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Design, synthesis and biological activity of thiazolidine-4-carboxylic acid derivatives as novel influenza neuraminidase inhibitors

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ABSTRACT

A series of thiazolidine-4-carboxylic acid derivatives were synthesized and evaluated for their ability to inhibit neuraminidase (NA) of influenza A virus. All the compounds were synthesized in good yields starting from commercially available L-cysteine hydrochloride using a suitable synthetic strategy. These compounds showed moderate inhibitory activity against influenza A neuraminidase. The most potent compound of this series is compound **4f** (IC_{50} = 0.14 μM), which is about sevenfold less potent than oseltamivir and could be used to design novel influenza NA inhibitors that exhibit increased activity based on thiazolidine ring.

1. Introduction

Influenza can cause serious public health and economic problems, which affects millions of people worldwide.[1] Despite advances in the understanding of molecular and cellular aspects of influenza, the disease remains the major cause of mortality and morbidity among patients with respiratory diseases.[2]

Influenza virus neuraminidase (NA) is thought to promote virus entry and release of virion progeny, thereby enhances infection efficiency. Rationally designed NA inhibitors (NAIs) that block the viral life cycle are proved to be effective for the treatment of influenza. The NAIs have currently emerged as promising therapeutics for influenza. The first approved neuraminidase inhibitor zanamivir (Relenza) is rarely used because it is administered by inhalation,[3] which causes inconvenience. Subsequently discovered oseltamivir (Tamiflu) is orally available.[4] As the predominant choice, oseltamivir is used worldwide for the treatment of influenza. However, the generation and circulation of oseltamivir-resistant mutants of seasonal influenza, as well as H5N1 avian influenza, have become major concerns.[5–11] Peramivir, the third neuraminidase inhibitor, displays only limited oral bioavailability.[12,13] Intravenous peramivir is currently undergoing a pre-emergency use authorization review for its use in cases of severe influenza outbreaks.

Neuraminidase remains an attractive anti-influenza drug target, while the emergence of viruses resistant to the currently available drugs has presented a new challenge. Noticeably, the crystal structures of the group-1 neuraminidase (N1, N4, and N8) revealed a novel cavity adjacent to the active site in group-1 but not in group-2 proteins crystallography, suggesting new opportunities for drug design that target this cavity in addition to the known active site.[14]

Earlier crystallographic and ensuing SAR studies have revealed that the active site of NA could be divided into four major binding sites. Steindl and Lange[15] described the development of highly selective pharmacophore models for inhibitors of viral NA within the Catalyst software package. All NA inhibitors on the market or in clinical phases possess strong structural resemblance in those parts, which correspond to the fact that the four pockets are critical for interaction with the active site of NA. The four pockets defining the binding site are displayed in Figure 1.

The pocket C1 is comprised of the positively charged guanidino groups of arginines 118, 292 and 371 and interacts with the carboxylate. In pocket C5, Arg 152 functions as the hydrogen-bond donor. Trp 178 and Ile 222 comprise a small hydrophobic region. In pocket C4, usually a guanidine or an amine group, participates in charge–charge interactions and hydrogen bonds to Glu 119, Asp 151, and/or Glu 227. Compared with the 4-hydroxy analogues developed earlier, these basic substituents are proved to increase inhibitory activity. In pocket C6, Glu 276, the side chain of Arg 152, the amidic carbonyl of Trp 178 and Asp 151 form a new hydrophobic binding pocket. Moreover, Glu277 and Tyr406 are believed to play a critical role in the catalytic activity of NA.[16,17]

According to the studies on NA active site and SAR of published NA inhibitors, inhibition of the NA is mainly determined by the relative positions of the four substituents of the central ring.[18] For example, in oseltamivir, the four substituents are carboxyl ethyl ester, amino, acetamino and alkyl. However, more and more recent

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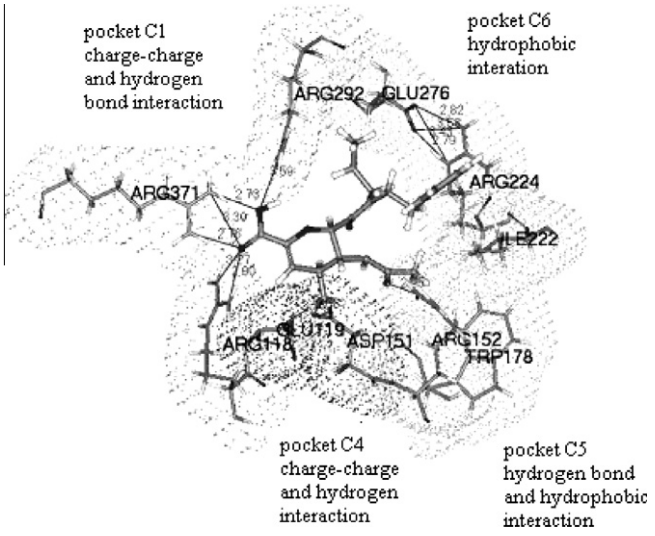


Figure 1. Display of important amino acids forming four pockets of NA binding site.[15]

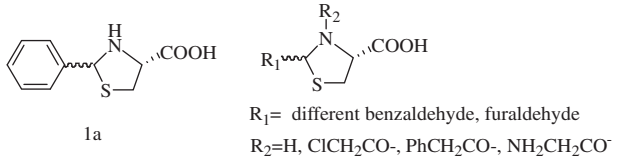


Figure 2. Structure of compound **1** and the modified thiazolidine derivarives.

studies revealed some untypical neuramnidase inhibitors (including flavonoids, diarylheptanoid, etc.) that beyond the 'airplane' model interaction with the NA active site,[19–26] thus we suppose that the aromatic group is suitable for the pocket of the NA active site.

