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Sugar-Based Synthesis of Tamiflu and Its Inhibitory Effects on Cell Secretion

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Abstract: Tamiflu is currently the most effective drug for the treatment of influenza, but the insufficient supply and side-effects of this drug demand urgent solutions. We present a practical synthesis of Tamiflu by using novel synthetic routes, cheap reagents, and the abundantly available starting material D-glucal. The strategy features a Claisen rearrangement of hexose to obtain the cyclohexene backbone and introduction of diamino groups through tandem intramolecular aziridination and ring opening. In addition, this synthetic protocol allows late-stage functionalization for the flexible synthesis of Tamiflu analogues. By using the synthesized Tamiflu and its active metabolite (oseltamivir carboxylate), we investigated their influences on neuroendocrine PC12 cells in various aspects. It was discovered that oseltamivir carboxylate significantly inhibits the vesicular exocytosis (regulated secretion) of PC12 cells, and suggests a mechanism underlying the Tamiflu side-effects, in particular its possible adverse influences on neurotransmitter release in the central nervous system.

Keywords: carbohydrates · cell secretion · inhibitors · tamiflu · total synthesis

Introduction

Influenza, a common and highly infectious disease, kills hundreds of thousands of people annually and millions during its outbreaks. At the time of writing, the recent outbreak of influenza A-H1N1 (swine flu) is rapidly reaching global pandemic stage (over 100 countries) and creating great panic worldwide, similar to that induced by SARS and bird flu a few years ago.

In the limited drug arsenal against influenza, neuraminidase (or sialidase) inhibitors, specifically Tamiflu (oseltamivir phosphate, **1**·H_3PO_4) and Relenza (Zanamivir), excel as effective weapons to combat various strains of influenza by preventing virus replication. Both of these drugs mimic sialic acid and competitively inhibit viral neuraminidase, the enzyme that removes terminal sialic acid from glycoconjugates on the host cell surface to allow release and spread of the influenza virus (Figure 1).[1] Compared with Relenza, which relies on an inconvenient inhalation administration, Tamiflu (prodrug) has high oral bioavailability and can be readily hydrolyzed by hepatic esterase to give the free carboxylate of oseltamivir (GS4071, OC, **2**) as the active competitive inhibitor to neuraminidase. Tamiflu is currently the most effective drug for the treatment of influenza, including H1N1.

The current commercial synthesis involves a 10-step process of complex chemical reactions and relies on (−)-shikimic acid,[2] and was thought to be the most proficient till now. However, Tamiflu is undoubtedly a popular molecule of high importance, and intensive efforts have been made to develop alternative routes to Tamiflu with various strategies for cyclohexenyl ring formation and to start from readily available and less-expensive starting materials.[3] For example, asymmetric Diels–Alder chemistry[4] and the Michael reaction[5] have been exploited to generate the cyclohexene moiety. Commercially available starting materials, such as (−)-quinic acid,[6] L-serine,[7] xylose,[8] mesoaziridine,[9] substituted cyclohexanediene,[10] lactone,[11] pyridine,[12] 2,6-dimethoxyphenol,[13] D-mannitol,[14] L-methionine,[15] and ethyl

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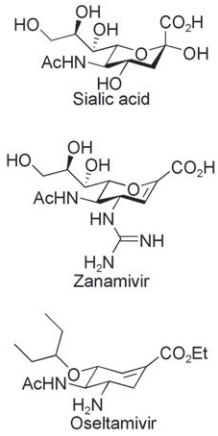


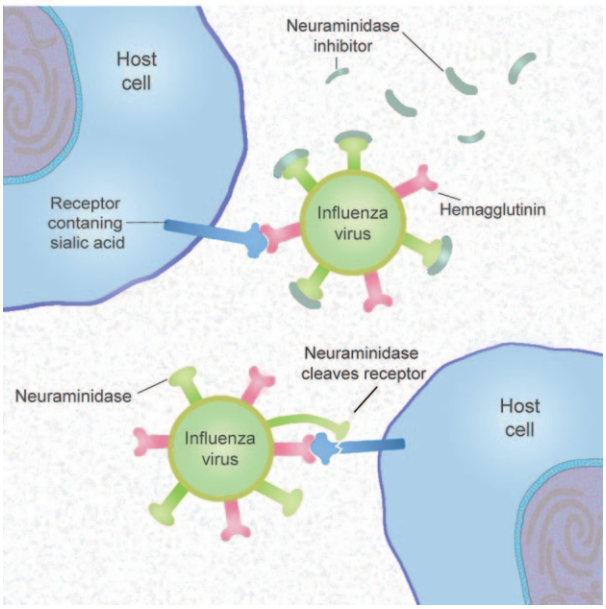


Figure 1. Top: Chemical structures of sialic acid and the two most popular neuraminidase inhibitors (Zanamivir and oseltamivir). Bottom: The working mechanism of neuraminidase inhibitors. The neuraminidase of the influenza virus cleaves the binding of hemagglutinin with the cellular receptor at the sialic acid residue to release the virus for further replication.

benzoate,[16] have been used as alternative starting materials for robust syntheses of Tamiflu.

