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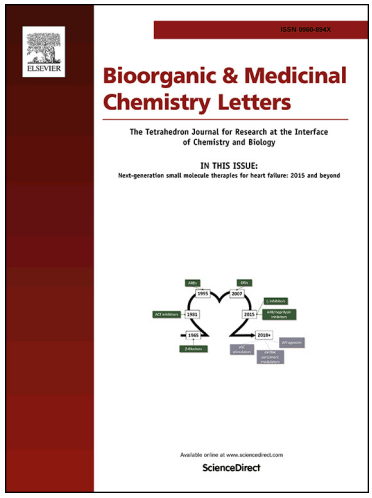

Design, synthesis, and evaluation of carboxyl-modified Oseltamivir derivatives with improved lipophilicity as neuraminidase inhibitors

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Design, synthesis, and evaluation of carboxyl-modified Oseltamivir derivatives with improved lipophilicity as neuraminidase inhibitors

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Abstract In this study, a series of carboxyl-modified oseltamivir analogs with improved lipophilicity were designed and synthesized, and their inhibitory activities against neuraminidase from influenza A virus H5N1 subtype were evaluated. The results demonstrated that compound **5m** exhibited potent inhibitory activity (IC_{50} = 1.30 ± 0.23 μM), and it targeted the recently discovered 430-cavity. Compound **5m** (Log D = -0.12) is more lipophilic than oseltamivir carboxylate (Log D = -1.69) at pH 7.4, which is potentially propitious to improved membrane permeability and oral drug absorption. Meanwhile, **5m** showed high stability in human liver microsomes. The findings of this study can be valuable in identifying neuraminidase inhibitors with optimal lipophilicity and in the exploration of 430-cavity.

Keywords Influenza virus · Neuraminidase inhibitors · Oseltamivir derivatives

1. Neuraminidase and inhibitors

Influenza is a highly contagious disease, and influenza A and B viruses cause seasonal epidemics. Novel influenza strains that emerge periodically could cause an influenza pandemic. In the past centuries, influenza epidemics have caused numerous human deaths.[1-4] Vaccines and drugs are available for the prevention and treatment of influenza, respectively. Vaccines are effective only for influenza caused by a matched virus. In cases where vaccines are ineffective, anti-influenza drugs are administered. There are two types of anti-influenza drugs, namely Matrix-2 (M2) proton channel inhibitors and neuraminidase (NA) inhibitors. M2 proton channel inhibitors[5-6] are no

longer recommended owing to severe side effects and development of drug resistance. [7] NA inhibitors (NAIs) are the mainstay of treatment for influenza.[8-9] NA, a viral surface glycoprotein, can facilitate the release of nascent viruses from host cells by cleaving the glycosidic bond formed between sialic acid and galactose.[10] By inhibiting NA, NAIs prevent the influenza virus from infecting normal cells, thus preventing their propagation and achieving success in the treatment of influenza.

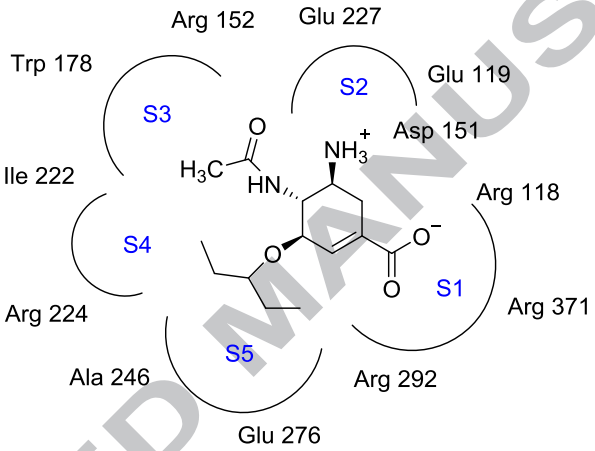


Fig. 1 Interaction of S1-S5 subsites of neuraminidase active site with oseltamivir carboxylate