In previous studies,[27–29] we have reported several kinds of novel neuraminidase inhibitors based on pyrrolidine, benzyl and thiazole scaffold. In our recent screening, we found that compound **1a** exhibited substantial NA inhibition (IC_{50} = 21.3 μM). Considering that no thiazolidine derivative has been reported as neuraminidase inhibitors, we chose 1a as the lead compound the lead compound for further modification and optimization (shown in Fig. 2).

2. Chemistry

To contain the different substituents to interact with the four binding pockets of the NA active site, we used the following chemical modifications as shown in Scheme 1. Benzaldehydes with different substituents and 2-furaldehyde were used to couple with L-cysteine hydrochloride in EtOH/H_2O 1:1 to obtain the thiazolidines **1** as diastereomers (*anti* and *syn* compounds). Then the thiazolidines were alkylated with chloroacetyl chloride or phenylacetyl chloride in the existence of $NaHCO_3$ in H_2O to yield chloroacetyl thiazolidines **2** and phenylacetyl thiazolidines **3**. Then the crude chloroacetyl thiazolidines were aminated in ammonia solution to get the crude compounds **4**. Subsequently by Boc-protected reaction and the Boc-group cleavage in HCl/EtOAc, compound **4** were obtained as hydrochloride salts.

3. Result and discussion

All the target compounds (27 compounds) were tested for their ability to inhibit NA. Preliminary result showed that most of the compounds (24 compounds) displayed enhanced inhibitory activities (IC_{50} = 20.3–0.14 μM) compared to the lead compound **1a** (Table 1). The amino-acetyl-thiazolidines showed the best activities, and the order of increasing activity in R2 is: NH_2– > $PhCH_2CO$– > $ClCH_2CO$– > H.

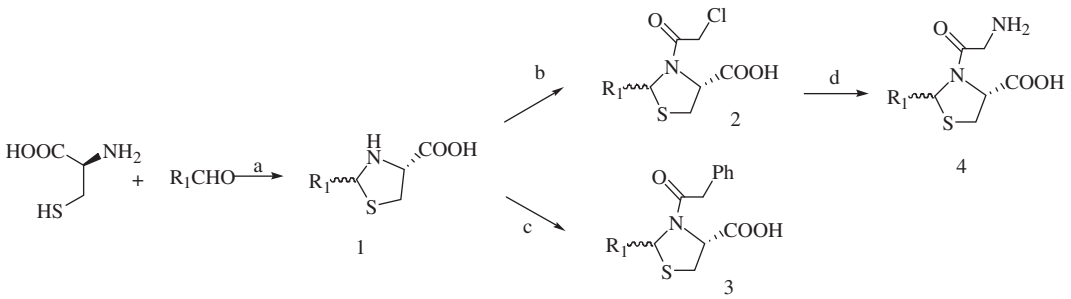
The most potent compound is **4f** (IC_{50} = 0.14 μM), which is the mixture of $\mathbf{4f}_{anti}$ (the major product) and $\mathbf{4f}_{syn}$ (the minor product). In order to determine the interaction between thiazolidines and the NA active site, compound $\mathbf{4f}_{anti}$ and $\mathbf{4f}_{syn}$ were docked into the active sites of NA separately.

The binding of compound **4f** in the active site of NA is shown in Figure 3, and we find that the –COOH group of the target compound interacts with the pocket C1 of NA active site by making charge–charge interactions with Arg 292, Arg 371, Tyr 406 of this subsite. The reason for which the –COOH group is important for the development of novel inhibitors is that it is well established that two or three Arg residues in the immediate vicinity of the carboxylic group of NA inhibitors play a key role in orienting and stabilizing various inhibitors.[30,31] The $–NH_2$ group binds to the pocket C4 by hydrogen bond interaction with Asp151. The –CO– group forms hydrogen bond with Arg 118 and Arg 371 of pocket C1.

Moreover, for compound $\mathbf{4f}_{anti}$, the $–OCH_3$ group occupies the hydrophobic region in pocket C6 formed by Glu 277, Glu 227 and Glu 226. The –OH group forms hydrogen bond with Glu 277. The hydrophobic region formed by Trp 178 and Ala 180 in pocket C5 accommodates the phenyl group.

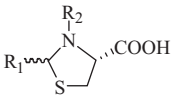
For compound $\mathbf{4f}_{syn}$, the phenyl group together with the $–OCH_3$ group of $\mathbf{4f}_{syn}$ occupies the hydrophobic region formed by Arg 224 and Glu 277 of the C6 pocket. The –OH group does not form any hydrogen bond with the active site, which may explain the reason that the predicted activity of the $\mathbf{4f}_{syn}$ is not so good as that of the $\mathbf{4f}_{anti}$.

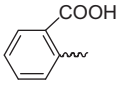
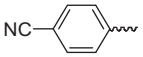
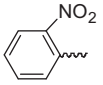
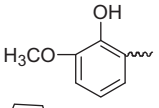
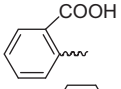
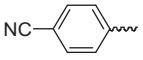
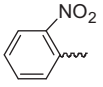
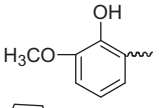
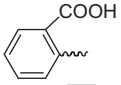
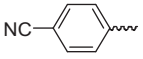
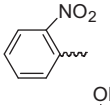
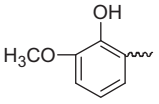
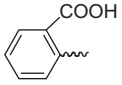
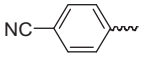
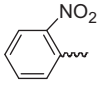
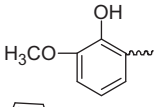
After all, the occupation of the phenyl group in the active site confirms our indication that the aromatic group is suitable for the pocket of the NA active site. And the docking result of **4f** compared with oseltmivir shown in Figure 4 also supports this suggestion.