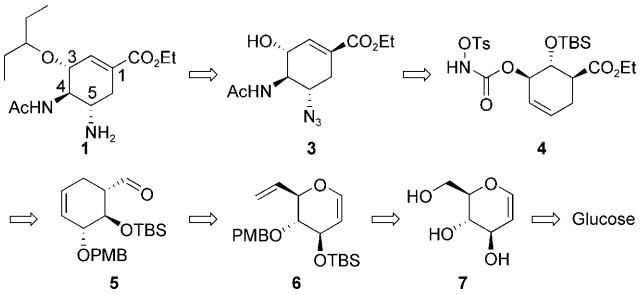
The efficacy of oseltamivir as a powerful anti-influenza drug notwithstanding, adverse side-effects have been reported, particularly the mysterious psychiatric consequences that have dismayingly led to 54 adolescent suicides in Japan as of 2007.[17] The molecular mechanisms underlying the Tamiflu side-effects are yet to be unveiled, but the side-effects of Tamiflu are not totally unexpected given that the Tamiflu inhibition of neuraminidase inherently lacks isoform or species selectivity and that neuraminidases are implicated in many essential biological processes, such as signal transduction,[18] cell proliferation and differentiation,[19] cell-to-cell interaction,[20] immune responses,[21] and neuronal functions.[22]

Herein we report an economical synthetic route to Tamiflu that uses cheap and abundant D-glucal as the starting material. Furthermore, we investigated the effects of Tamiflu and its active form (OC) on the morphology, differentiation, cytoskeleton organization, and vesicular exocytosis (regulated secretion) of neuroendocrine PC12 cells. It was discovered that OC significantly inhibits the vesicular exocytosis (regulated secretion) of PC12 cells. Given that exocytosis is a fundamental and ubiquitous function found in many cell types and is responsible for various critical biological processes, such as hormone secretion from endocrine cells and neurotransmitter release from neurons, our results postulate a mechanism for the side-effects of Tamiflu, in particular its possible adverse influences on neurotransmitter release in the central nervous system.

Results and Discussion

Sugar-based synthesis of Tamiflu

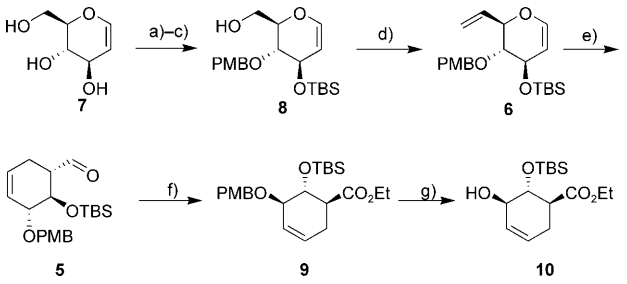
Retrosynthetic analysis: Carbohydrates are structurally diverse and contain a wealth of stereochemical properties. They have been serving as chiral pools for the total synthesis of numerous bioactive natural products for decades. Herein D-glucal, which is commercially available, was chosen as the starting material for the Tamiflu synthesis to take advantage of its naturally occurring stereocenters. As shown in Scheme 1, compound **4** in our retrosynthetic analysis was



Scheme 1. Retrosynthetic analysis of (−)-oseltamivir (**1**).

identified as the pivotal intermediate that could be used to synthesize **3**, the prototype of the target molecule, through tandem intramolecular aziridination, ring-opening by *N*-nucleophiles, and olefin formation (Scheme 1). We envisioned that the configurations at C4 and C5 of compound **3** could be set by taking advantage of the regioselectivity and stereoselectivity in the formation and ring-opening of the aziridine. Carbamate **4** could be derived from aldehyde **5** through known transformations. Aldehyde **5** could be generated from functionalized glucal **6** by employing a 3,3-sigmatropic rearrangement as a critical step to form the carbocycle with the desired chiral configurations at C2 and C3. Glucal **7** is commercially available or can be readily synthesized from D-glucose.

