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Synthesis and anti-influenza activities of carboxyl alkoxyalkyl esters of 4-guanidino-Neu5Ac2en (zanamivir)

Zong-ying Liu, Bo Wang, Li-xun Zhao, Yu-huan Li, Hua-yi Shao, Hong Yi, Xue-fu You and Zhuo-rong Li*

Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China

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Abstract—Three alkoxyalkyl 2-carboxylate ester derivatives related to zanamivir were synthesized. All of the analogs of zanamivir modified at carboxylic moiety with alkoxyalkyl esters **1a**–**c** showed higher activities than ribavirin on influenza A and B virus in the MDCK cells. Oral treatment or intraperitoneal administration of compound **1c** showed significantly protective effects in mice infected with influenza A virus with low toxicities.

Despite the advances in understanding of the molecular and cellular aspects of influenza, the disease remains the major cause of mortality and morbidity among patients with respiratory diseases. Several viral molecular targets have been identified for drug intervention including hemagglutinin, neuraminidase, M2 protein, and endonuclease.[1] Among these antiviral targets, neuraminidase (NA) has been extensively investigated for drug design. The role of NA is to cleave the terminal sialic acid residues from the receptors to allow the release of progeny viral particles from the infected cells for next infection. The NA is also thought to play a role in facilitating passage of the virus through the mucin layer in the respiratory tract. Therefore, compounds that inhibit neuraminidase protect the host from viral infection and retard its propagation.[2] Indeed, the effectiveness of NA inhibitors as anti-influenza agents has been demonstrated both in animals and in human clinical trails by several research groups and highlighted by FDA approval of zanamivir[3] and tamiflu (GS4104).[4] Zanamivir was delivered by inhalation because of its low oral bioavailability, small volume of distribution, and rapid renal elimination. Masuda et al.[5] reported the synthesis and anti-influenza effect of orally active bicyclic ether derivatives related to zanamivir. However, no report related to zanamivir 2-carboxyl alkoxyalkyl esters modified as prodrugs of zanamivir was found. Poor oral bioavailability derived from the high hydrophilicity of the molecular framework was also observed in nucleoside antiviral compounds. Masking the phosphonate functionality with alkoxyalkyl esters potentially increased their potency to allow passage through a membrane. It was reported[6] that phosphonate monoalkoxyalkyl esters of cidofovir had improved oral bioavailability and were active orally in animal models as compared to the original parent compound. Mendel et al.[7] reported that oral administration of tamiflu, an ethyl ester prodrug of GS4071, resulted in a dramatic therapeutic response in the mouse and ferret models of influenza virus infection. Zanamivir is a high hydrophilic compound with carboxyl and alkalescent guanidino groups in the structure. Short-chain carboxyl ester would not make marked improvement on its liposolubility. In order to improve its oral activity, three zanamivir alkoxyalkyl ester derivatives instead of short-chain esters were synthesized in this work using a similar methodology that was for nucleoside compounds and is described in Scheme 1. Compounds **1a**–**c** were prepared by esterifying zanamivir in dimethylsulfoxide (DMSO) with different alkoxyalkyl bromides in the presence of triethylamine.[8] The starting material zanamivir was synthesized according to the reported method,[9] alkoxyalkyl bromides used in Scheme 1 were prepared following a similar method developed by Kern et al. All of the synthetic compounds were well characterized through the spectral characteristics.[10]

Keywords: Alkoxyalkyl ester; Influenza; Zanamivir.
* Corresponding author. Tel./fax: +86 10 63027185; e-mail: l-z-r@263.net

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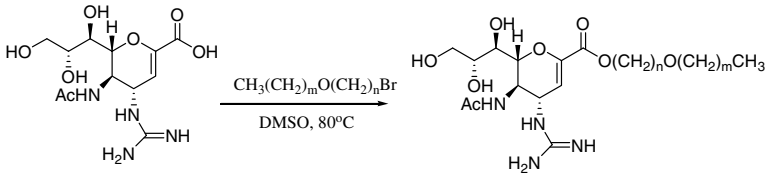

Scheme 1. Synthesis of zanamivir alkoxyalkyl ester derivatives.

Influenza virus inhibitory activity of the synthesized compounds was evaluated by inhibition of visually discerned viral cytopathic effect (CPE) assay[11] in the MDCK cells and the results are summarized in Table 1. For the four tested influenza A strains, the IC_{50} of compounds **1a–c** was in the ranges of 0.315–18.9, 0.301–18.1, and 0.226–26.8 μM, respectively; and the IC_{50} of zanamivir and ribavirin serving as controls was in the ranges of 0.075–16.7 and 5.04–176 μM accordingly. For the three tested influenza B strains, the IC_{50} of compounds **1a–c** was in the ranges of 23.3–37.8, 22.3–23.4, and 21.8–247 μM; the IC_{50} of zanamivir and ribavirin was in the ranges of 223–5015 and 15.2–164 μM. The in vitro anti-influenza evaluation results showed that compounds **1a–c** were more potent to influenza A strains than to B strains. All of the three tested compounds had stronger antiviral activities on influenza A and B than ribavirin did, and showed stronger antiviral activities on influenza B than zanamivir. However, their activities on influenza A strains were less as compared to that of zanamivir. The results were consistent with the expectation that the in vitro activities of the prodrug analogs **1a–c** were reduced in respect to their leading compound zanamivir.