Scheme 1. Reagents: (a) $NaHCO_3$, EtOH/H_2O; (b) $ClCH_2COCl$, $NaHCO_3$/H_2O; (c) $PhCH_2COCl$, $NaHCO_3$/H_2O; (d) $NH_2{\cdot}H_2O$; BOC_2O, THF; HCl/EtOAc.

Table 1
The structures and in vitro NA inhibitory activity of the target compounds and oseltamivir



Compound	R1	R2	IC_{50} (μM)
1a		H	21.3
1b		H	20.2
1c		H	18.1
1d		H	23.4
1e		H	22.5
1f		H	12.3
1g		H	43.1
2a		$ClCH_2CO$–	7.53
2b		$ClCH_2CO$–	5.84
2c		$ClCH_2CO$–	10.7
2d		$ClCH_2CO$–	8.64
2e		$ClCH_2CO$–	7.65
2f		$ClCH_2CO$–	7.92
2g		$ClCH_2CO$–	12.9
3a		$PhCH_2CO$–	1.21
3b		$PhCH_2CO$–	0.65
3c		$PhCH_2CO$–	1.92
3d		$PhCH_2CO$–	2.47
3e		$PhCH_2CO$–	1.87
3f		$PhCH_2CO$–	1.62
3g		$PhCH_2CO$–	2.89
4a		NH_2CH_2CO–	0.53
4b		NH_2CH_2CO–	0.21
4c		NH_2CH_2CO–	0.28
4d		NH_2CH_2CO–	0.81
4e		NH_2CH_2CO–	1.02
4f		NH_2CH_2CO–	0.14
4g		NH_2CH_2CO–	0.98
Oseltamivir carboxylate			0.02

4. Conclusion

A series of novel influenza neuraminidase inhibitors based on thiazolidine core were synthesized and evaluated for their ability to inhibit neuraminidase (NA) of influenza A virus.

Several compounds were shown to possess moderate influenza NA inhibitory activity, although in all cases, measured activities were lower than that of oseltamivir. The most potent compound of the series is compound **4f** (IC_{50} = 0.14 μM), about sevenfold less potent than oseltamivir. The binding of compound **4f** in the active site of NA showed that the four pockets of the active site of NA were all occupied, although not so well as oseltamivir to establish a consistent binding orientation and more potent activity.

In summary, our study indicated that thiazolidine derivatives could show potent NA inhibitory activity and this finding could be used to design novel influenza NA inhibitors that exhibit increased activity based on thiazolidine ring. Additionally, in the following study, we will perform the separation of *anti* and *syn* diastereomers to find more active compounds.

5. Experimental

5.1. Synthetic methods and spectroscopic details

All the reactions were carried out by the standard techniques for the exclusion of moisture. Solvents were distilled prior to use and flash chromatography was performed using silica gel (60 Å, 200 ± 300 mesh). All the reactions were monitored by thin-layer chromatography on 0.25 mm silica gel plates (60GF-254) and visualized with UV light, or iodine vapour. Melting points were obtained on an electrothermal melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were determined on a Brucker Avace 300 spectrometer using TMS as an internal standard. Chemical shifts are reported in delta (δ) units, parts per million (ppm) downfield from TMS. High-resolution mass spectral (HRMS) data are reported as *m/z* (relative intensity).

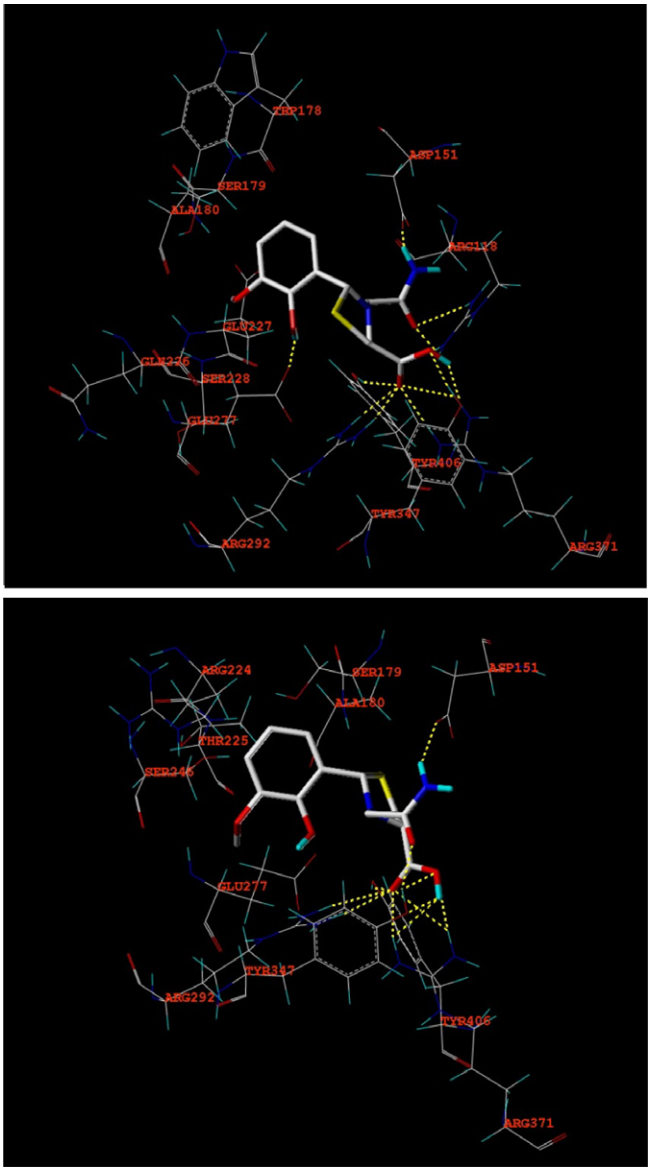


Figure 3. Detailed view of the docking result of compound **4f** with some key amino acid residues in the active site of neuraminidase (PDB ID: 2hu4) through the Surflex-Dock module of Sybyl 8.1. The yellow lines and numbers show the potential hydrogen bonds and bond length. The first one is $\mathbf{4f}_{anti}$, and the second one is $\mathbf{4f}_{syn}$.