*Construction of cyclohexene core **10** (a precursor to **4**)*: Scheme 2 shows the construction of a six-membered carbon backbone starting from commercially available D-glucal. In-

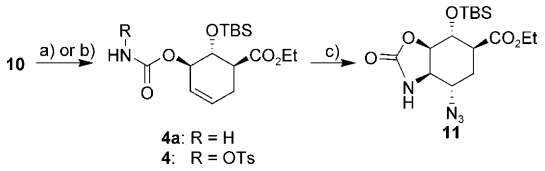


Scheme 2. Synthesis of compound **10**. Reagents and conditions: a) *p*-anisaldehyde diethyl acetal (1.5 equiv), PPTS (0.1 equiv), DMF, 25 °C, 2 h; b) TBSCl (1.2 equiv), imidazole (2.4 equiv), DMAP (0.1 equiv), DMF, 25 °C, 3 h; c) DIBAL-H (1.2 equiv), dichloromethane, −15–0 °C, 2 h, 65 % (3 steps); d) DMP (1.2 equiv), dichloromethane, 25 °C, 2 h followed by methyltriphenylphosphonium bromide (1.8 equiv), *n*BuLi (1.5 equiv), THF, −78–25 °C, 1 h, 67 % (2 steps); e) diphenyl ether, 210 °C, 2 h, 88 %; f) $NaClO_2$ (3 equiv), NaH_2PO_4 (3 equiv), 2-methyl-2-butene (5 equiv), *t*BuOH/H_2O, 25 °C, 2 h followed by EtI (2 equiv), K_2CO_3 (1.5 equiv), DMF, 25 °C, 3 h, 87 % (2 steps); g) DDQ (1.5 equiv), dichloromethane/H_2O, 92 %. Abbreviations: PPTS = pyridinium *p*-toluenesulfonate; DMF = *N*,*N*-dimethylformamide; TBSCl = *tert*-butyldimethylsilyl chloride; DMAP = 4-dimethylaminopyridine; DIBAL-H = diisobutylaluminium hydride; DMP = Dess–Martin periodinate; DDQ = 2,3-dichloro-5,6-dicyano-*p*-benzoquinone.

stallation of 4,6-benzylidene acetal and silylation of 3-hydroxyl gave fully protected D-glucal, which underwent selective opening of the benzylidene acetal with DIBAL-H in dichloromethane at −15 °C to give free primary alcohol **8** (65 % yield starting from D-glucal). The primary hydroxyl group in **8** was oxidized to the aldehyde by using Dess–Martin periodinate or Swern reagents and then subjected to Wittig methylenation to give terminal olefin **6** in 67 % yield. The next step was the critical Claisen rearrangement reaction, which allowed ready access to a carbocycle from the sugar ring. This reaction was conducted in a sealed reaction vessel at 210 °C in diphenyl ether, and aldehyde **5** was diastereoselectively achieved in an excellent yield of 88 %. As discussed by Büchi and Powell,[23] this rearrangement is controlled by a facial preference via a boat-like transition state that results in the formation of single isomer **5**. The oxidation of **5** to ethyl ester **9** was found to be best carried out by using $NaClO_2/NaH_2PO_4$ in the presence of 2-methyl-2-butene, followed by esterification (87 % yield). Use of Oxone or I_2/KOH in ethanol gave poor yields of approximately 30 %. The *p*-methoxybenzyl (PMB) group was successively removed with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) to give alcohol **10** in 92 % yield.

Copper-catalyzed intramolecular aziridination: The installation of the three contiguous chiral centers in the molecule of Tamiflu, which is probably the most difficult part in all Tamiflu synthesis methods proposed thus far, was carried out by using synthesized carbocycle **10**. Our strategy was to take advantage of the hydroxyl group on C4 position, the configuration of which is originally from the C4 of the sugar ring. We first followed the strategy of intramolecular nitrogen delivery for the unnatural aminosugar synthesis, which was successfully developed in our laboratory.[24] Specifically, a sulfonamide ester was tethered onto the 4-hydroxy group and generated rhodium-stabilized nitrene by using $Rh_2(OAc)_4$/PhI=O. The active nitrene species was intramolecularly delivered into a carbon–carbon double bond, followed by an aziridine ring-opening reaction by using nitrogenous nucleophiles. Unfortunately, this endeavor did not lead to formation of the desired product, probably due to insurmountable hindrance. Inspired by the work of Padwa et al.,[25] we attempted to form carbamate **4a** by treating alcohol **10** with $Cl_3CCONCO/K_2CO_3$ (yield 87 %). Disappointingly, intramolecular aziridination could not take place despite the extensive screening of catalysts, such as $[Cu(MeCN)_4PF_6]$, $(CuOTf)_2$·toluene, $Rh_2(OAc)_4$, and $[Rh_2(tfacam)_4]$ (tfacam = CF_3CONH).