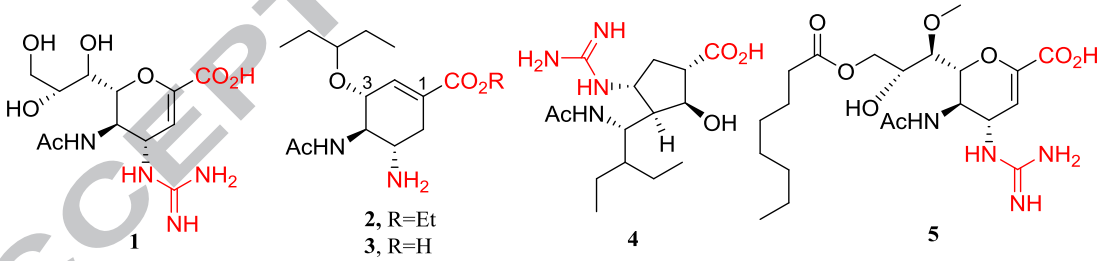


Fig. 2 Active ingredients of four neuraminidase inhibitors

The active site of NA is empirically divided into five subsites (S1-S5) (**Fig. 1**). S1 containing three basic amino acids (Arg118, Arg292, and Arg371) and S2 comprising three acidic amino acids (Glu119, Asp151, and Glu227) could form ionic bonds with the acidic and basic fragments of ligands, respectively, which facilitate potent inhibition. Therefore, the clear majority of potent NAIs, including the active

ingredients of four drugs (**Fig. 2**), carried both acidic and basic moieties. Compounds with a zwitterionic structure coupled with low molecular weight generally have poor lipophilicity, which results in poor bioavailability of drugs. Thus, zanamivir hydrate (**1**, **Fig. 2**)[11] and laninamivir octanoate (**5**, **Fig. 2**) were approved for inhalation and peramivir hydrate (**4**, **Fig. 2**) was used for injection. Oseltamivir (OS) (**2**, **Fig. 2**) phosphate, a prodrug of oseltamivir carboxylate (OC) (**3**, **Fig. 2**), is the only oral agent with suitable lipophilicity and good bioavailability.[12]

Recently, 371- and 430-cavity located near S1 were found (**Fig. 3**), suggesting new opportunities for NAI design.[13-15] The 430-cavity is a relatively positively-charged pocket consisting of R430-T439. The 371-cavity comprising R371, W403, I427, P431, and K432 is a hydrophobic cavity. Several NAIs interacting with 371- or 430-cavity have been reported. Hong et al. grafted an indole ring fragment on the carboxyl group of oseltamivir to obtain the highly active compound **A** (**Fig. 4**) (A/WSN/33 H1N1 IC_{50}= 6.4 nM, OC: IC_{50}= 1.78 nM), and the elongated groups at the C-1-position were projected toward the 371-cavity region. In addition, they observed additional interactions.[16] Recently, Ju et al. modified the carboxyl group of oseltamivir to obtain compound **B** (**Fig. 4**), which exhibits good inhibitory effects with IC_{50} value of 0.088 μM (A/chicken/China/1220/2012 H5N1 OC: IC_{50}= 0.023 μM).[17] Feng et al. modified the carboxyl group of zanamivir and obtained compound **C** (A /Indonesia/5/2005 H5N1 IC_{50}= 0.001 μM, OC: IC_{50}= 0.012 μM) (**Fig. 4**), and the elongated groups at the C-1-position were projected toward the 430-loop region. Moreover, they mentioned that aryl group facilitates interaction with 430-cavity.[18]

Currently, only a few compounds acting on the new active cavities near the S1 region have been reported, and the activity of compound **A** is slightly weaker than that of the control drug. Compound **C** showed potent activity. Owing to its high polarity, it is highly soluble in water, leading to poor drug absorption. Because 430-cavity is relatively positively charged, it can interact with phenyl or hydrophobic substituents. The 371-cavity can interact with hydrophobic substituents. Therefore, the addition of lipophilic substituents with new active cavities to the listed NAIs can lead to additional interactions and increase the inhibitory effects on the virus. It can also improve the lipophilicity of compounds as well as the absorption of drugs. Therefore, it is necessary to develop potent NAIs acting on the new active sites near the S1 region.