5.1.1. (2*RS*,4*R*)-2-Phenyl-thiazolidine-4-carboxylic acid (1a)

To the solution of L-cysteine hydrochloride hydrate and $NaHCO_3$ (1.1 equiv) in water (200 mL), benzaldehyde (1.1 equiv) in 95% ethanol (200 mL) was added in one portion. The reaction mixture was stirred for 6 h. The product was filtered, washed with ethanol, and dried to afford as a white solid. Yield: 95%. Mp 159–160 °C (lit. 159–160 °C). ^{1}H NMR (DMSO-d_6, 300 MHz): 3.049–3.166 (m, 0.9H, 5-H), 3.294–3.406 (m, 1.1H, 5-H), 3.902 (q, J = 7.2 Hz, J = 1.5 Hz, 0.45H, 4-H), 4.234 (t, J = 4.8 Hz, J = 2.4 Hz, 0.55H, 4-H), 5.504 (s, 0.45H, 2-H), 5.669 (s, 0.55H, 2-H), 7.242–7.533 (m, 5H).HRMS: m/z calcd for $C_{12}H_{14}N_2O_3S$ $[M+H]^+$ 210.0588; found: 210.0587.

Compounds **1b**–**1g** were prepared following the general procedure as described above.

5.1.2. (2*RS*,4*R*)-2-(2-Hydroxy-phenyl)-thiazolidine-4-carboxylic acid (1b)

White solid. Yield: 95%. Mp 160–161 °C (lit. 167 °C). ^{1}H NMR (DMSO-d_6, 300 MHz): 2.946–3.046 (m, 0.9H, 5-H), 3.176–3.367 (m, 1.1H, 5-H), 3.834(q, J = 6.9 Hz, J = 2.1 Hz, 0.45H, 4-H), 4.213 (t, J = 6 Hz, 0.55H, 4-H) 5.649 (s, 0.45H, 2-H), 5.838 (s, 0.55H, 2-H), 7.033–7.360 (m, 4H). HRMS: m/z calcd for $C_{10}H_{11}NO_3S$ $[M+H]^+$ 226.0538; found: 226.0532.

5.1.3. (2*RS*,4*R*)-2-(2-Carboxy-phenyl)-thiazolidine-4-carboxylic acid (1c)

White solid. Yield: 90%. Mp 160–161 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): 2.870–3.022 (m, 1.5H, 5-H), 3.124–3.360 (m, 0.25H, 5-H), 3.906 (q, J = 6.9 Hz, J = 3 Hz, 0.75H, 4-H), 4.080 (t, J = 6.9 Hz, 0.25H, 4-H) 6.158 (s, 0.25H, 2-H), 6.508 (s, 0.75H, 2-H), 7.296–7.780 (m, 4H). HRMS: m/z calcd for $C_{11}H_{11}NO_4S$ $[M+H]^+$ 254.0487; found: 254.0486.

5.1.4. (2*RS*,4*R*)-2-(4-Cyano-phenyl)-thiazolidine-4-carboxylic acid (1d)

White solid. Yield: 96%. Mp 145–147 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): 3.065–3.133 (m, 0.75H, 5-H), 3.284–3.396 (m, 1.25H, 5-H), 3.902 (q, J = 6.6 Hz, J = 2.1 Hz, 0.375H, 4-H), 4.140 (t, J = 6 Hz, 0.625H, 4-H) 5.606 (s, 0.375H, 2-H), 5.807 (s, 0.625H, 2-H), 7.598–7.854 (m, 4H). HRMS: m/z calcd for $C_{11}H_{10}N_2O_2S$ $[M+H]^+$ 235.0541; found: 235.0534.

5.1.5. (2*RS*,4*R*)-2-(2-Nitro-phenyl)-thiazolidine-4-carboxylic acid (1e)

White solid. Yield: 96%. Mp 145–147 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): 3.324–3.810 (m, 2H, 5-H), 5.088–5.198 (m, 1H, 4-H), 6.324 (s, 0.7H, 2-H), 6.768 (s, 0.3H, 2-H) 7.729–8.285 (m, 4H). HRMS: m/z calcd for $C_{10}H_{10}N_2O_4S$ $[M+H]^+$ 255.0439; found: 255.0437.

5.1.6. (2*RS*,4*R*)-2-(2-Hydroxy-3-methoxy-phenyl)-thiazolidine-4-carboxylic acid (1f)

Light yellow solid. Yield: 92%. Mp 137–138 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): 2.931–3.040 (m, 1H, 5-H), 3.171–3.368 (m, 1H, 5-H), 3.777–3.858 (m, 3H, –OCH3), 3.836 (t, 0.46H, 4-H), 4.207 (t, J = 5.4 Hz, 0.54H, 4-H) 5.671 (s, 0.46H, 2-H), 5.865 (s, 0.54H, 2-H), 6.704–6.971 (m, 3H). HRMS: m/z calcd for $C_{11}H_{13}NO_4S$ $[M+H]^+$ 256.0645; found: 256.0643.

5.1.7. (2*RS*,4*R*)-Furan-2-yl-thiazolidine-4-carboxylic acid (1g)

Brown solid. Yield: 98%. Mp 140–142 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): 2.965–3.381 (m, 2H, 5-H), 3.876 (q, J = 6.9 Hz, J = 2.1 Hz, 0.33H, 4-H), 4.114 (t, J = 6.3 Hz, 0.67H, 4-H) 5.607 (s, 0.33H, 2-H), 5.742 (s, 0.67H, 2-H), 6.348 (d, J = 3.3 Hz, 0.67H), 6.382 (dd, J = 1.8 Hz, 3.3 Hz, 0.67H), 6.447 (dd, J = 1.8 Hz, 3.3 Hz, 0.33H), 6.508 (d, J = 3.3 Hz, 0.33H), 7.591 (d, J = 1.8 Hz, 0.67H), 7.663 (d, J = 1.8 Hz, 0.33H). HRMS: m/z calcd for $C_8H_9NO_3S$ $[M+H]^+$ 200.0381; found: 200.0388.