To enhance the electrophilicity of the nitrene species, substituted carbamate **4** was prepared in 77 % yield by treating alcohol **10** with CDI/NH_2OH, followed by $TsCl/Et_3N$ (Scheme 3).[26] The $Rh_2(OAc)_4$-catalyzed aziridination in di-



Scheme 3. Synthesis of compound **11**. Reagents and conditions for **4a**: a) $Cl_3CCONCO$ (2 equiv), K_2CO_3 (5 equiv), dichloromethane/MeOH, 87 %; for **4**: b) CDI (1.5 equiv), dichloromethane, 25 °C, 2 h; hydroxylamine hydrochloride (2 equiv), pyridine, 25 °C, 3 h followed by TsCl (1.1 equiv), Et_3N (1.05 equiv), Et_2O, 25 °C, 12 h, 77 % (2 steps); c) $(CuOTf)_2$·toluene (0.05 equiv), K_2CO_3 (3 equiv), MeCN, 25 °C, 12 h; $TMSN_3$ (2 equiv), TBAF (1 equiv), THF, 0–25 °C, 3 h, 82 %. CDI = 1,1′-carbonyldiimidazole; TBAF = tetra-*n*-butylammonium fluoride.

chloromethane at room temperature proceeded in moderate yield (63 %). Further optimization showed that $(CuOTf)_2$·toluene was an ideal catalyst for this transformation, giving the highest yield (94 %) as determined by crude ^1H NMR spectroscopy (Table 1). With these optimized aziridination conditions, ring opening with *N*-containing nucleophiles was sequentially conducted in a one-pot manner. Unsatisfactorily, *p*-methoxybenzylamine and allyl amine were introduced to compound **4** in a one-pot process that gave two diastereomers (approximate ratio: *anti*/*syn* 3:1) after ring-opening. To our surprise, when $TMSN_3$ was used as the nucleophile, compound **11** was formed stereoselectively and regioselectively in 82 % yield. Notably, this aziridine intermediate has the provision for synthesis of Tamiflu analogues by using different N-, O-, or S-containing nucleophiles. Optimization of the reaction conditions is under investigation to

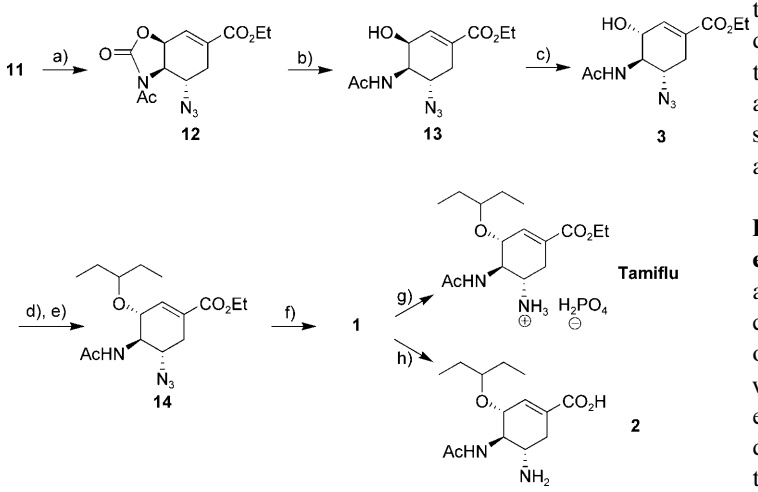
Table 1. Optimization of aziridination of tosyl-carbamate **4**.[a]

Catalyst	Solvent	Yield [%][b]
$Rh_2(OAc)_4$	CH_2Cl_2	63
$Cu(OTf)_2$	CH_3CN	78
IprCuCl[c]	CH_3CN	83
$Cu(MeCN)_4PF_6$	CH_3CN	86
$(CuOTf)_2$toluene	CH_3CN	94

[a] All reactions were conducted at RT. [b] Yields, judged from crude ^1H NMR data. [c] 1,3-bis(2,6-diisopropylphenyl)imidazolium copper chloride.[26]

improve the stereoselectivity when other nucleophiles are applied.

Completion of synthesis of Tamiflu: Treatment of **11** with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile at room temperature for 24 h failed to generate the desired α,β-unsaturated carboxylate. Chirality at the C1 position was partially inverted to give a pair of diastereoisomers through a deprotonation/protonation process. Interestingly, aromatization occurred when **11** was treated with DBU at higher temperature. However, the elimination was successful after acetylating the nitrogen in carbamate **11** with AcCl/NaH, with an overall yield of 67% for the two steps (Scheme 4). Substituted carbamate **12** was then hydrolyzed by using Cs_2CO_3 in EtOH to provide alcohol **13** in 72% yield.