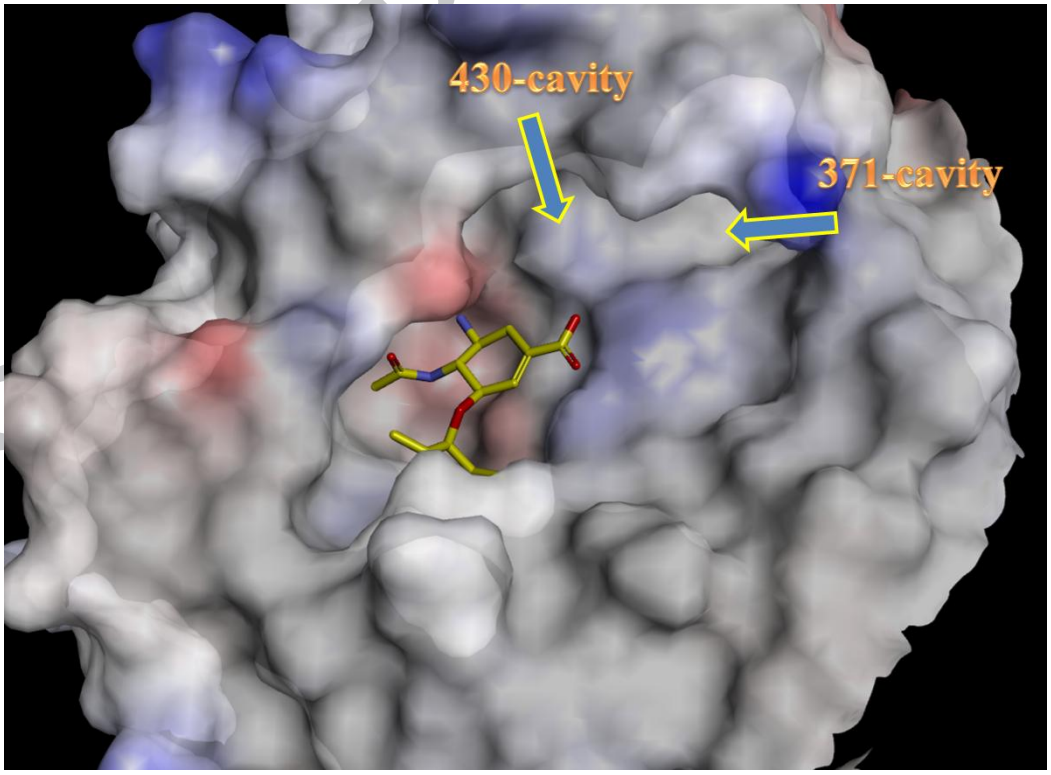


Fig. 3 Structure of compound **OC** and the active cavities in H5N1 (PDB code: 2HU4)

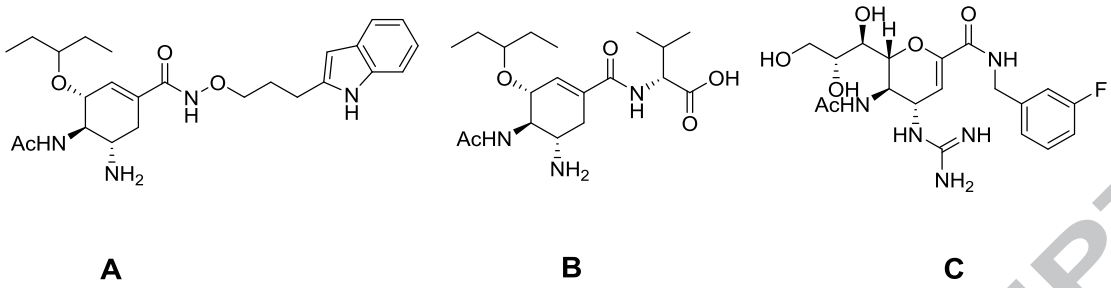


Fig. 4 Structure of compounds A, B, and C

2. Synthesis route of target compounds

Intrigued by the newly discovered cavities near S1, two types of C-1-modified OC analogs with improved lipophilicity were designed (**Fig. 5**). One was a lipophilic substituent with aromatic and/or alkyl groups to produce additional hydrophobic and π-π interactions. The other type contained a carboxyl group to retain electrostatic interactions with Arg118, Arg292, and Arg371.