5.1.8. 3-(2-Chloro-acetyl)-(2*RS*,4*R*)-2-phenylthiazolidine-4-carboxylic acid (2a)

To the solution of compound **1a** with $NaHCO_3$ (2 equiv) in 150 mL H_2O, $ClCH_2COCl$ (1.2 equiv) was added dropwise to the solution and stirred at room temperature until no insoluble material existed. The mixture was adjusted to pH 8–9 with $NaHCO_3$, and washed with DCM (50 mL × 3). The aqueous layer was then acidified with citric acid to pH 2–3, and extracted with DCM (50 mL × 3). The organic phase was dried and evaporated to give the crude product, which was purified by column chromatography to give the title compound.

White solid. Yield: 72.0%. Mp 121–123 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 3.389–3.410 (m, 2H, 5-H), 3.729–3.883 (m, 2H, Cl–CH_2), 5.151 (t, 1H, 4-H), 6.215 (s, 1H, 2-H), 7.354–7.630 (m, 5H). HRMS: m/z calcd for $C_{12}H_{12}ClNO_4S$ $[M+H]^+$ 286.0304; found: 286.0297.

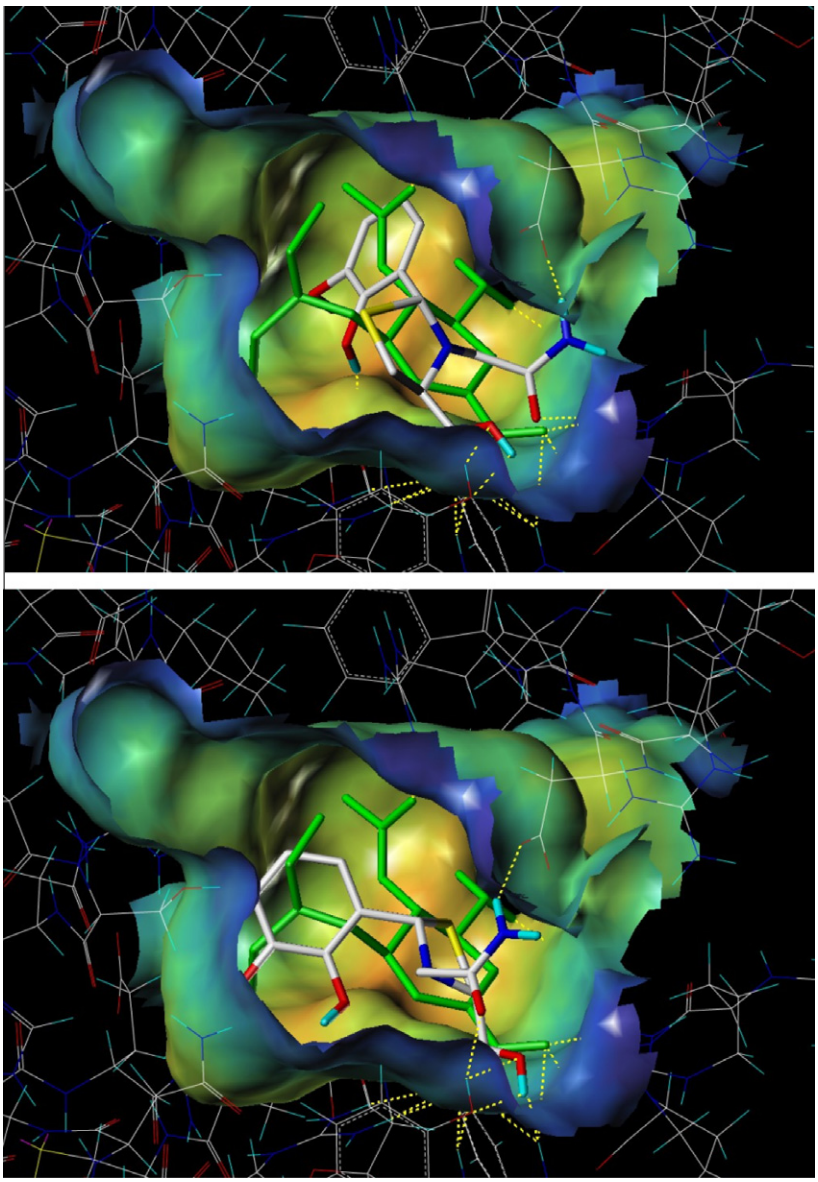


Figure 4. Docking result of compound **4f** and oseltamivir (**4f** is displayed by atom type while oseltamivir is shown in lime line). The active pocket is displayed using Channel pattern (Type: Cavity Depth) with the program MOLCAD Surfaces of Sybyl8.1. The first one is $\mathbf{4f}_{anti}$, and the second one is $\mathbf{4f}_{syn}$.

Compounds **2b**–**2g** were prepared following the general procedure as described above.

5.1.9. 3-(2-Chloro-acetyl)-(2*RS*,4*R*)-2-(2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (2b)

Whitle solid. Yield: 78.5%. Mp 139–141 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 2.998–3.482 (m, 4H, 5-H, Cl–CH_2), 4.656–5.223 (m, 1H, 4-H), 6.332 (s, 0.25H, 2-H), 6.427 (s, 0.75H, 2-H), 6.667–6.862 (m, 2H), 7.016–7.175 (m, 1H), 7.474 (d, J = 7.2 Hz, 0.25H), 7.885 (d, J = 7.2 Hz, 0.75H). HRMS: *m/z* calcd for $C_{12}H_{12}ClNO_4S$ $[M+H]^+$ 302.0254; found: 302.0252.