Scheme 4. Complete synthesis of Tamiflu. Reagents and conditions: a) AcCl (2 equiv), NaH (1.5 equiv), THF, 0–25 °C, 2 h followed by DBU (2 equiv), MeCN, 25 °C, 24 h, 67% (2 steps); b) Cs_2CO_3 (0.1 equiv), EtOH, 25 °C, 3 h, 72%; c) DMP (1.5 equiv), dichloromethane, 25 °C, 2 h followed by $LiAlH(tBuO)_3$ (3 equiv), THF, −20–25 °C, 3 h, 70% (2 steps); d) MsCl (1.5 equiv), Et_3N (3 equiv), CH_2Cl_2, 0–25 °C, 3 h; e) BF_3Et_2O (1 equiv), 4 Å MS, 3-pentanol, −20 °C, 2 h, 52% (2 steps); f) Ph_3P (1.5 equiv), THF/H_2O (1:1), reflux, 3 h, 90%; g) 85% H_3PO_4 (1 equiv), ethanol, 55 °C, 30 min, 85%; h) LiOH (1.5 equiv), THF/H_2O (10:1), 25 °C, 3 h, 80%. MS = molecular sieve.

The chirality of C3 was reversed by Dess–Martin oxidation followed by reduction with $LiAlH(OtBu)_3$ to stereospecifically give intermediate **3** in 70% yield. It was then treated with $MsCl/Et_3N$ to produce an aziridine intermediate, which was subjected to regioselective ring-opening with 3-pentanol/BF_3•Et_2O to generate ether **14** in 52% yield. The stereochemistry of **14** was confirmed by using X-ray crystallography (see the Supporting Information). The azido group in **14** was reduced to an amine with PPh_3 in THF/H_2O (90% yield). Finally, the oseltamivir phosphate salt (Tamiflu) was obtained by treating **1** with H_3PO_4 in EtOH (85% yield). Hydrolysis of **1** with LiOH in water afforded oseltamivir acid **2**.

Our synthetic route to Tamiflu is based on the cheap and abundantly available starting material D-glucal. By taking advantage of the naturally occurring chiral centers and innate stereochemistry of the D-glucal scaffold, most of the reactions in our synthesis occur in a regio- and stereoselective manner to readily produce the desired products in high yields. Other highlights of this work include the 3,3-sigmatropic rearrangement reaction, which allows the construction of a cyclohexene core with a conjugated carboxylate moiety. Similar strategies can be employed to form other functionalized six-membered carbocycles from abundantly available *O*-containing sugar scaffolds. In our method, vicinal diamino groups on C4 and C5 were introduced by stereoselective intramolecular nitrogen delivery with a tethered carbamate at the C3 position, followed by regio- and stereoselective ring opening of aziridine. Elegantly, this strategy fixes the stereochemistry at both C4 and C5. The third key step is the installation of a pentyloxy group at C3, which was achieved through regio- and stereoselective ring opening of the aziridine intermediate. Furthermore, the functionalization on the three contiguous chiral centers, C3, C4, and C5, which occur at the late stage of our synthesis, allow prompt and efficient synthesis of Tamiflu analogues by varying the nucleophiles and alkyloxy groups.

Influences of Tamiflu and oseltamivir carboxylate on neuroendocrine PC12 cells: We examined the effects of Tamiflu and OC on neuroendocrine PC12 cells, which are a popular cell model to study vesicular exocytosis (regulated secretion) of neurotransmitters/hormones and neural differentiation. It was found that both Tamiflu and OC had no obvious influences on cell morphology, proliferation, differentiation induced by nerve growth factor, or organization of cytoskeleton networks (see the Supporting Information). These observations support the view that Tamiflu is generally well tolerated. On the other hand, we discovered that OC exerts significant inhibitory effects on vesicular exocytosis of PC12 cell. This suggests that Tamiflu treatment may adversely affect neurotransmitter release in the central nervous system and may thus explain the neuropsychiatric consequences and other side-effects observed in some patients.

OC impairs vesicle trafficking: The large dense core secretory vesicles (LDCVs) in PC12 cells were selectively labeled by overexpression of enhanced green fluorescence protein (EGFP)-conjugated neuropeptide-Y (NPY-EGFP).[27] Fluorescently labeled vesicles in the subplasmalemmal region

were resolved by using total internal reflection fluorescence microscopy (TIRFM), which is instrumental in observing phenomena that occur near the cell membrane by selectively illuminating the thin section (<200 nm) just above the interface between the glass coverslip and the adhered cell membrane. Figure 2a presents typical TIRFM images of an OC-treated (left) and an untreated PC12 cell.[28] Each bright fluorescent dot indicates the footprint of a subplasmalemmal vesicle. There was no appreciable difference in the total number of visible vesicles between the treated and untreated cells.