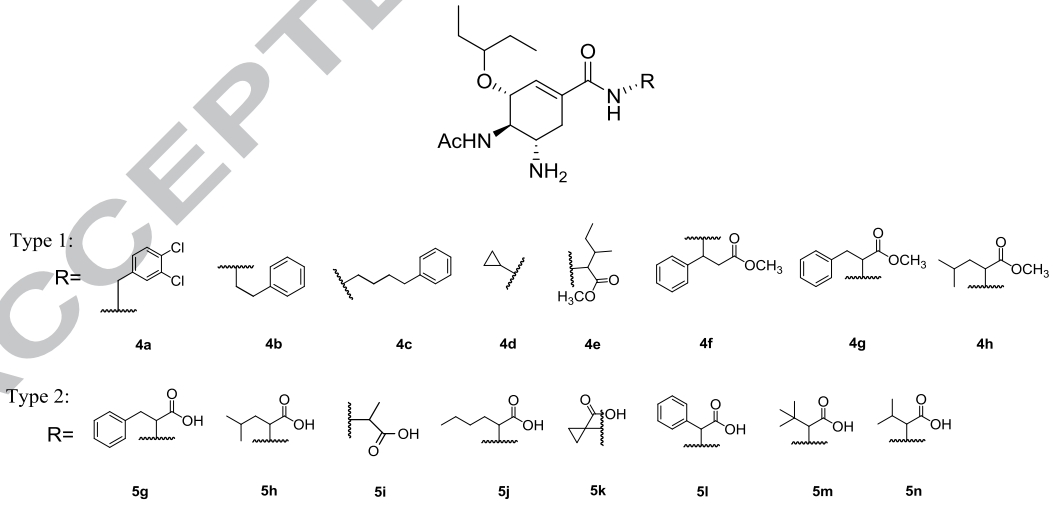
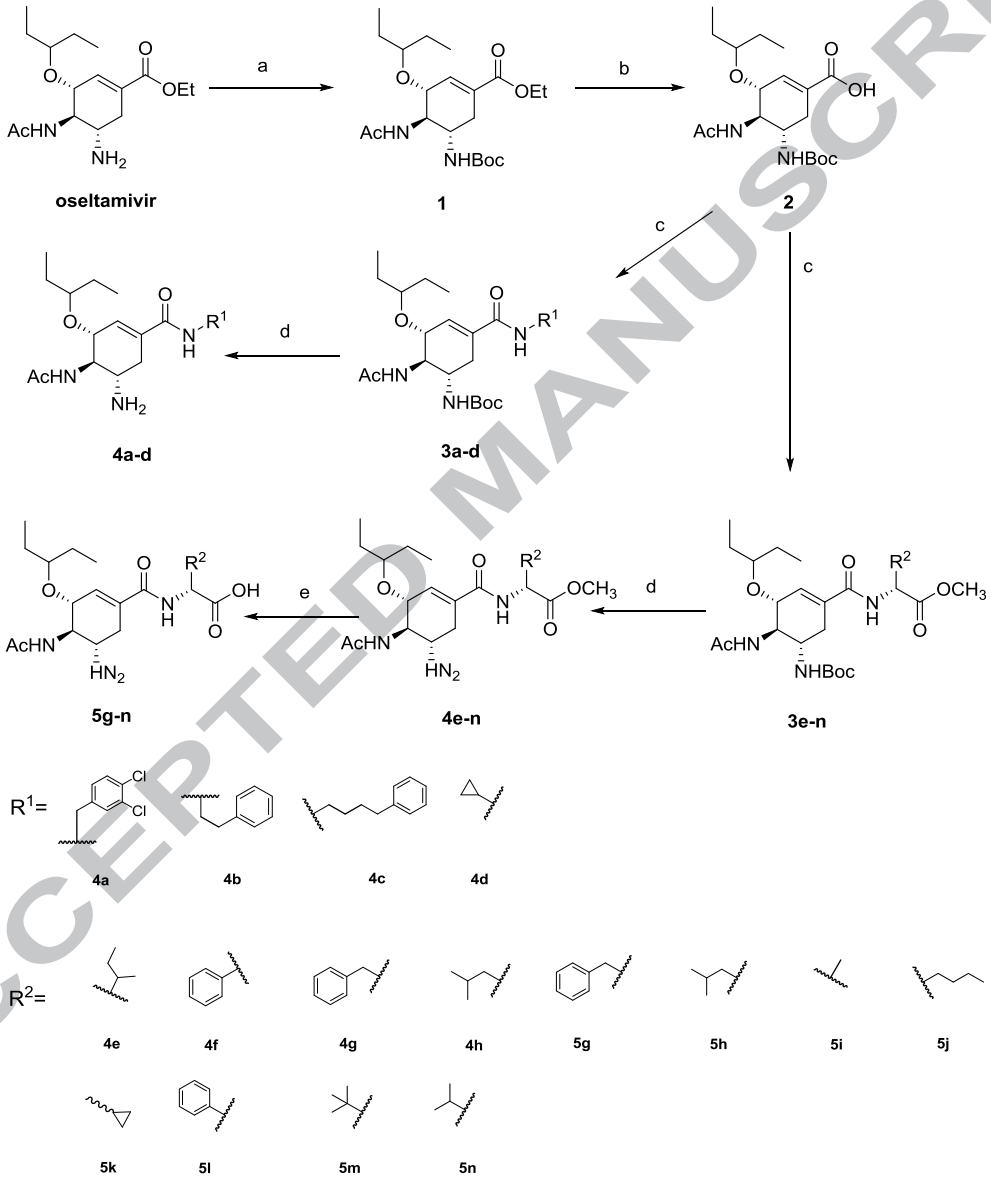