The influenza A/Jingfang/FM1 (H1N1) virus infected mice[7,12] were treated with multiple dosing of compound **1c** orally (po) or intraperitoneally (ip). Ribavirin was used as a known control agent. The results are summarized in Table 2. For the mice infected with influenza A/

Table 1. Anti-influenza activities of synthesized compounds[a]

Virus	IC_{50} (μM)				
	1a	**1b**	**1c**	Zanamivir	Ribavirin
A/Yuefang/243/72 (H3N2)	0.315	0.301	0.226	0.075	5.85
A/Jifang/15/90 (H3N2)	2.49	3.01	2.94	0.469	5.04
A/Jingfang/262/95 (H1N1)	18.9	18.1	26.8	16.7	176
A/Hanfang/359/95 (H3N2)	1.06	3.06	5.89	0.385	7.01
B/Jifang/13/97	31.5	23.4	247	5015	96.2
B/Jingfang/76/98	23.3	22.3	66.6	223	15.2
B/Sichuan/83/2000	37.8	22.3	21.8	1579	164

[a] The viral tissue culture infective dose ($TCID_{50}$) values varied depending on the experiments, the $TCID_{50}$ values are 10–50 for A/Jifang/15/90 (H3N2), A/Jingfang/262/95 (H1N1), A/Hanfang/359/95 (H3N2), B/Jifang/13/97, and >100 for others.

Table 2. Effects of oral and intraperitoneal treatment[a] with **1c** on influenza A/Jingfang/FM1 (H1N1) virus infections[b] in mice

Compound	Dosage (mg/kg/day)	Toxicity controls		Infected, treated mice			
		Survivors/total	Host wt change[c] (g)	% Survivors	Mean day to death[d]	Lung consolidation[e]	Mean lung score
1c	125 (po)	5/5	+12.5	60 (6/10)*	11.2 **	1.14*	1.49 ± 0.38
	62.5 (po)	5/5	+13.5	40 (4/10)	10.2**	1.14*	1.31 ± 0.26*
	31.25 (po)	5/5	+12.7	10 (1/10)	9.1**	2.00	1.56 ± 0.21
	62.5 (ip)	5/5	+13.9	60 (6/10)*	11.5**	0.57**	0.92 ± 0.14**
Ribavirin	100 (ip)	5/5	+11.5	100 (10/10)**	14.0**	0.43**	1.09 ± 0.13**
Vehicle		5/5	+14.4	0 (0/10)	6.0	3.0	1.91 ± 0.64
Normal controls		5/5	+13.9	100 (5/5)**	14.0**	0**	0.72 ± 0.09**

[a] b.i.d. × 5, beginning 2 h after viral infection.
[b] The viral infection dose was 15.8 LD_{50}.
[c] Weight change = day 14 weight minus day 0 weight.
[d] Mean survival time of mice dying on or before day 14.
[e] Lungs were removed on day 6 post-virus exposure. Lung scores were determined based on the percentage of the lung displaying signs of consolidation, with 0 being normal and 4 indicative of 100% consolidation.
* $P < 0.05$.
** $P < 0.01$ as compared to 5% Tween 80 treated control. % Survivors were evaluated using crosstable chi-square analysis; mean day to death was evaluated using Kaplan–Meier analysis; lung consolidation was evaluated using Radit analysis; and mean lung score was compared with the values of vehicle controls using Student's *t*-test.

Jingfang/FM1 (H1N1) virus, ribavirin treatment at a single dose of 100 mg/kg/day (ip) resulted in significant increases in mean survival time and a moderate decrease in the mean of the lung score. Compound **1c** at 125 mg/kg/day (oral administration) and 62.5 mg/kg/day (intraperitoneal administration) yielded significantly increased numbers of survivors and prolonged mean survival time, whereas all of the control animals died. Lower doses of **1c** (62.5 and 31.25 mg/kg/day, orally) also increased the survival rates for the infected animals. Oral administration of **1c** provided protection against the lethal effects of influenza A/Jingfang/FM1 (H1N1) virus in a dose-dependent fashion. Effects of **1c** on the development of lung score on day 6 are also shown in Table 2. Compound **1c** at all doses used decreased the lung consolidation as compared with that of the vehicle controls. The lung scores at 125, 62.5 mg/kg/day (oral) and 62.5 mg/kg/day (intraperitoneal) were statistically improved in respect to the untreated control ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively). Compound **1c** appeared to be well tolerated by the animals, with all surviving and gaining weight.