All the secretory vesicles in neuroendocrine or neuron cells move constantly. They move laterally (parallel to the cell membrane) when in the subplasmalemmal region, in which they travel around and interact with various secretory factors and eventually fuse with the plasma membrane to discharge their vesicular cargoes into the extracellular fluid upon being triggered. In addition, they transit vertically between the subplasmalemmal region and the inner cytosol, in which a large reserve pool of vesicles resides. The dynamics of vesicle trafficking directly correlates with the competence and kinetics of vesicular exocytosis.[27a,29] The motion of individual LDCVs was tracked at 0.5 s time intervals over 2 min to obtain information about the velocity and area coverage of vesicle lateral movement, the time that vesicles dwell in the subplasmalemmal region, and the rate of vesicle arrival from the inner cytosol. Figure 2b depicts the typical motion trajectories of a vesicle in the OC-treated cell (top)[30] and the untreated cell (bottom). It is evident that vesicles undertake confined Brownian motion.[27a]

Interestingly, the velocity of vesicle movement in OC-treated cells is dramatically reduced (587 vesicles in 11 treated cells: $(55.1\pm1.7)\ nm\,s^{-1}$ vs. 657 vesicles from 14 untreated cells: $(183.1\pm5.5)\ nm\,s^{-1}$; $p<0.001$; Figure 2c). The dashed rectangles (Figure 2b) that just encase the total vesicle footprint give a first-order estimation of the coverage of vesicle random motion and reflect how vesicles are confined by physical barriers or molecular tethering. In OC-treated cells the vesicle movements were more severely confined than in the untreated control ($(0.27\pm0.02)\ \mu m^2$ vs. $(0.58\pm0.07)\ \mu m^2$; $p<0.001$; Figure 2d). The reduction in motion area cannot be attributed to a decrease in the vesicle dwell time. On the contrary, vesicles stay in the subplasmalemmal region for much longer in the treated cells compared with the control ((63.8 ± 1.8) s vs. (41.2 ± 1.5) s; $p<0.001$; Figure 2e). In addition to its inhibitory influences on vesicle lateral trafficking, OC also causes a large decrease in the rate of vesicle arrival from the inner cytosol ($(23.3\pm3.1)\ vesicles\,cell^{-1}$ arrived in 2 min vs. $(52.1\pm3.6)\ vesicles\,cell^{-1}$; $p<0.001$; Figure 2f). The rate of vesicle retrieval back into the inner cytosol, which is in balance with vesicle arrival to keep the total number of subplasmalemmal vesicles constant, was similarly affected. Taken together, it can be seen that OC severely impairs both lateral and vertical trafficking of LDCV vesicles in