Fig. 5 Modification of oseltamivir at the carboxylic moiety by adding amido groups

Two kinds of oseltamivir derivatives were synthesized via the process outlined in **Scheme 1**. The amino group of oseltamivir was protected by *t*-Boc to yield compound **1**. Hydrolysis of compound **1** with NaOH yielded compound **2**. Compound **2** was

reacted with different amines to afford compounds **3a-n**. The *t*-Boc group of compounds **3a-n** was then hydrolyzed in the presence of trifluoroacetic acid (TFA) to yield compounds **4a-n**. The hydrolysis of compounds **4g-n** with NaOH yielded the target compounds **5g-n**. The complete data are provided in supplemental material.



Scheme 1. Reagents and conditions: **a** $(Boc)_2O$, Et_3N, rt; **b** NaOH, CH_3OH:H_2O (5:1), rt; **c** HATU, DIPEA, CH_2Cl_2, R-NH_2, rt; **d** TFA, rt; **e** NaOH, CH_3OH:H_2O (5:1), rt.

3. Biological activity of the target compounds

The compounds were tested for their abilities to inhibit NA from H5N1 subtype

using OC as a positive control (see Supplementary Data for details). First, we tested the inhibition rates of compounds **4a-h** and 5**g-n** at concentrations of 1 and 10 μM. Then, the IC_{50} values of compounds **5g-k** and **5m-n** with good inhibition rates were assayed. The results are presented in **Tables 1** and **2**, respectively.

As shown in **Table 1**, the inhibitory effects of compounds **4a-d** were less potent than that of OC. This may be due that compounds **4a-d** lost the electrostatic interactions with S1 in the absence of carboxyl fragment. Compounds **4e-h** exhibited better inhibitory activities than compounds **4a-d**. The additional ester moieties of compounds **4e-h** acting as hydrogen bond acceptors might form additional H-bond interactions with the three basic residues (Arg118, Arg292, and Arg371), resulting in improved inhibitory activities. Compounds **5g-h**, obtained by changing the ester fragments of compounds **4g-h** to the corresponding carboxyl fragments, showed general improvement in inhibitory activities. This can be attributed to the ability of carboxyl fragment to form electrostatic interactions with basic amino acids. The improvement in inhibitory activities could also be attributed to the enhanced capacity to generate hydrogen bonds both as an acceptor and a donor. Meanwhile, substituents other than carboxyl fragments also played a vital role in the inhibitory activities, as shown by compounds **5g-n**. Compounds **5g** and **5l**, containing one benzene ring, showed dissatisfactory inhibitory activities, and compound **5g** exhibited the most powerful activity with inhibition rates of 21.1 and 80.4% at concentrations of 1 and 10 μM, respectively. The biological activities of compounds **5h-k** and **5m-n** improved to some extent owing to the absence of phenyl groups, with the inhibition rates at 10 μM

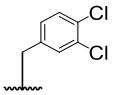
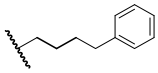
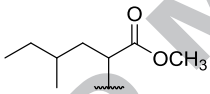
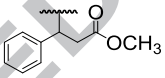
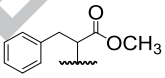
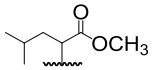
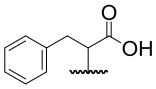
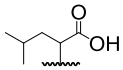
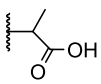
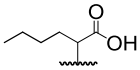
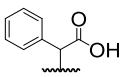
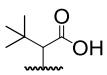
exceeding 80.4%. It is worth noting that the activities of **4f** and **4g** containing phenyl groups were significantly higher than those of **4e** and **4h**, which is contradictory to the results obtained from the activity data of **5g-n**. We considered that the carboxyl group, on the one hand, formed strong ionic and hydrogen bond interactions with the basic amino acids Arg292, Arg371, and Arg118 in the S1 region. However, the anchoring of carboxyl group to S1 region limits the binding of rigid groups such as phenyl group to NA, resulting in unsatisfactory effects for compounds **5g** and **5l** containing phenyl group. The IC_{50} values of compounds **5m** and **5n**, which showed the most potent activities among compounds **4a-h** and **5g-n**, were 1.30 and 4.18 μM, respectively. The *t*-butyl fragment in compound **5m** favored potent inhibition, because *t*-butyl formed new interactions with NA. Fragments larger or smaller than the optimum *t*-butyl fragment led to unfavorable biological activities. Although compound **5m** exhibited weaker inhibitory activity than OC, its lipophilicity was likely to be higher, which is propitious to improved membrane permeability, oral bioavailability, and even therapeutic effect.