5.1.10. 3-(2-Chloro-acetyl)-(2*RS*,4*R*)-2-(2-carboxy-phenyl)-thiazolidine-4-carboxylic acid (2c)

Whitle solid. Yield: 70.8%. Mp 117–118 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 3.112–3.434 (m, 2H, 5-H), 4025–4.223 (m, 2H, Cl–CH_2–), 4.964 (t, J = 8.1 Hz, 1H, 4-H), 6.273 (s, 1H), 6.353 (dd, 1H), 6.632 (d, 1H), 7.428 (d, 1H). HRMS: *m/z* calcd for $C_{13}H_{14}NO_5S$ $[M+H]^+$ 329.0125; found: 329.0117.

5.1.11. 3-(2-Chloro-acetyl)-(2*RS*,4*R*)-2-(4-cyano-phenyl)-thiazolidine-4-carboxylic acid (2d)

Whitle solid. Yield: 71.5%. Mp 68–70 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 3.276–3.588 (m, 2H, 5-H), 4.096–4.167 (m, 2H, Cl–CH_2–), 5.088–5.111 (m, 0.7H, 4-H), 5.133–5.209 (m, 0.3H, 4-H), 6.273 (s, 1H, 2-H), 7.639–7.816 (m, 4H). HRMS: *m/z* calcd for $C_{13}H_{11}ClN_2O_3S$ $[M+H]^+$ 311.0257; found: 311.0249.

5.1.12. 3-(2-Chloro-acetyl)-(2*RS*,4*R*)-2-(2-nitroh-phenyl)-thiazolidine-4-carboxylic acid (2e)

Whitle solid. Yield: 70.1%. Mp 71–73 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 3.324–4.161 (m, 4H, 5-H, Cl–CH_2–), 5.088–5.109 (m, 0.65H, 4-H), 5.198–5.132 (m, 0.35H, 4-H), 6.324 (s, 1H, 2-H), 7.729–7.75 (m, 0.7H), 7.863–7.889 (m, 1.3H), 8.161–8.187 (m,

0.7H), 8.258–8.285 (m, 1.3H). HRMS: *m/z* calcd for $C_{12}H_{11}ClN_2O_5S$ $[M+H]^+$ 331.0155; found: 331.0149.

5.1.13. 3-(2-Chloro-acetyl)-(2*RS*,4*R*)-2-(2-hydroxy-3-methoxy-phenyl)-thiazolidine-4-carboxylic acid (2f)

Whitle solid. Yield: 70.5%. Mp 130–132 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 3.325–3.464 (m, 2H, 5-H), 3.773–3.818 (d, 1H, *J* = 13.5 Hz, Cl–CH_2–), 3.916 (s, 3H, –OCH_3), 4.010–4.055 (d, 1H, *J* = 13.5 Hz, Cl–CH_2–), 5.043 (t, 1H, *J* = 6.9 Hz, 4-H), 6.486 (s, 1H, 2-H), 6.849–6.942 (m, 2H), 7.352 (dd, 1H). HRMS: *m/z* calcd for $C_{13}H_{14}ClNO_5S$ $[M+H]^+$ 332.0359; found: 332.0357.

5.1.14. 3-(2-Chloro-acetyl)-2-(2*RS*,4*R*)-furan-2-yl-thiazolidine-4-carboxylic acid (2g)

White solid. Yield: 71.2%. Mp 140–142 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 3.385–3.406 (m, 2H, 5-H), 3.729–3.885 (m, 2H, Cl–CH_2–), 5.125–5.167 (m, 1H, 4-H), 6.353 (s, 1H, 2-H), 6.273 (dd, 1H, *J* = 1.8 Hz, 3.3 Hz), 6.632 (d, *J* = 3.3 Hz, 1H), 7.428(d, *J* = 1.8 Hz, 1H). HRMS: *m/z* calcd for $C_{10}H_{10}ClNO4S$ $[M+H]^+$ 271.0097; found: 276.0094.

5.1.15. 3-Phenylacetyl-(2*RS*,4*R*)-2-phenylthiazolidine-4-carboxylic acid (3a)

To the solution of compound (**2a**) with $NaHCO_3$ (2 equiv) in 150 mL H_2O, $PhCH_2COCl$ (1.2 equiv) was added dropwise to the solution and stirred at room temperature until no insoluble material existed. The mixture was adjusted to pH 8–9 with $NaHCO_3$, and washed with DCM (50 mL × 3). The aqueous layer was then acidified with citric acid to pH 2–3, and extracted with DCM (50 mL × 3). The organic phase was dried and evaporated to give the crude product, which was purified by column chromatography to give compound. White solid. Yield: 77.1%. Mp 163–165 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 3.241–3.470 (m, 2H, 5-H), 3.354 (m, 2H, Ph–CH_2–), 5.078 (t, 1H, *J* = 6.9 Hz, 4-H), 6.087 (s, 1H, 2-H), 7.063–7.507 (m, 9H). HRMS: *m/z* calcd for $C_{18}H_{17}NO3S$ $[M+H]^+$ 328.1007; found: 328.1005.

Compounds **3b–3g** were prepared following the general procedure as described above.

5.1.16. 3-Phenylacetyl-(2*RS*,4*R*)-2-(2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (3b)

White solid. Yield: 82.5%. Mp 152–154 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 3.209–3.668 (m, 4H, 5-H, Ph–CH_2–), 4.990–5.032 (m, 1H, 4-H), 6.338, 6.405 (2s, 1H, 2-H), 6.821–6.929 (m, 2H), 7.024–7.260 (m, 6H), 7.563 (d, *J* = 7.5 Hz, 1H). HRMS: *m/z* calcd for $C_{18}H_{17}NO4S$ $[M+H]^+$ 344.0957; found: 344.0959.