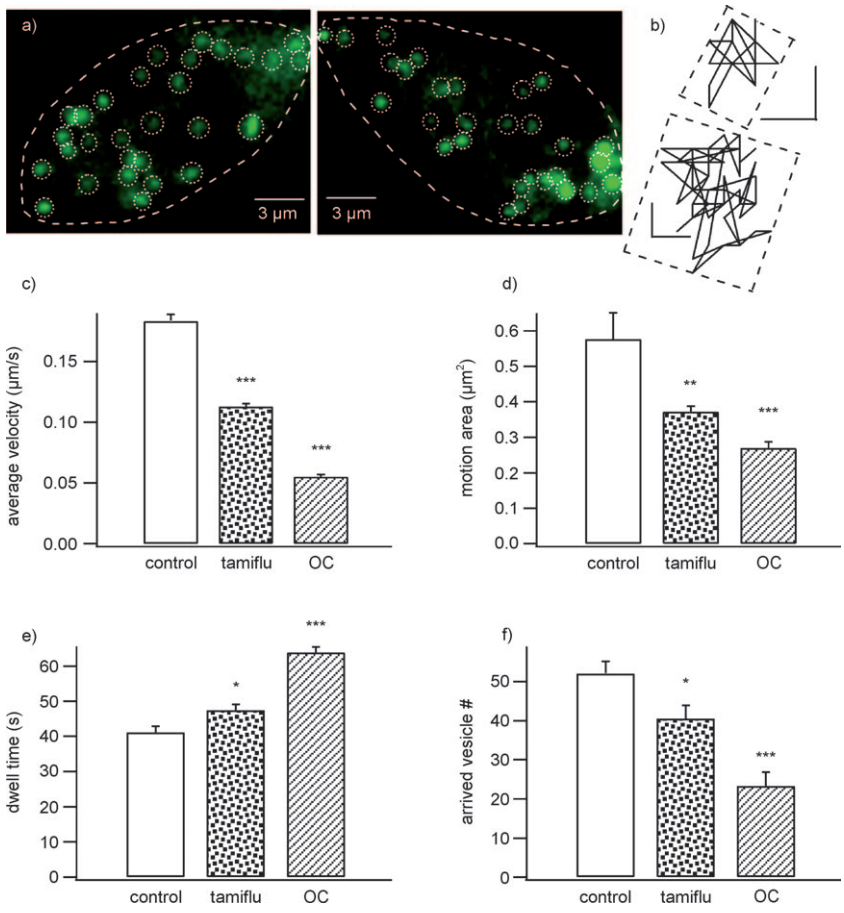


Figure 2. Oseltamivir carboxylate (OC) and Tamiflu reduce vesicle trafficking (both lateral and vertical movement) in PC12 cells. a) Typical TIRFM images of a PC12 cell with (left) or without (right) OC treatment (0.5 mM for 20 h). Individual footprints of the subplasmalemmal vesicles can be resolved as bright green dots (highlighted by circles). The cell contours are outlined by the dashed lines. b) Motion trajectories of a vesicle in an OC-treated cell (top) and a vesicle in an untreated cell (bottom). The dashed rectangle that exactly encases the total vesicle footprints gives a first-order estimation of the area coverage of vesicle lateral motion (scale bars = 200 nm). c), d), e), and f) The statistics of the vesicle average velocity, motion area, dwell time, and total number of vesicles arrived from the inner cytosol during 2 min imaging, respectively. The statistics, shown as mean ±SEM (standard error of the mean), are from 587 vesicles in 11 OC-treated cells, 564 vesicles in 10 Tamiflu-treated cells, or 657 vesicles from 14 untreated control cells. Student t test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs. control.

PC12 cells. In comparison with OC, Tamiflu also similarly suppressed vesicle trafficking, but to a lesser extent (Figure 2). Sufficient mobility is believed to be important to ensure vesicle fusion competence and fast replenishment of readily releasable vesicles during a continuous stimulation.[27a,29] Therefore, we reasoned that oseltamivir carboxylate may inhibit vesicular exocytosis.

OC inhibits vesicular exocytosis: Vesicular exocytosis was assayed by carbon fiber microelectrode (CFM)-based amperometry, which is able to detect exocytosis with single vesicle sensitivity and millisecond resolution.[31] Figure 3a[30] shows a typical amperometric trace, recorded by using a voltage-biased (700 mV) CFM positioned on the surface of a PC12 cell, in response to a local perfusion of high-K^+ solution that invokes a voltage-activated Ca^{2+} current and in turn triggers Ca^{2+}-dependent exocytosis. Each current spike corresponds to a single vesicular release of catecholamine molecules (including dopamine, epinephrine, and norepinephrine). As compared with the control, the average number of amperometric spikes in response to high-K^+ stimulation for 2 min was significantly inhibited by OC treatment (19 OC-treated cells: (22.8 ± 3.8) spikes cell^{-1} vs. 40 control cells: (45.4 ± 4.1) spikes cell^{-1}; $p<0.001$; Figure 3a, bottom), whereas Tamiflu did not appreciably affect the extent of exocytosis (21 Tamiflu-treated cells: (41.9 ± 4.7) spikes cell^{-1}; $p>0.05$).

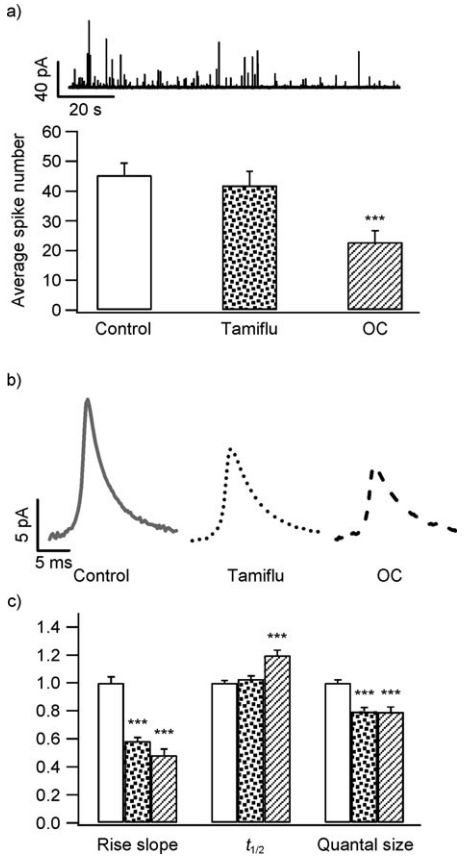


Figure 3. Oseltamivir carboxylate inhibits vesicular exocytosis in PC12 cells. a) A representative amperometric recording from a untreated PC12 cell in response to high-K^+ stimulation for 2 min (top)[30] and the statistics of the average total spike number (bottom) from the control cells (40 cells: (45.4 ± 4.1) spikes cell^{-1}), Tamiflu-treated cells (21 cells: (41.9 ± 4.7) spikes cell^{-1}), and OC-treated cells (19 cells: (22.8 ± 3.8) spikes cell^{-1}). b) The average amperometric spikes averaged from 1814 fusion events from the control cells (——), 880 fusion events recorded from Tamiflu-treated cells (•••••), and 434 fusion events recorded from OC-treated cells (-----). c) Statistics of the rise slope, half-width time ($t_{1/2}$), and quantal size (Q) of the amperometric spike. The data is normalized to the control, for which rise slope $=(10.58\pm0.46)$ pA ms^{-1}; $t_{1/2}=(5.13\pm0.09)$ ms; $Q=(87.87\pm2.03)$ fC. In a) and c), the statistics are represented as mean $\pm$SEM; white column: control cells, dotted column: Tamiflu-treated cells, slashed column: OC-treated cells. Student t test: *** $p<0.001$ vs. control.

