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Tamiphosphor monoesters as effective anti-influenza agents

Chun-Lin Chen [a], Tzu-Chen Lin [a], Shi-Yun Wang [b], Jiun-Jie Shie [b], Keng-Chang Tsai [c], Yih-Shyun E. Cheng [b], Jia-Tsrong Jan [b], Chun-Jung Lin [d,**], Jim-Min Fang [a,b,*], Chi-Huey Wong [b,**]

[a] Department of Chemistry, National Taiwan University, Taipei 106, Taiwan, ROC
[b] The Genomics Research Center, Academia Sinica, Taipei 115, Taiwan, ROC
[c] National Research Institute of Chinese Medicine, Taipei 112, Taiwan, ROC
[d] School of Pharmacy, National Taiwan University, Taipei 100, Taiwan, ROC

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ABSTRACT

Oseltamivir is a potent neuraminidase inhibitor for influenza treatment. By replacing the carboxylate group in oseltamivir with phosphonate monoalkyl ester, a series of tamiphosphor derivatives were synthesized and shown to exhibit high inhibitory activities against influenza viruses. Our molecular modeling experiments revealed that influenza virus neuraminidase contains a 371-cavity near the S1-site to accommodate the alkyl substituents of tamiphosphor monoesters to render appreciable hydrophobic interactions for enhanced affinity. Furthermore, guanidino-tamiphosphor (TPG) monoesters are active to the oseltamivir-resistant mutant. TPG monohexyl ester **4e** having a more lipophilic alkyl substituent showed better cell permeability and intestinal absorption than the corresponding monoethyl ester **4c**, but both compounds showed similar bioavailability. Intranasal administration of TPG monoesters at low dose greatly improved the survival rate of mice infected with lethal dose of H1N1 influenza virus, whereas **4c** provided better protection of the infected mice than oseltamivir and other phosphonate congeners by oral administration.

1. Introduction

Influenza is a highly contagious disease that occurs in seasonal epidemics and also emerges periodically to global pandemics. Influenza viruses are negative-sense single-stranded RNA viruses of the family Orthomyxoviridae. The 8 RNA gene segments replicate at least 11 proteins [1,2]. Hemagglutinin (HA) and neuraminidase (NA) are the most significant surface glycoproteins for influenza virulence. Depending on the specific strain of influenza virus, HA selectively binds to human or avian respiratory epithelial cells. Influenza NA catalyzes the hydrolysis of the terminal sialic acid residue from the sialo-receptors of host cells to facilitate the release of progeny viruses for propagation and infectivity [3].

NA is a good drug target in view of its rather conserved active site [4–6]. Four NA inhibitors zanamivir (Relenza™) [7,8], oseltamivir (Tamiflu™ as the phosphate salt) [9,10], peramivir (Rapiacta™) [11,12] and laninamivir (Inavir™) [13,14] have been approved for use as anti-influenza drugs. The phosphate salt of oseltamivir (OS, **1b**), is currently the most popular oral medication for influenza therapy. Oseltamivir is converted by endogenous esterase to oseltamivir carboxylate (OC, **1a**) which is the active component for NA inhibition [3,15,16]. The carboxylic acid in OC provides multiple electrostatic interactions with the three arginine residues (R118, R292 and R371) in NA [4–6]. However, oseltamivir-resistant viruses have occurred over the years. New anti-influenza drugs that can also inhibit oseltamivir-resistant strains, such as

Abbreviations: AUC, area under the concentration-versus-time curve; CC_{50}, 50% cytotoxicity concentration; CL, clearance; CPE, cytopathic effect; *D*, distribution coefficient; DMEM, Dulbecco's modified Eagle medium; EC_{50}, half maximal effective concentration; EDTA, ethylenediaminetetraacetic acid; *F* (%), oral bioavailability (fraction absorbed); GOC, guanidino-oseltamivir carboxylic acid; GOS, guanidino-oseltamivir; HBTU, *o*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; IC_{50}, half maximal inhibitory concentration; i.p., intraperitoneal; i.v., intravenous; LD_{50}, median lethal dose; MDCK, Madin–Darby canine kidney; MUNANA, 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid; NA, neuraminidase; OC, oseltamivir carboxylic acid; OS, oseltamivir; *P*, partition coefficient; $TCID_{50}$, 50% cell culture infectious dose; TP, tamiphosphor; TPG, guanidino-tamiphosphor.