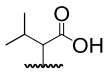
Coincidentally, compound **B** (against A/chicken/China/1220/2012 H5N1, IC_{50} =0.088 μM, OC: IC_{50}= 0.023 μM) found by Zhu et al.[18] has the same structure as a compound in our earlier patent application,[19] and this compound was identified as compound **5n** in this paper (against A/Anhui/2005 H5N1, IC_{50} =4.18±0.33 μM, OC: IC_{50}= 0.21±0.02 μM). However, in our study, a new compound **5m** (against A/Anhui/2005 H5N1, IC_{50} =1.30±0.23 μM) showed stronger inhibitory activity than **5n**. It can be suggested that compound **5m** has better activity than compound **B** found

by Zhu et al. To some extent, the discovery of compound **5m** contributed to the discovery of carboxyl-modified potent NAIs.

Table 1

Structures and *in vitro* inhibition rates[a] of compounds against neuraminidase from H5N1 subtype

Compound	R	@ 1 μM	@ 10 μM
4a		N.D.[b]	21.4%
4b		13.5%	19.3%
4c		24.7%	29.6%
4d		5.0%	55.3%
4e		23.6%	50.5%
4f		26.5%	56.7%
4g		24.9%	63.2%
4h		13.1%	41.7%
5g		21.1%	80.4%
5h		38.9%	84.4%
5i		27.8%	81.1%
5j		N.D.	85.5%
5k		N.D.	85.7%
5l		N.D.	8.3%
5m		58.3%	91.0%

5n		57.6%	91.4%
OC		66.7%	91.0%

[a]: Inhibition rates of compounds against NA from A/Anhui/2005 H5N1 subtype at this concentration.

[b]: Not Detected.

Table 2

IC_{50} of compounds against neuraminidase from H5N1 subtype

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
5g	21.89±1.1	**5h**	6.55±2.8
5i	24.44±1.0	**5j**	30.74±2.1
5k	46.44±0.7	**5m**	1.30±0.23
5n	4.18±0.33	**OC**	0.21±0.02

4. Lipophilicity of compound 5m

Compounds with appropriate lipophilicity can readily traverse a membrane and then distribute to their sites of action, which is conducive to improving bioavailability and therapeutic effects. Most of the potent NAIs have poor bioavailability owing to poor lipophilicity and membrane permeability. Based on the above consideration, we adopted octanol/phosphate-buffered saline (PBS) distribution coefficient (Log D) to explore the lipophilicity of compound **5m** (see Supplementary Data for details). As shown in **Table 3**, compound **5m** showed the highest lipophilicity at approximately pH 6.8. Compound **5m** (Log D = -0.1159) was approximately fifteen times more lipophilic than OC (Log D = -1.69) at pH 7.4, which led to potentially improved membrane permeability, oral bioavailability, and therapeutic effect.

Table 3

Distribution coefficients of compound **5m** between octanol and PBS buffer

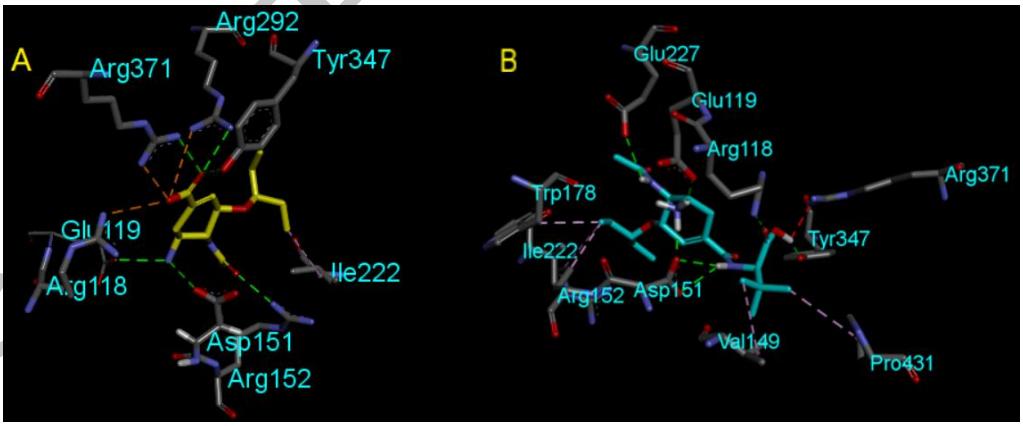
pH	D	Log D
5.8	0.0028	-2.5
6.8	0.8998	-0.0458
7	0.6812	-0.1667
7.4	0.7658	-0.1159
7.6	0.6183	-0.2087
7.4 (**OC**)		-1.69

