J = 8.0 Hz); 4.26 (2H, q, J = 7.2 Hz), 3.96–3.91(1H, m), 3.87–3.80 (1H, m), 3.55 (1H, t, J = 5.2 Hz), 2.89 (1H, dd, J = 17.2, 4.8 Hz), 2.43 (1H, dd, J = 18.0, 10 Hz), 2.05 (3H, s), 1.62–1.43 (4H, m), 1.31 (3H, t, J = 6.8 Hz), 0.93–0.84 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 174.7, 167.7, 160.9 (CO_2 of TFA, q, J = 41.2 Hz), 159.0, 139.3, 130.0, 117.3 (CF_3 of TFA, q, J = 285.3 Hz), 84.3, 76.5, 62.6, 56.2, 52.0, 31.7, 27.6, 27.3, 23.2, 14.9, 10.2, 10.1; ^{19}F NMR (400 MHz, CD_3OD) δ −76.9; ESI-HRMS calcd for $C_{17}H_{30}N_4O_4$: 355.2344, found: m/z 355.2345 $[M+H]^+$.

4.3.8. 3-Hydroxypropyl 4-acetamido-5-[N^2,N^3-bis(*tert*-butoxy carbonyl)guanidino]-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate (13)

To a solution of **12** (221 mg, 0.42 mmol) in MeOH (5 mL) was added KOH (26 mg, 0.46 mmol). The mixture was stirred at room temperature for 30 min, and then concentrated under reduced pressure to give a potassium salt, which was dissolved in DMF (5 mL) and treated with 3-iodo-1-propanol (52 μL, 0.55 mmol). After stirring at room temperature for 5 h, the mixture was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (20 mL) and extracted with 1 M HCl and brine. The organic phase was dried over $MgSO_4$, concentrated under reduced pressure, and purified by flash chromatography on a silica gel column (EtOAc/hexane = 1:1) to afford ester **13** (137 mg, 55%). $C_{28}H_{48}N_4O_9$; colorless solid, mp 95–97 °C; TLC (EtOAc/hexane = 3:1) R_f = 0.28; $[\alpha]_D^{20}$ −68.0 (c 1.4, $CHCl_3$); IR ν_{max} (neat) 3278, 2970, 2932, 1724, 1643, 1610, 1252, 1144, 1055 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.33 (1H, s), 8.58 (1H, d, J = 8.4 Hz), 6.75 (1H, s), 6.51 (1H, d, J = 8.8 Hz), 4.34–4.28 (1H, m), 4.26–4.21 (2H, m), 4.07 (1H, m), 3.97 (1H, d, J = 7.6 Hz), 3.64 (2H, t, J = 6.4 Hz), 3.29 (1H, t, J = 5.6 Hz), 2.73–2.68 (1H, m), 2.33 (1H, dd, J = 17.6, 9.2 Hz), 1.88–1.82 (5H, m), 1.50–1.43 (22H, m), 0.86–0.78 (6H, m); ^{13}C

NMR (100 MHz, $CDCl_3$) δ 170.0, 166.0, 162.9, 156.7, 152.3, 138.4, 128.2, 83.5, 82.7, 79.5, 76.1, 61.8, 58.9, 54.3, 48.2, 31.8, 30.7, 28.4 (3×), 28.1 (3×), 26.1, 25.8, 23.4, 9.7, 9.4; ESI–HRMS calcd for $C_{28}H_{48}N_4O_9$: 585.3496, found: *m/z* 585.3500 $[M+H]^+$.