5.1.17. 3-Phenylacetyl-(2*RS*,4*R*)-2-(2-carboxy-phenyl)-thiazolidine-4-carboxylic acid (3c)

Whitle solid. Yield: 86.8%. Mp 185–187 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 3.165–3.627 (m, 4H, 5-H, Ph–CH_2), 4.565–4.181 (m, 1H, 4-H), 6.153, 6.216 (2s, 1H, 2-H), 6.990–8.081 (m, 9H). HRMS: *m/z* calcd for $C_{19}H_{17}NO_5S$ $[M+H]^+$ 372.0906; found: 372.0910.

5.1.18. 3-Phenylacetyl-(2*RS*,4*R*)-2-(4-cyano-phenyl)-thiazolidine-4-carboxylic acid (3d)

White solid. Yield: 89.0%. Mp 117–119 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 3.234–3.833 (m, 4H, 5-H, Ph–CH_2–), 5.035–5.086 (m, 1H, 4-H), 6.141 (s, 0.75H, 2-H), 6.337 (s, 0.25H, 2-H), 7.041–7.772 (m, 9H). HRMS: *m/z* calcd for $C_{19}H_{16}N_2O3S$ $[M+H]^+$ 353.0960; found: 353.0966.

5.1.19. 3-Phenylacetyl-(2*RS*,4*R*)-2-(2-nitroh-phenyl)-thiazolidine-4-carboxylic acid (3e)

White solid. Yield: 87.5%. Mp 124–126 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 3.221–3.370 (m, 2H, Ph–CH_2–), 3.519–3.821 (m, 2H, 5-H), 5.041 (t, 1H, *J* = 8.1 Hz, 4-H), 6.094 (s, 0.75H, 2-H), 6.296 (s, 0.25H, 2-H), 7.041–7.715 (m, 9H). HRMS: *m/z* calcd for $C_{18}H_{16}N_2O_5S$ $[M+H]^+$ 373.0858; found: 373.0847.

5.1.20. 3-Phenylacetyl-(2*RS*,4*R*)-2-(2-hydroxy-3-methoxy-phenyl)-thiazolidine-4-carboxylic acid (3f)

Whitle solid. Yield: 88.9%. Mp 148–150 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 3.244–3.685 (m, 4H, 5-H, Ph–CH_2–), 3.914 (s, 3H, –OCH_3), 4.981 (t, 1H, *J* = 6.9 Hz, 4-H), 5.977(s, 0.5H, 2-H), 6.473 (s, 0.5H, 2-H), 6.840–7.316 (m, 8H). HRMS: *m/z* calcd for $C_{19}H_{19}NO_5S$ $[M+H]^+$ 374.1062; found: 374.1053.

5.1.21. 3-Phenylacetyl-2-(2*RS*,4*R*)-furan-2-yl-thiazolidine-4-carboxylic acid (3g)

Whitle solid. Yield: 89.2%. Mp 163–165 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): 3.302–3.366 (m, 2H, Ph–CH_2–), 3.747–3.776 (m, 2H, 5-H), 4.941 (t, 1H, *J* = 8.1 Hz, 4-H), 6.162 (s, 1H, 2-H), 6.335 (dd, *J* = 3 Hz, *J* = 3.3 Hz, 1H), 6.632 (d, *J* = 3 Hz, 1H), 7.177–7.411 (m, 6H). HRMS: *m/z* calcd for $C_{16}H_{15}NO_4S$ $[M+H]^+$ 318.0800; found: 318.0793.

5.1.22. 3-(2-Amino-acetyl)-(2*RS*,4*R*)-2-phenylthiazolidine-4-carboxylic acid (4a)

The crude product **2a** was dissolved in 50 mL $NH_3{\cdot}H_2O$ and mixed for 3 days. Then the mixture was evaporated and dissolved in 50 mL distilled water with $NaHCO_3$ (2 equiv), then BOC_2O (1.1 equiv) in 50 mL THF was added dropwise to it. Stir at room temperature for 12 h. The mixture was evaporated and acidified with 1 M citric acid to pH 2–3, then extracted with EtOAC. The combined organic phase was dried, evaporated and purified by column chromatography to give the Boc-protected thiazolidine, which was added to 20 mL HCl/EtOAc, and stirred overnight. The precipitate was collected through filtration and washed with EtOAc to obtain the product.

Yield: 45.3%. Mp 159–161 °C, ^{1}H NMR (H_2O-d_6, 300 MHz): 3.171–3.295 (m, 1.2H, 5-H), 3.371–3.941 (m, 2.8H, 5-H, NH_2–CH_2–), 4.954–4.997 (m, 1H, 4-H), 6.137, 6.145 (2s, 1H, 2-H), 7.220–7.404 (m, 3H), 7.447–7.467 (m, 1H), 7.571–7.593 (m, 1H).

HRMS: *m/z* calcd for $C_{12}H_{14}N_2O_3S$ $[M+H]^+$ 267.0785; found: 267.0793.

Compounds **4b**–**4g** were prepared following the general procedure as described above.

5.1.23. 3-(2-Amino-acetyl)-(2*RS*,4*R*)-2-(2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (4b)

Yield: 41.2%. Mp 125–127 °C, ^{1}H NMR (H_2O-d_6, 300 MHz): 3.732–3.810 (m, 2H, 5-H), 3.981–4.188 (m, 2H, NH_2–CH_2–), 5.401–5.414 (m, 1H, 4-H), 6.343, 6.357 (2s, 1H, 2-H), 6.838–6.932 (m, 2H), 7.144–7.171 (m, 1H), 7.417–7.447 (m, 1H). HRMS: *m/z* calcd for $C_{12}H_{14}N_2O_4S$ $[M+H]^+$ 283.0753; found: 283.0746.