* Corresponding author. Department of Chemistry, National Taiwan University, Taipei, 106, Taiwan, ROC.
** Corresponding authors.
E-mail addresses: clementumich@ntu.edu.tw (C.-J. Lin), jmfang@ntu.edu.tw (J.-M. Fang), chwong@gate.sinica.edu.tw (C.-H. Wong).

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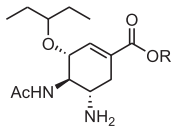
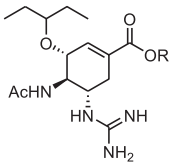
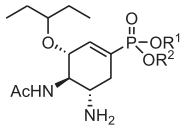
the clinically relevant H275Y mutant, are in urgent need for our fight against the threat of pandemic influenza. Compound **2a** (GOC) shows higher inhibitory activity than OC by replacing the C-5 amino group with a more basic guanidino group, which is considered to exert stronger interactions with the acidic residues (E119, D151, and E227) in the active site of influenza NA [9]. However, compound **2a** and its ethyl ester (**2b**, GOS) have not been developed for therapeutic use.



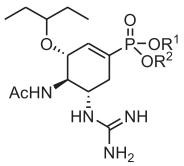
1a (OC), R = H
1b (OS), R = C_2H_5



2a (GOC), R = H
2b (GOS), R = C_2H_5



3a (TP), $R^1 = R^2 = H$
3b, $R^1 = R^2 = CH_2CH_3$
3c, $R^1 = H$, $R^2 = CH_2CH_3$
3d, $R^1 = H$, $R^2 = (CH_2)_3CH_3$
3e, $R^1 = H$, $R^2 = (CH_2)_5CH_3$
3f, $R^1 = H$, $R^2 = (CH_2)_3Ph$
3g, $R^1 = H$, $R^2 = (CH_2)_3$-(3-indole)



4a (TPG), $R^1 = R^2 = H$
4b, $R^1 = R^2 = CH_2CH_3$
4c, $R^1 = H$, $R^2 = CH_2CH_3$
4d, $R^1 = H$, $R^2 = (CH_2)_3CH_3$
4e, $R^1 = H$, $R^2 = (CH_2)_5CH_3$
4f, $R^1 = H$, $R^2 = (CH_2)_3Ph$

Formation of phosphonate–guanidinium complex is thermodynamically favored [17]. Tamiphosphor (TP, **3a**), a phosphonate congener of OC, is thus designed to have strong electrostatic interactions with the three arginine residues (R118, R292 and R371) in the active site of NA [18,19]. The phosphonate ion in **3a** is also topologically complementary to bind with the three arginine residues. Compound **3a** has high NA inhibitory activities to suppress the replication of human and avian influenza viruses. Our previous study [19] indicates that phosphonate monoethyl ester **3c** is also very active to protect human 293T and canine MDCK cells from infection by influenza viruses because **3c** still contains a negative charge at the phosphonate group to render the necessary electrostatic interactions with the three arginine residues in NA. Monoester **3c** is a drug, instead of a prodrug, because it does not metabolize to the free phosphonic acid **3a** in animal experiments [19]. In another approach, Streicher and coworkers have demonstrated that the phospha-oseltamivir monoesters carrying various functional groups can be of versatile uses [20–23]. For example, the phospha-oseltamivir monoesters equipped with aglycone mimetics or linked to gold nanoparticles are good for treatment and detection of influenza viruses.