4.3.9. Compound 14

A mixture of compound **13** (177 mg, 0.30 mmol) acid **10** (90 mg, 0.39 mmol), EDCI (114 mg, 0.45 mmol) and 4-dimethylaminopyridine (34 mg, 0.30 mmol) in CH_2Cl_2 (5 mL) was stirred at room temperature for 12 h. The mixture was extracted with 1 M HCl, saturated $NaHCO_3$ and brine. The organic phase was dried over $MgSO_4$, concentrated under reduced pressure, and purified by flash chromatography on a silica gel column (EtOAc/hexane = 1:3 to 1:1) to afford compound **14** (144 mg, 60%). $C_{42}H_{58}N_4O_{11}$; colorless solid, mp 75–77 °C; TLC (EtOAc/hexane = 2:1) R_f = 0.54; $[\alpha]_D^{20}$ −60.6 (*c* 1.1, $CHCl_3$); IR ν_{max} (neat) 3278, 2973, 2933, 1723, 1644, 1614, 1416, 1336, 1232, 1141, 1054 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 11.36 (1H, s), 8.61 (1H, d, *J* = 8.0 Hz), 8.24 (1H, d, *J* = 8.0 Hz), 7.84–7.81 (2H, m), 7.62–7.51 (3H, m), 6.79 (1H, s), 6.26 (1H, d, *J* = 9.2 Hz), 6.24–6.14 (1H, m), 5.47 (1H, d, *J* = 17.2 Hz), 5.29 (1H, d, *J* = 10.4 Hz), 4.64 (2H, d, *J* = 5.6 Hz), 4.45 (2H, t, *J* = 5.6 Hz), 4.40–4.32 (3H, m), 4.13–4.07 (1H, m), 3.90 (1H, d, *J* = 6.8 Hz), 3.31–3.25 (1H, m), 2.77 (1H, dd, *J* = 19.6, 5.2 Hz), 2.38 (1H, dd, *J* = 17.2, 9.6 Hz), 2.20–2.35 (2H, m), 1.90 (3H, s), 1.47–1.48 (22H, m), 0.94–0.79 (6H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.0, 165.8, 165.5, 162.9, 156.7, 156.6, 152.3, 138.3, 136.6, 133.4, 128.6, 128.2, 128.1, 127.7, 126.4, 123.7, 123.6, 119.4, 117.6 (2×), 83.5, 82.6, 79.5, 77.3, 76.1, 61.9, 61.8, 54.4, 48.1, 30.6, 28.4, 28.2 (3×), 28.1 (3×), 26.1, 25.8, 23.4, 9.7, 9.4; ESI-HRMS calcd for $C_{42}H_{58}N_4O_{11}$: 795.4180, found: *m/z* 795.4178 $[M+H]^+$.

4.3.10. GO–HNAP conjugate 6

To a solution of **4** (74 mg, 0.09 mmol) in anhydrous THF (5 mL) was added $Pd(PPh_3)_4$ (35 mg, 0.03 mmol) and morpholine (0.1 mL, 1.4 mmol). The mixture was stirred at room temperature for 12 h, and then extracted with 1 M HCl and brine. The organic phase was dried over $MgSO_4$, concentrated under reduced pressure, and purified by flash chromatography on a silica gel column (EtOAc/hexane = 1:2). The intermediate product was dissolved in CH_2Cl_2 (3 mL) and treated with TFA (0.1 mL). After stirring at room temperature for 4 h, the mixture was evaporated under reduced pressure. The residue was triturated with Et_2O, and the precipitate was collected by centrifugation to give conjugate **6** (26 mg, 56 %). $C_{29}H_{38}N_4O_7$; white solid, mp 136–138 °C; $[\alpha]_D^{20}$ −36.4 (*c* 0.4, MeOH); UV–vis (MeOH) λ_{max} 315 nm (ε = 29,550), 270 nm (ε = 106,930); UV–vis (PBS) λ_{max} 270 nm (ε = 13,490); IR ν_{max} (neat) 3413, 2967, 1703, 1663, 1253, 1205 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 8.34 (1H, d, *J* = 8.0 Hz), 7.80 (1H, d, *J* = 8.0 Hz), 7.75 (1H, d, *J* = 8.8 Hz), 7.65–7.60 (1H, m), 7.56–7.50 (1H, m), 7.33 (1H, d, *J* = 8.4 Hz), 6.82 (1H, d, *J* = 2.0 Hz), 4.56–4.51 (2H, m), 4.40 (2H, t, *J* = 6.4 Hz), 4.17 (1H, d, *J* = 6.4 Hz), 3.91–3.84 (2H, m), 3.35–3.30 (1H, m), 2.84 (1H, dd, *J* = 16.8, 4.0 Hz), 2.38–2.33 (1H, m), 2.23 (2H, m), 1.98 (3H, s), 1.51–1.40 (4H, m), 0.09–0.84 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 172.0, 177.0, 158.4, 139.2, 138.6, 133.1, 133.0, 130.6, 130.0, 129.3, 128.7, 126.9, 125.1, 124.5, 119.9, 106.6, 83.8, 76.1, 63.7, 63.3, 55.9, 51.7, 31.5, 29.3, 27.4, 27.0, 23.0, 10.1, 9.8; ESI–HRMS calcd for $C_{29}H_{38}N_4O_7$: 555.2819, found: *m/z* 555.2819 $[M+H]^+$.