5. Molecular docking

To understand the interactions between compound **5m** and NA further, molecular docking was performed using MAESTRO. Images depicting the proposed binding modes were generated by Discovery Studio 2016 Client. The resolved NA (PDB code: 2HU4) from H5N1 subtype was downloaded from RCSB protein data bank (PDB).

As shown in Figure 6, the binding model of compound **5m** and NA was similar to that of OC and NA. Compared with the binding model of OC and NA, the OC part of **5m** retained C3-(pentan-3-yloxy) to form hydrophobic interactions with Trp178 and IIe222, and retained C5-NH_2 to form H-bond interactions with Asp151 and Glu119. Although the hydrogen bond interactions between C4-acetamido of OC and Arg152 did not occur in the new compound **5m**, acetamido of **5m** formed new hydrogen bond interactions with Glu227. In **5m**, the same structure as OC maintains the original interactions between OC and NA. However, the two binding models do

not coincide completely; the carboxyl group at C1 site of OC have three ionic bonds and two hydrogen bonds with Arg292, Arg371, and Arg118. Compared with OC, the carboxyl group of **5m** forms only one ionic bond interaction and one hydrogen bond interaction with Arg118 and Arg371, because the carboxyl group of **5m** is pushed to the deeper region of NA. Although the carboxyl group of **5m** forms a new π-cation interaction with Tyr347, the loss of several interactions was observed in **5m** when compared with the interactions between OC and NA. The *t*-butyl group of **5m** was projected toward the newly discovered 430-cavity and formed new hydrophobic interactions with Val149 and Pro431. The new hydrophobic interactions make up for the lost interactions between the carboxyl group of OC and NA to some extent. Therefore, **5m** shows good activity, and may have beneficial effects *in vivo* owing to the introduction of lipophilic group.



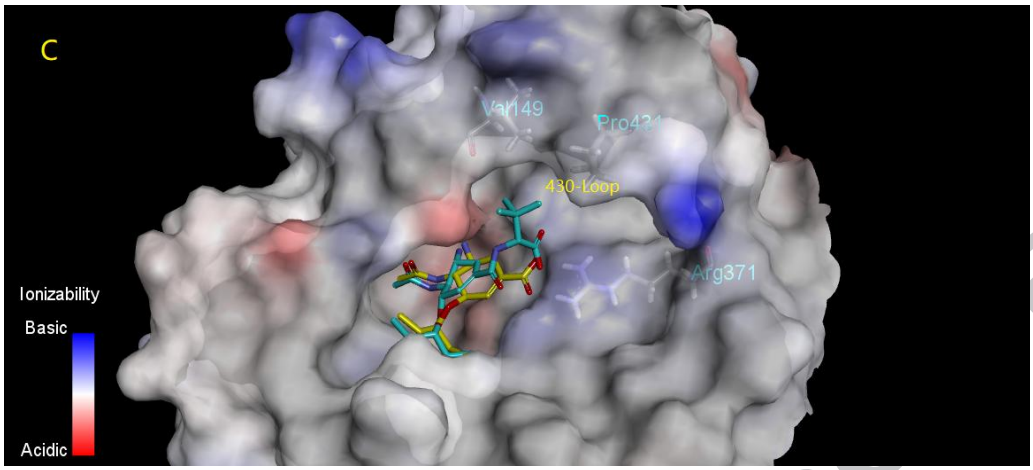


Fig. 6 A and **B:** Docking modes of compounds OC and **5m** with neuraminidase (PDB code: 2HU4), respectively. **C**: Compound **5m** (blue) and **OC** (yellow) are superposed within NA (PDB code: 2HU4). This image was obtained using Discovery Studio 2016 Client.

6. Metabolic stability assay in vitro

Table 4

Human liver microsomal stability of compound **5m**

Compounds	$\mathbf{T_{1/2}}$ (min)	Remaining (**T** = 60 min)
5m	>145	84.1%
Testosterone	14.5	5.2%
Diclofenac	11.6	2.4%
Propafenone	6.1	0.1%

Liver weight: 20 g/kg for humans.