It is well known that zanamivir has a poor bioavailability in oral administration, with only 4–17% of the agent absorbed after inhalation. This work demonstrated that the alkoxyalkyl ester prodrugs of zanamivir **1a**–**c** potentially inhibit influenza virus in cell culture and in mice. All of these compounds showed higher activities on influenza virus A and B in MDCK cells as compared to ribavirin. In influenza-infected mice, compound **1c** administered in different routes showed significantly protective effects and low toxicities. Intraperitoneal administration of **1c** with 62.5 mg/kg/day resulted in a promising therapeutic response in the mice. Tamiflu demonstrated a 10-fold potency against the influenza A/NWS/33 (H1N1) virus-induced infection in comparison with GS4071.[13] The present work showed that alkoxyalkyl esters of zanamivir, both oral and intraperitoneal administration, have potent protective effect in the influenza (H1N1) infected mice. The results indicated that alkoxyalkyl ester modification improved the bioavailabilities, because zanamivir was inactive in oral or intraperitoneal administration. On the basis of these results, it could be concluded that the alkoxyalkyl esters of zanamivir are a new class of anti-influenza agents with a potential of being effective for the treatment of human influenza A and B viral infections in oral administration.

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8. General procedure for the preparation of zanamivir alkoxyalkyl esters: zanamivir (10.5 mmol) suspended in 70 ml dimethylsulfoxide was added triethylamine (29.8 mmol). After the mixture was stirred for 30 min, alkoxyalkyl bromide (16.4 mmol) was added and the reaction mixture was stirred at 80 °C for 12 h. Then concentrated in vacuum, and the residue was purified by chromatography on silica gel column elut with 17% methanol–83% ethyl acetate to give the corresponding zanamivir alkoxyalkyl esters (**1a**–**c**) in good yields (65–80%).
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10. Physical and spectral characteristics of compound (**1a**): mp 163–165 °C. ^{1}H NMR (400 MHz, CD_3OD), δ 0.84 (t, $J = 6.8$ Hz, 3H), 1.23 (s, 22H), 1.46–1.52 (m, 2H), 1.84–1.91 (m, 2H), 1.96 (s, 3H), 3.37 (t, $J = 6.4$ Hz, 2H), 3.46 (t, $J = 6.4$ Hz, 2H), 3.61–3.65 (m, 2H), 3.74–3.83 (m, 2H), 4.15 (t, $J = 8.8$ Hz, 1H), 4.23 (t, $J = 6.4$ Hz, 2H), 4.36 (d, $J = 9.6$ Hz, 1H), 4.44 (dd, $J = 2.4$, 8.8 Hz, 1H), 5.81 (d, $J = 2.4$ Hz, 1H). ESIMS $C_{29}H_{55}N_4O_8$, calcd ($M+H^+$): 587.40199; meas.: 587.40112. $[\alpha]_D^{20}$: +9.60 (*c* 1.00, MeOH).

Compound (**1b**): mp 166–169 °C. ^{1}H NMR (400 MHz, CD_3OD), δ 0.83 (t, J = 6.8 Hz, 3H), 1.23 (s, 26H), 1.48–1.52 (m, 2H), 1.87–1.90 (m, 2H), 1.97 (s, 3H), 3.37 (t, J = 6.4 Hz, 2H), 3.46 (t, J = 6.4 Hz, 2H), 3.62–3.66 (m, 2H), 3.74–3.82 (m, 2H), 4.16 (t, J = 5.6 Hz, 1H), 4.23 (t, J = 6.4 Hz, 2H), 4.38 (d, J = 10 Hz, 1H), 4.55 (dd, J = 2.4, 8.8 Hz, 1H), 5.81 (d, J = 2.4 Hz, 1H). ESIMS $C_{31}H_{59}N_4O_8$, calcd ($M+H^+$): 615.43329; meas.: 615.42938. $[\alpha]_D^{20}$: +6.50 (*c* 1.01, MeOH).

Compound (**1c**): mp 179–182 °C. ^{1}H NMR (400 MHz, CD_3OD), δ 0.84 (t, J = 6.8 Hz, 3H), 1.23 (s, 30H), 1.49–1.52 (m, 2H), 1.97 (s, 3H), 3.43 (t, J = 6.4 Hz, 2H), 3.62–3.66 (m, 4H), 3.77–3.82 (m, 2H), 4.16 (t, J = 9.6 Hz, 1H), 4.27 (t, J = 6.4 Hz, 2H), 4.39 (d, J = 10.8 Hz, 1H), 4.52 (dd, J = 2.8, 8.8 Hz, 1H), 5.85 (d, J=2.8 Hz, 1H). ESIMS $C_{32}H_{61}N_4O_8$, calcd ($M+H^+$): 629.44894; meas.: 629.44443. $[\alpha]_D^{20}$: +6.57 (*c* 0.99, MeOH).

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