We have also prepared guanidino-tamiphosphor (TPG, **4a**) and its monoethyl ester **4c** to show that they possess high inhibitory activities against the oseltamivir-resistant mutant of influenza virus. In contrast, phosphonate diethyl esters **3b** and **4b** are inactive to influenza viruses. The phosphonate compounds **3a**, **3c**, **4a** and **4c** have similar pharmacokinetic properties in mice, rats and dogs. TPG monoester **4c** in saline solution shows better oral bioavailability ($F = 12\%$) than TPG **4a** ($F = 7\%$) in mice [19]. We therefore aim to investigate whether the bioavailability and pharmacokinetic properties can be further improved in the TP and TPG monoalkyl esters bearing more lipophilic alkyl groups, such as **3d**–**3g** and **4d**–**4f**.

In another aspect, a flexible 150-loop adjacent to the S2 active site is found from the X-ray crystallographic studies of the apo and inhibitor-bound structures of NAs [6,24]. The A-type influenza virus NAs are divided into two categories: group-1 including N1, N4, N5 and N8 subtypes, and group-2 comprising N2, N3, N6, N7 and N9 subtypes. It is proposed that the 150-loop of group-2 NA always exists in the closed form, whereas that of group-1 NA would change from the open conformation to the closed form on binding with substrate or inhibitor. In contrast, molecular dynamics simulations suggest that both group-1 and group-2 NAs are able to adopt an open 150-cavity within their solution-phase structural ensemble [25]. Molecular dynamics simulations of influenza NAs further indicate the existence of a 430-loop comprising the residues R430–T439 adjacent to the S1 active site [26,27]. The conformational change of 150-loop may be coupled with motion of the neighboring 430-loop to form a much larger binding pocket than that shown in the static crystal structures. In this study, we carried out molecular modeling experiments to reveal that influenza virus also contains a hydrophobic 371-cavity near the NA active site that may provide additional interactions with the alkyl groups in our designed inhibitors of phosphonate monoesters.

2. Results and discussion

2.1. Molecular modeling

The structure-based design of NA inhibitors has been a successful strategy in discovery of anti-influenza drugs [4]. The phosphonate group has been used as a bioisostere of carboxylate in drug design [29–31]. In comparison with carboxylate ion, a phosphonate ion exhibits stronger electrostatic interactions with guanidinium ion [17]. Formation of phosphonate–guanidinium complex is thermodynamically favored [17]. Consistent with this rationale, compound **3a** containing a phosphonic acid binds strongly with the three arginine residues in the NA active site, thus showed higher inhibitory activity than the corresponding carboxylate **1a** against various human and avian influenza viruses, including A/H1N1 (wild-type and H275Y mutant), A/H5N1, A/H3N2 and type B viruses [19]. Using the known N1 crystal structure (PDB code: 2HU4) [6], our molecular docking experiments indicated that the phosphonate group of **3a** formed 8-pair hydrogen bonds with three arginine residues (Fig. 1A), greater than the carboxyl group of **1a** (6 pairs of hydrogen bonds). The monoalkyl phosphonate ion, e.g. **3f**, exhibited 7 pairs of hydrogen bonds with the three arginine residues in the S1 site of NA (Fig. 1B), in addition to the appreciable interactions of the C_3-pentoxy, C_4-acetamido and C_5-amino groups in the S2–S5 sites [5]. Our molecular modeling revealed that the 3-phenylpropyl moiety in **3f** also exhibited considerable interactions with I427, P431 and K432 residues in the 430-loop. This result is in agreement with the prediction by ensemble-based virtual screening [32], which shows that the 430-cavity of NA favors to accept aromatic rings and hydrophobic substituents.

Moreover, we found a hydrophobic cleft, namely 371-cavity (Fig. 1D), was enclosed by the R371, P431, I427, K432 and W403 residues located between the 430- and 371-loops near the S1 site. The 3-phenylpropyl substituent of **3f** extended to the 371-cavity, so that the phenyl group was disposed in a manner to exert a significant π–cation interaction with R371, and a T-shape interaction with the indole ring of the W403 residue in an edge-to-face configuration [33]. In a similar fashion, the (indol-3-yl)propyl substituent of **3g** was located in the region of 430- and 371-loops to attain additional hydrophobic, π–π and π–cation interactions (Fig. 1C). Thus, phosphonate monoesters **3f** and **3g** were predicted to possess high affinity to influenza virus NAs.