Metabolic stability of drugs can affect pharmacodynamic properties such as oral efficacy and duration of action. Generally, the degree to which a compound is metabolized within the liver is expressed as the metabolic half-time ($\mathbf{T_{1/2}}$) *in vitro*.

Compound **5m** with the most potent inhibitory activity was selected to perform *in vitro* metabolic stability study in human liver microsomes. The control compounds

were testosterone, diclofenac, and propafenone.[20-23] The data are shown in **Table 4** (see Supplementary Data for details). After incubation with human liver microsomes for 1 h, **5m** (84.1%) was detected. The $\mathbf{T_{1/2}}$ was more than 145 min, which indicated that **5m** showed good stability in human liver microsomes.

In summary, we designed 16 oseltamivir analogs by modification at the C-1 position to make use of the newly discovered cavities. Compound **5m** exhibited a slightly weaker inhibitory activity than OC. Molecular modeling showed that the introduced *t*-butyl moiety of **5m** interacted with NA and was projected toward the 430-loop region. Compound **5m** showed high metabolic stability in human liver microsomes. Notably, the lipophilicity of compound **5m** was improved, which favored membrane permeability and oral drug absorption. To a certain extent, we achieved beneficial anti-NA activity and lipophilicity, which will be of value in the rational design of new types of NAIs with potent inhibitory activity under the precondition of optimal Log D.

Acknowledgements This work was financially supported by Foundation of Shenyang Science and Technology Bureau (NO. F13-196-9-00).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Abbreviation list

NA, neuraminidase; OS, oseltamivir; OC, oseltamivir carboxylate; TFA, trifluor-

oacetic acid; HATU, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; DIPEA, N,N-diisopropylethylamine; PDB, Protein Data Bank

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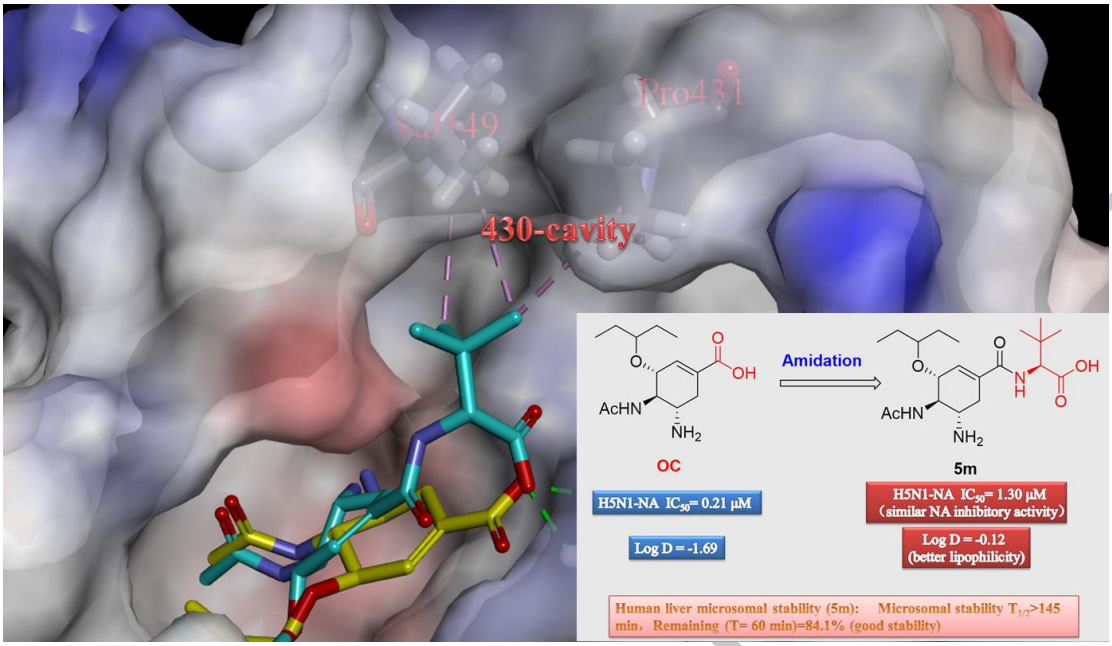

Highlights

- A series of carboxyl-modified oseltamivir analogs with improved lipophilicity were designed and synthesized.
- Inhibitory activities of compounds against neuraminidase from influenza A virus H5N1 subtype were evaluated.
- The molecular docking studies of **5m** was performed.
- Compound **5m** targeted the recently discovered 430-cavity.
- Lipophilicity and metabolic stability of compound **5m** were evaluated.