The characteristic waveform of the amperometric spikes reveals the kinetics of quantal vesicle fusion catalyzed by secretory proteins, such as SNAREs.[31] Individual amperometric spikes were extracted and analyzed to investigate the effects of Tamiflu and OC on vesicle fusion kinetics. The mean amperometric spikes averaged from all the recorded signals from the control cells (1814 spikes from 40 cells), Tamiflu-treated cells (880 spikes from 21 cells), and OC-treated cells (434 spikes from 19 cells) are displayed in Figure 3b. The mean amperometric amplitude from the OC-treated cells was much smaller than that from the control cells ((8.83 ± 0.49) pA vs. (15.91 ± 0.47) pA; $p<0.001$). In comparison, the amplitude reduction caused by Tamiflu was less ((10.53 ± 0.33) pA; $p<0.001$).

The rise slope, which is defined by the slope of the amperometric current as it rises from 35 to 90% of its peak, reflects how fast the initial fusion pore formed between the vesicular and the plasma membrane expands to quickly discharge the molecules inside. It was observed that the rise slope in the OC-treated cells was much less steep than that in the control cells ((5.11 ± 0.46) pA ms^{-1} vs. (10.58 ± 0.46) pA ms^{-1}; $p<0.001$; Figure 3c). The half-width time ($t_{1/2}$) of the amperometric signal indicates the time of the fusion process. It was found that the $t_{1/2}$ of the OC-treated cells was significantly longer than that of the control ((6.15 ± 0.19) ms vs. (5.13 ± 0.09) ms; $p<0.001$; Figure 3c). From these observations, it can clearly be seen that the fusion kinetics are greatly slowed by treatment with OC. Furthermore, the size of the quantal vesicular release (Q), which is the charge integration of the amperometric spike and the indicator of the total number of released molecules, was reduced by OC treatment ((69.69 ± 3.19) fC vs. (87.87 ± 2.03) fC; $p<0.001$; Figure 3c). Similarly, Tamiflu treatment also led to reduction in the rise slope and quantal size (Figure 3c). Taken together, OC significantly impaired not only the vesicular fusion competence (Figure 3a) but also the efficiency of quantal vesicular release (Figure 3b and c).

It should not be surprising that Tamiflu, particularly after being hydrolyzed to its active form (OC), could have profound influences in cell functions because its target, neuraminidases, are implicated in many essential cellular processes in which they remove the terminal sialic acid from glycoproteins and glycolipids.[18–19] The influences of Tamiflu and OC on the central nervous system have been investigat-

ed by using rat brain synaptosomes, and it was found that neither Tamiflu nor OC affected the release of monoamine neurotransmitters.[32] In another study, it was reported that Tamiflu increased dopamine levels in the rat medial prefrontal cortex.[33] For the reasons that require further investigation, these observations obtained from different experimental preparations by using biochemical assays are seemingly discrepant with our findings on PC12 cells obtained by single-cell recording or imaging with single vesicle sensitivity and millisecond resolution. Our experiments demonstrate that OC significantly inhibits exocytosis, which is a fundamental process in neurons and many other secretory cells. Therefore, it is possible that, once the hydrolyzed Tamiflu (OC) passes the blood–brain barrier,[34] it might inhibit the release of neurotransmitters from presynaptic neurons and consequently modulate neurotransmission in the central nervous system. Excitation or depression of the central nervous system may result depending on the types of neurons and neurotransmitters being affected. The ability of Tamiflu and OC to affect synaptic function and neuronal excitability has been reported in two recent studies.[35]

Conclusion

We have developed an economic synthetic route to Tamiflu by using cheap and abundantly available starting materials and reagents. In addition, our method allows late-stage functionalization for ready and flexible synthesis of Tamiflu analogues that may be able to overcome virus resistance to Tamiflu and to avoid the side-effect problems of Tamiflu. The practical synthetic strategy presented here may provide not only an alternative to the synthesis of Tamiflu but also the opportunity of designing Tamiflu-like molecular tools to study the cellular functions mediated by neuraminidases.

Herein we show that the active Tamiflu metabolite (OC) significantly inhibits the vesicular exocytosis in neuroendocrine PC12 cells. This inhibition on the ubiquitous exocytotic process may explain why Tamiflu causes a variety of problems, including nausea, vomiting, diarrhea, headaches, vertigo, insomnia, somnolence, and behavioral excitement. Further investigations will be carried out to reveal the detailed molecular mechanisms of how Tamiflu interferes with the exocytotic pathways and possibly other cell functions that involve neuraminidases.

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