5.1.24. 3-(2-Amino-acetyl)-(2*RS*,4*R*)-2-(2-carboxy-phenyl)-thiazolidine-4-carboxylic acid (4c)

Yield: 43.5%. Mp 156–158 °C, ^{1}H NMR (H_2O-d_6, 300 MHz): 3.739–3.807 (m, 2H, 5-H), 3.974–4.082 (m, 2H, NH_2–CH_2–), 5.128–5.324 (m, 1H, 4-H), 6.306, 6.357 (2s, 1H, 2-H), 6.807–8.200 (m, 4H). HRMS: *m/z* calcd for $C_{13}H_{14}N_2O_5S$ $[M+H]^+$ 311.0701; found: 311.0703.

5.1.25. 3-(2-Amino-acetyl)-(2*RS*,4*R*)-2-(4-cyano-phenyl)-thiazolidine-4-carboxylic acid (4d)

Yield: 42.9%. Mp 131–134 °C, ^{1}H NMR (H_2O-d_6, 300 MHz): 3.245–3.313 (m, 0.8H, 5-H) 3.851–4.053 (m, 2.2H, 5-H, NH_2–CH_2–), 4.869–4.981 (m, 1H, 4-H), 6.234, 6.264 (2s, 1H, 2-H), 7.693–7.919 (m, 4H). HRMS: *m/z* calcd for $C_{13}H_{13}N_3O_3S$ $[M+H]^+$ 292.0756; found: 292.0752.

5.1.26. 3-(2-Amino-acetyl)-(2*RS*,4*R*)-2-(2-nitroh-phenyl)-thiazolidine-4-carboxylic acid (4e)

Yield: 43.8%. Mp 111–113 °C, ^{1}H NMR (DMSO-d_6, 300 MHz): 3.194–3.296 (m, 1.1H, 5-H), 3.383–3.997 (m, 2.9H, 5-H, NH_2–CH_2–), 4.923–4.971 (m, 0.45H, 4-H), 5.011–5.041 (m, 0.55H, 4-H), 6.173 (s, 0.45H, 2-H), 6.227 (s, 0.55H, 2-H), 7.533–7.795 (m, 4H). HRMS: *m/z* calcd for $C_{12}H_{13}N_3O_5S$ $[M+H]^+$ 312.0654; found: 312.0650.

5.1.27. 3-(2-Amino-acetyl)-(2*RS*,4*R*)-2-(2-hydroxy-3-methoxy-phenyl)-thiazolidine-4-carboxylic acid (4f)

Yield: 44.5%. Mp 152–155 °C, ^{1}H NMR (H_2O-d_6, 300 MHz): 3.204–3.305 (m, 2H, 5-H), 3.702 (s, 3H), 3.996–4.107 (m, 2H, NH_2–CH_2–), 5.407 (m, 1H, 4-H), 6.280, 6.331 (2s, 1H, 2-H), 6.829–7.235 (m, 3H). HRMS: *m/z* calcd for $C_{13}H_{13}N_2O_5S$ $[M+H]^+$ 313.0858; found: 313.0851.

5.1.28. 3-(2-Amino-acetyl)-2-(2*RS*,4*R*)-furan-2-yl-thiazolidine-4-carboxylic acid (4g)

Yield: 42.0%. Mp 161–163 °C, ^{1}H NMR (H_2O-d_6, 300 MHz): 3.401–3.774 (m, 4H, 5-H, NH_2–CH_2–), 4.921 (m, 1H, 4-H), 6.271 (s, 0.6H, 2-H), 6.280(s, 0.4H, 2-H), 6.357–6.428 (m, 1.8H), 6.508–6.594 (m, 0.8H), 7.375–7.532 (m, 0.4H). HRMS: *m/z* calcd for $C_{10}H_{12}N_2O_4S$ $[M+H]^+$ 257.0596; found: 257.0587.

5.2. Neuraminidase inhibition assay

NA inhibitory activity was determined by the commercial NA inhibitory screening kit (Beyotime Institute of Biotechnology, Jiangsu, China). Although the NA for enzyme assay is not originated from avian influenza A/H5N1 strain, their sequences are highly conserved. Therefore, this kit is suitable for the high-throughput screening of NA inhibitors in vitro.

The compound 2'-(4-methylumbelliferyl)-a-D-acetylneuraminic acid (MUNANA) is the substrate of NA. And cleavage of this substrate by NA produces a fluorescent product, which can emit an emission wavelength of 460 nm with an excitation wavelength of 355 nm. The intensity of fluorescence can reflect the activity of NA sensitively.

The reaction mixture containing the buffer, NA enzyme, test compounds or oseltamivir carboxylate (which was prepared according to literature method[32] and the substrate, was incubated at 37 °C. Terminate the reaction by adding 150 μL stop solution to all wells including the blank row. Read the plate within 20 min of adding stop solution detecting fluorescence using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The IC_{50} was calculated by plotting percent inhibition versus the inhibitor concentration and determination of each point was performed in duplicate. The data are expressed as the mean of three independent experiments.[33]

5.3. Binding modes of the inhibitors

The binding modes were clarified by docking based on the co-crystal complex of N1 neuraminidase in complex with corresponding ligand oseltamivir downloaded from PDB database (http://www.rcsb.org/pdb/home/home.do), the Protein Data Bank ID codes were 2hu4.pdb. Before docking, the pre-existing ligand was extracted out and hydrogen atoms and charges were added. The docking studies were performed using the Surflex-Dock module of Sybyl 8.1, and the maximum number of poses per ligand was set to 10. The active site of the protein was automatically explored and created based on the previous ligand oseltamivir by the Surflex-Dock Protomol Generation Programme, and other parameters were set as default.

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