4.4. Determination of octanol-buffer partition coefficients

Solutions of each compound (100 μg/mL) were prepared in octanol saturated phosphate buffered saline (0.01 M, pH 7.4). These aqueous solutions were then equilibrated at 37 °C with an equivalent volume (0.5 mL) of buffer saturated octanol using magnetic stirring at 1200 rpm for 24 h. Five replicates of each determination were carried out to assess reproducibility. The octanol and aqueous phases were then separated by centrifugation at 3000 rpm for 15 min. Each sample (300 μL) of aqueous layer was diluted with PBS to 3 mL, and organic layer was diluted with MeOH to 3 mL. The concentration of drug was measured using UV spectrophotometry at λ_{max} (MeOH) 290 nm (ε = 14,440) and λ_{max} (PBS) 290 nm (ε = 4610) at pH 7.4 for the chromophore of 1-hydroxy-2-naphthoic acid. From these data, the apparent octanol/buffer (pH 7.4) partition coefficient, DB = [Bt]oct/[Bt]aq, is determined, where [Bt]oct and [Bt]aq are the concentrations of the drug in organic and aqueous phases,, respectively.

4.5. Incubation of conjugates in plasma and mass spectrometric analyses

Sprague Dawley® rat plasma (550 μL) and 50 μL 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 10,000 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-TOFMS.

Alternatively, the mixture resulted from incubation was subjected to LC–QTOF MS analysis. The sample was loaded onto a Sep-Pak® Plus tC18 cartridge (Waters Corp.), and flushed with deionized water (2 × 2 mL) to remove excess salts and proteins, followed by elution with MeOH/H_2O (1:1, 4 × 2 mL) to collect analyte. The analyte was evaporated to dryness, and re-dissolved in CH_3CN/H_2O (1:9, 100 μL) for LC–QTOF MS analysis on a nano Acquity UPLC BEH C18 column (1.7 μm, 75 μm × 250 mm, Waters Corp.). The injection volume was 2–5 μL and the total run time was 50 min. A mixture of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) was used with a gradient program at a flow rate of 0.3 μL/min at ambient temperature. To analyze the product mixture formed by incubation of ZA–HNAP in plasma, the gradient program of mobile phase was set: a linear increase from 10:90 to 85:15 B/A in 25 min, and kept at 85:15 B/A for 10 min. The column was washed for 15 min before returning to original conditions. To analyze the product mixture formed by incubation of GO–HNAP in plasma, the gradient program of mobile phase was set: a linear increase from 8:92 to 70:30 B/A in 25 min, from 70:30 to 85:15 B/A in 5 min, and kept at 85:15 B/A for 5 min.

4.6. Determination of influenza virus $TCID_{50}$

The $TCID_{50}$ (50% tissue culture infectious dose) was determined by serial dilution of the influenza virus stock solution onto 100 μL MDCK cells at 1 × 10^5 cells/mL in 96-well microplates. The infected cells were incubated at 37 °C under 5.0% CO_2 for 48 h and added to each wells with 100 μL per well of CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay reagent (Promega). After incubation at 37 °C for 15 min, absorbance at 490 nm was read on a plate reader. Influenza virus $TCID_{50}$ was determined using Reed-Muench method.[24,25]

4.7. Determination of neuraminidase activity by a fluorescent assay

The neuraminidase 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 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA; Sigma).

The fluorescence of the released 4-methylumbelliferone was measured in Envision plate reader (Perkin–Elmer, Wellesley, MA) using excitation and emission wavelengths of 365 and 460 nm, respectively. Neuraminidase activity was determined at 200 μM of MUNANA. Enzyme activity was expressed as the fluorescence increase during 15 min incubation at room temperature.

4.8. Determination of IC_{50} of neuraminidase inhibitor

Neuraminidase inhibition was determined by mixing inhibitor and neuraminidase for 10 min at room temperature followed by the addition of 200 μM of substrate. Inhibitor IC_{50} value were determined from the dose-response curves by plotting the percent inhibition of NA activity versus inhibitor concentrations using Graph Pad Prism 4.

4.9. Determination of EC_{50} of neuraminidase inhibitor

The anti-flu activities of neuraminidase inhibitors were measured by the EC_{50} values that were the concentrations of NA inhibitor for 50% protection of the H1N1 CPE activities. Fifty microliters diluted H1N1 at 100 $TCID_{50}$ were mixed with equal volumes of NA inhibitors at varied concentrations. The mixtures were used to infect 100 μL of MDCK cells at 1×10^5 cells/mL in 96-wells. After 48 h incubation at 37 °C under 5.0% CO_2, the cytopathic effects (CPE) were determined with CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay reagent as described above. Inhibitor EC_{50} value were determined by fitting the curve of percent CPE versus the concentrations of NA inhibitor using Graph Pad Prism 4.

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Supplementary data

Supplementary data (MS, ^1H, ^{13}C, ^{19}F NMR spectra) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.06.080.

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